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Geranylgeranylacetone, an Inducer of the 70-kDa Heat Shock Protein (HSP70), Elicits Unfolded Protein Response and Coordinates Cellular Fate Independently of HSP70

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ABSTRACT

Geranylgeranylacetone (GGA), an antiulcer agent, has the ability to induce 70-kDa heat shock protein (HSP70) in various cell types and to protect cells from apoptogenic insults. However, little is known about effects of GGA on other HSP families of molecules. We found that, at concentrations ≥100 μM, GGA caused selective expression of 78-kDa glucose-regulated protein (*GRP78*), an HSP70 family member inducible by endoplasmic reticulum (ER) stress, without affecting the level of *HSP70* in various cell types. Induction of ER stress by GGA was also evidenced by expression of another endogenous marker, CCAAT/enhancer-binding protein-homologous protein (CHOP); decreased activity of ER stress-responsive alkaline phosphatase; and unfolded protein response (UPR), including activation of the activating transcription factor 6 (ATF6) pathway and the inositol-requiring ER-to-nucleus signal kinase 1-X-box-binding protein 1 (IRE1-XBP1) pathway. Incubation of mesangial cells with GGA caused significant apoptosis, which was attenuated by transfection with inhibitors of caspase-12 (i.e., a dominant-negative mutant of caspase-12 and MAGE-3). Dominant-negative suppression of IRE1 or XBP1 significantly attenuated apoptosis without affecting the levels of CHOP and *GRP78*. Inhibition of c-Jun NH_2-terminal kinase, the molecule downstream of IRE1, by 1,9-pyrazoloanthrone (SP600125) did not improve cell survival. Blockade of ATF6 by 4-(2-aminoethyl)benzenesulfonyl fluoride enhanced apoptosis by GGA, and it was correlated with attenuated induction of both *GRP78* and CHOP. Overexpression of *GRP78* or dominant-negative inhibition of CHOP significantly attenuated GGA-induced apoptosis. These results suggested that GGA triggers both proapoptotic (IRE1-XBP1, ATF6-CHOP) and antiapoptotic (ATF6-GRP78) UPR and thereby coordinates cellular fate even without induction of *HSP70*.

Geranylgeranylacetone (GGA) has been used in clinics for many years as a therapeutic agent for gastric ulcer and gastritis. However, recent investigation revealed the novel potential of this agent as a general cytoprotective compound. GGA induces 70-kDa heat shock protein (HSP70) selectively, and it protects various cells from apoptosis in vitro triggered by ethanol, reactive oxygen species, proteasome inhibitors, and cytotoxic drugs (Hirakawa et al., 1996; Ikeyama et al., 2001; Kikuchi et al., 2002; Nishida et al., 2006). The therapeutic utility of GGA in vivo has also been documented by several investigators. For example, administration with GGA attenuated not only gastric mucosal damage but also ischemic brain injury, viral infection, and endotoxin shock (Unoshima et al., 2003; Nakada et al., 2005; Uchida et al., 2006). However, it is currently unclear whether these therapeutic effects of GGA are ascribed only to up-regulation of HSP70. GGA might exert the beneficial effects through other mechanisms (e.g., induction of other HSP family of mole-

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ABBREVIATIONS: GGA, geranylgeranylacetone; HSP, heat shock protein; ER, endoplasmic reticulum; GRP, glucose-regulated protein; UPR, unfolded protein response; CHOP, CCAAT/enhancer-binding protein-homologous protein; JNK, c-Jun NH_2-terminal kinase; PERK, RNA-dependent protein kinase-like ER kinase; ATF6, activating transcription factor 6; IRE1, inositol-requiring ER-to-nucleus signal kinase 1; eIF2α, eukaryotic translation initiation factor 2α; XBP1, X-box-binding protein 1; FBS, fetal bovine serum; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; DTT, dithiothreitol; SEAP, secreted alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; ES-TRAP, ER stress-responsive alkaline phosphatase; ERAI, ER stress-activated indicator; nt, nucleotide(s); ASK, apoptosis signal-regulating kinase; DN, dominant-negative; TRAF, tumor necrosis factor receptor-associated factor; SP600125, 1,9-pyrazoloanthrone.

cules). However, information is very limited regarding how GGA affects the levels of other HSP family members.

According to molecular mass, HSPs are classified into five major families: the HSP100 family, the HSP90 family, the HSP70 family, the HSP60 family, and the small HSP family. Among these, the HSP70 family is the largest family, and it has been extensively studied during the past decades. Previous reports showed that several HSPs are induced not only by thermal stress but also under glucose deprivation. These include glucose-regulated protein (GRP)34, GRP47, GRP56, GRP75, GRP78, GRP94, and GRP174. Many of these, especially GRP78, act as molecular chaperones in the endoplasmic reticulum (ER), and they are regarded as biomarkers for ER stress (Lee, 2001). We have found that, in some cell types, GGA selectively up-regulates GRP78 without affecting the level of HSP70. We hypothesized that, under particular situations, GGA might induce ER stress and thereby influence cellular function independently of HSP70.

The ER plays an important role in appropriate folding of newly synthesized proteins by the resident chaperones. Various chemical, physical, and nutritional stress, including glucose deprivation, hypoxia, altered redox state, inhibition of protein glycosylation, and disturbed calcium homeostasis, perturbs the function of ER, leading to accumulation of unfolded/misfolded proteins within the ER (Rutkowski and Kaufman, 2004). The ER stress triggers several cascades of signal transduction pathways, known as unfolded protein response (UPR). ER stress triggers survival signals through UPR, leading to reduced translation, enhanced expression of ER chaperones, and accelerated degradation of unfolded/misfolded proteins through the proteasome pathway (Rutkowski and Kaufman, 2004). However, ER stress also causes apoptosis through 1) activation of the ER resident cysteine protease caspase-12 (in rodents) or caspase-4 (in humans), 2) induction of growth-arrested and DNA damage-inducible protein 153 [also called CCAAT/enhancer-binding protein-homologous protein (CHOP)], or 3) activation of c-Jun NH_2-terminal kinase (JNK) (Kim et al., 2006). GGA per se might induce apoptosis via induction of these proapoptotic molecules.

The UPR is initiated through three major transducers for ER stress. These are RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring ER-to-nucleus signal kinase 1 (IRE1). PERK and IRE1 have cytoplasmic serine/threonine kinase domains, and ER stress induces luminal domain-driven homodimerization, autophosphorylation, and activation of these transducers. Activation of PERK leads to phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which causes general inhibition of protein synthesis. In contrast, in response to ER stress, p90ATF6 transits to the Golgi where it is cleaved by the proteases S1P and S2P, yielding a free cytoplasmic domain that is an active transcription factor p50ATF6. Likewise, the endoribonuclease domain of the activated IRE1 catalyzes the removal of a small intron from the mRNA of the gene encoding X-box-binding protein 1 (XBP1). This splicing event creates a translational frameshift in XBP1 mRNA to produce an active transcription factor. Activated p50ATF6 and XBP1 heterodimerized with nuclear factor Y, subsequently bind to the ER stress response element and/or the UPR element (UPRE), leading to expression of target genes including GRP78 (Rutkowski and Kaufman, 2004).

In the present report, we first examine whether GGA, a "selective" inducer of HSP70, has the ability to induce GRP78, a marker of ER stress. We next investigate whether GGA can induce ER stress and how individual UPRs regulate cellular fate. Our current results disclosed that 1) GGA selectively up-regulates GRP78 without affecting the level of HSP70, 2) GGA unexpectedly induces apoptosis via induction of ER stress, and 3) GGA triggers both proapoptotic and antiapoptotic UPR and thereby coordinates cellular fate.

Materials and Methods

Cells and Reagents. The rat mesangial cell clone SM43 was established as described previously (Kitamura et al., 1994). The porcine renal proximal tubular cell line LLC-PK1, the rat renal tubular epithelial cell line NRK-52E, the murine hepatoma cell line Hepa-1c1c7, and the rat alveolar macrophage NR8383 were purchased from the American Type Culture Collection (Manassas, VA). The murine neuroblastoma cell Neuro2A was provided by Dr. K. Nagai (University of Yamanashi, Yamanashi, Japan). The normal human mesangial cell NHMC was purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD), and the human glioma T98G was from Cell Resource Center for Biomedical Research (Tohoku University, Miyagi, Japan). The human synoviocyte MH7A was purchased from Riken BRC Cell Bank (Ibaraki, Japan). Hepa-1c1c7 cells were maintained in α-minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% feral bovine serum (FBS). Other cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (Gibco-BRL, Gaithersburg, MD) supplemented with 5% FBS. Medium containing 1% FBS was generally used for the experiments. GGA was provided by Eisai Co. Ltd. (Tokyo, Japan). Tunicamycin, thapsigargin, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and SP600125 were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Dithiothreitol (DTT) was from Wako Pure Chemicals (Osaka, Japan). Salubrinal was from Calbiochem (San Diego, CA).

Establishment of Stable Transfectants. SM/SV-SEAP cells, which stably express secreted alkaline phosphatase (SEAP) under the control of the simian virus 40 early promoter and enhancer, were established as described previously (Hiramatsu et al., 2005). Using electroporation, SM43 cells were stably transfected with pcDNA3.1-GRP78 (Watson et al., 2003), pcDNA3-C12DN encoding a dominant-negative mutant of caspase-12 (Rao et al., 2002), pcDNA3.1-MAGE-3 (Morishima et al., 2002), pcDNA3.1-IRE1β-K536A encoding a dominant-negative mutant of IRE1β, pcDNA3.1-dnXBP1 encoding a dominant-negative mutant of XBP1 (Lee et al., 2003a), or pCAX-F-XBP1-ΔDBD-Venus encoding fluorescent protein Venus (Iwawaki et al., 2004), and SM/GRP78, SM/C12DN, SM/MAGE-3, SM/IRE1βDN, SM/XBP1DN, and SM/XBP1-Venus cells were established. pCAX-F-XBP1-ΔDBD-Venus consists of 1) the cytomegalovirus enhancer fused to the chicken β-actin promoter, 2) a Kozak sequence and FLAG-tagged coding sequence of XBP1 but lacking its DNA-binding domain, and 3) the fluorescent protein Venus. SM/Neo cells transfected with pcDNA3.1 (Invitrogen) alone were used as mock-transfected, control cells.

Transient Transfection. Using electroporation, SM43 cells were transiently transfected with pCMV-3xFLAG-ATF6 encoding FLAG-tagged ATF6 (Shen and Prywes, 2005). After incubation overnight, the cells were seeded in six-well plates in the presence of 1% FBS. After incubation for 24 h, the cells were stimulated with GGA for 1 to 3 h, and then they were subjected to Western blot analysis, as described below. SM43 cells were also cotransfected with pcDNA3.1 or pcDNA3.1-Myc-CHOPΔLZ encoding a dominant-negative mutant of CHOP (Ohoka et al., 2005) together with pEGFP-N1 (Clontech, Mountain View, CA) encoding enhanced green fluorescent protein at 4:1 ratio. After transfection, the cells were seeded and incubated in 24-well plates in the presence of 1% FBS, stimulated with GGA for 9 h, and subjected to fluorescence microscopy to evaluate the per-

centages of fluorescence-positive round cells versus total fluorescence-positive cells, as described previously (Yokouchi et al., 2007). Assays were performed in quadruplicate.

Northern Blot Analysis. Total RNA was extracted by a single-step method, and Northern blot analysis was preformed as described previously (Yokouchi et al., 2007). For preparation of radiolabeled probes, cDNAs encoding HSP70, GRP78 (Katayama et al., 2001), GRP94 (Gazit et al., 1999), SEAP (BD Biosciences, San Jose, CA), a dominant-negative mutant of caspase-12 (Rao et al., 2002), MAGE-3 (Morishima et al., 2002), IRE1β, and a dominant-negative mutant of XBP1 (Lee et al., 2003a) were used. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was shown as a loading control. Densitometric analysis was performed using Scion Image (Scion Corporation, Frederick, MD).

Formazan Assay. The number of viable cells was evaluated by a formazan assay using Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) (Hiramatsu et al., 2006). In brief, cells in 96-well plates were incubated at 37°C for 2 h in medium containing 10% Cell Counting Kit-8 assay solution. Absorbance (450 nm) of formazan generated from WST-8 (Dojindo Laboratory) was measured by Spectra Max 340 (Nihon Molecular Devices, Tokyo, Japan).

Hoechst Staining. Cells fixed in 4% formaldehyde were stained by Hoechst 33258 (10 μg/ml; Sigma-Aldrich Japan) for 2 h. Apoptosis was identified using morphological criteria including shrinkage of the cytoplasm (round shape) and nuclear condensation (Yokouchi et al., 2007). Fluorescence microscopic analysis was performed using an IX71 microscope (Olympus, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay. TUNEL assay was performed using DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions. To stain nuclei, 3 μg/ml 4′,6-diamidino-2-phenylindole (Sigma-Aldrich Japan) was used.

ER Stress-Responsive Alkaline Phosphatase Assay. Induction of ER stress was evaluated by ES-TRAP assay (Hiramatsu et al., 2006). It was based on the fact that activity of SEAP constitutively produced by transfected cells is rapidly and selectively down-regulated by ER stress independently of transcriptional regulation. Using this method, culture media from GGA-exposed cells were subjected to chemiluminescent assay to evaluate SEAP activity. The SEAP assay was performed using Great EscAPe SEAP detection kit (BD Biosciences), as described previously (Hiramatsu et al., 2006). To examine whether GGA can induce ER stress in vivo, ES-TRAP mice that systemically produce SEAP (Hiramatsu et al., 2007) were used. ES-TRAP mice (25 g b.wt.) were orally administered with 50 mg of GGA using feeding needles. Serum was collected periodically from the tail vein, and activity of SEAP was evaluated by chemiluminescent assay. The animals were treated humanely, and the experiments were performed following the regulations and guidelines of the University of Yamanashi.

ER Stress-Activated Indicator Assay. ER stress-activated indicator assay (ERAI) is a method for the assessment of XBP1 mRNA splicing caused by IRE1 during ER stress. The stress indicator was constructed by fusing XBP1 and Venus, a variant of green fluorescent protein. During stress, the spliced indicator mRNA is translated into XBP1-Venus fusion protein, which can be detected by its fluorescence (Iwawaki et al., 2004). SM/XBP1-Venus cells were established as described under Establishment of Stable Transfectants. After exposure to GGA, intensity of Venus was examined by fluorescence microscopy.

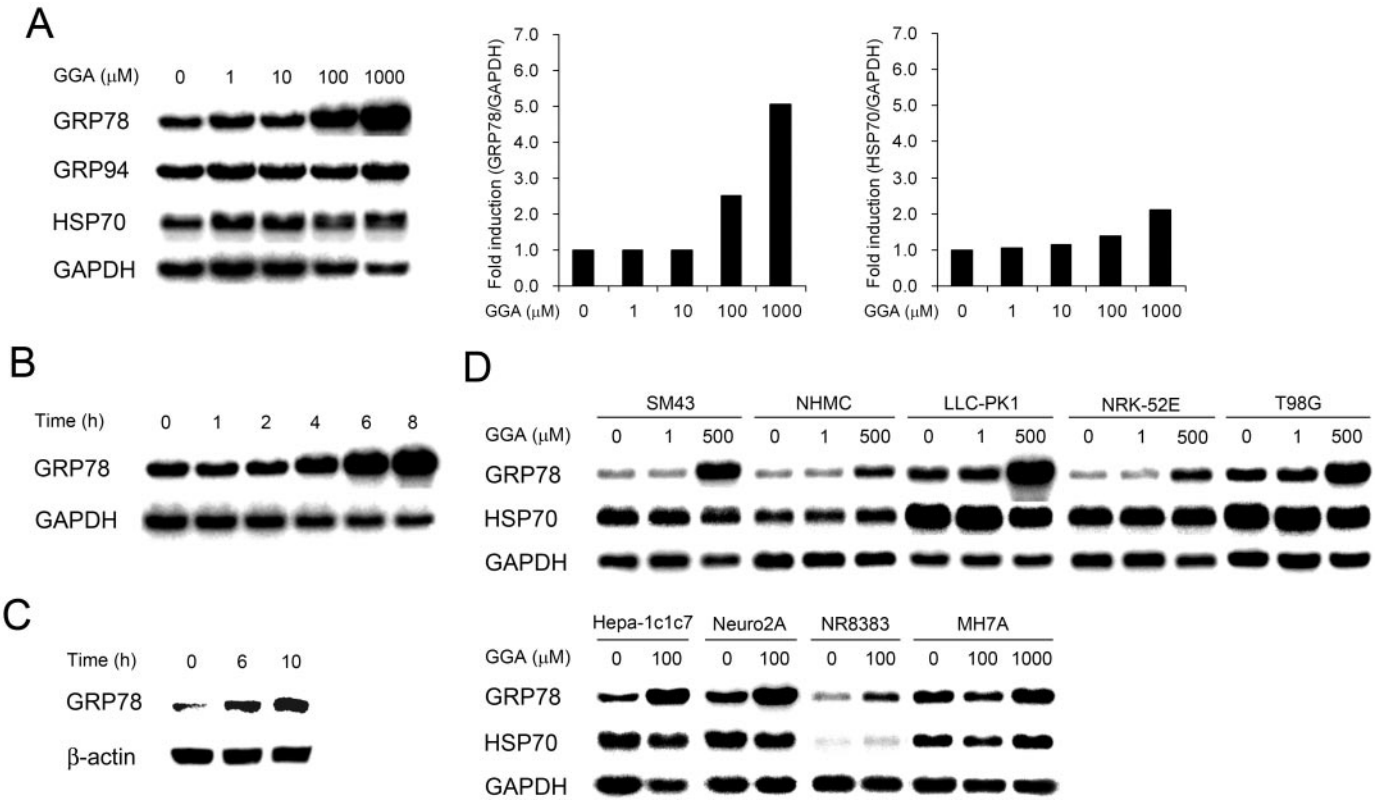


Fig. 1. Selective induction of GRP78 by GGA. A and B, rat mesangial cells (SM43) were treated with GGA for 8 h at indicated concentrations (A) or with 1 mM GGA for indicated times (B), and then they were subjected to Northern blot analysis of GRP78, GRP94, and HSP70. Expression of GAPDH is shown at the bottom as a loading control. A, densitometric analyses of GRP78 and HSP70 are shown as graphs. C, SM43 cells were exposed to 500 μM GGA for 6 or 10 h, and then the level of GRP78 protein was examined by Western blot analysis. The level of β-actin is shown as a loading control. D, SM43, NHMC, LLC-PK1, NRK-52E, T98G, Hepa-1c1c7, Neuro2A, NR8383, and MH7A cells were treated with 1 μM to 1 mM GGA for 6 to 8 h. After the treatment, cells were subjected to Northern blot analysis of GRP78 and HSP70.

Western Blot Analysis. Western blot analysis was performed, as described previously (Hayakawa et al., 2006). Primary antibodies used were anti-KDEL antibody (1:1000 dilution; Stressgen, Victoria, BC, Canada), anti-FLAG antibody (1:1000 dilution; Sigma-Aldrich Japan) and anti-phospho-PERK antibody (1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). As a loading control, levels of β-actin were evaluated using anti-β-actin antibody (1:30,000 dilution; Sigma-Aldrich Japan). Blots were visualized using the enhanced chemiluminescence system (Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, UK).

Statistical Analysis. Assays were performed in quadruplicate. Data were expressed as means ± S.E. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. $p < 0.05$ was considered to be a statistically significant difference.

Results

Selective Induction of GRP78 by GGA. GGA is known to induce HSP70 (Hirakawa et al., 1996; Ikeyama et al., 2001; Kikuchi et al., 2002). However, effects of GGA on other HSPs have not been well understood. We found that in cultured rat mesangial cell SM43, GGA up-regulated GRP78, a HSP70 family member that is inducible selectively by ER stress (Fig. 1A, left and middle). It is noteworthy that GGA induced neither GRP94 nor HSP70, although the HSP70/GAPDH ratio was slightly increased at the highest concentration, because of decrease in the level of GAPDH (Fig. 1A, left and right). Time-lapse studies revealed that induction of GPR78 was detectable at 4 h after the treatment with GGA and that expression increased progressively up to 8 h (Fig. 1B). The transcriptional induction of GRP78 was associated with a substantial increase in the protein level of GRP78 (Fig. 1C). To examine whether the induction of GRP78 is species- and/or cell type-specific, effects of GGA were tested in various cell types. These included the human mesangial cell NHMC, the porcine renal tubular cell LLC-PK1, the rat renal tubular cell NRK-52E, the human glioma cell T98G, the mouse hepatoma cell Hepa-1c1c7, the mouse neuroblastoma cell Neuro2A, rat alveolar macrophage NR8383, and the human synoviocyte MH7A. These cells were treated with GGA for 6 to 8 h, and then they were subjected to Northern blot analysis. Like in SM43 cells, substantial induction of GRP78 was observed in all cells tested, except for MH7A cells that showed only slight induction at 1 mM (Fig. 1D, top row). In contrast, similar to SM43 cells, none of the cells exhibited up-regulation of HSP70 in response to GGA (Fig. 1D, middle row). These results suggested that GGA generally induces GRP78 not only in SM43 cells but also in other cell types originated from different species, without induction of HSP70.

Induction of ER Stress by GGA. GRP78 is the most popular endogenous biomarker for ER stress (Lee, 2001). Induction of ER stress by GGA was further confirmed using

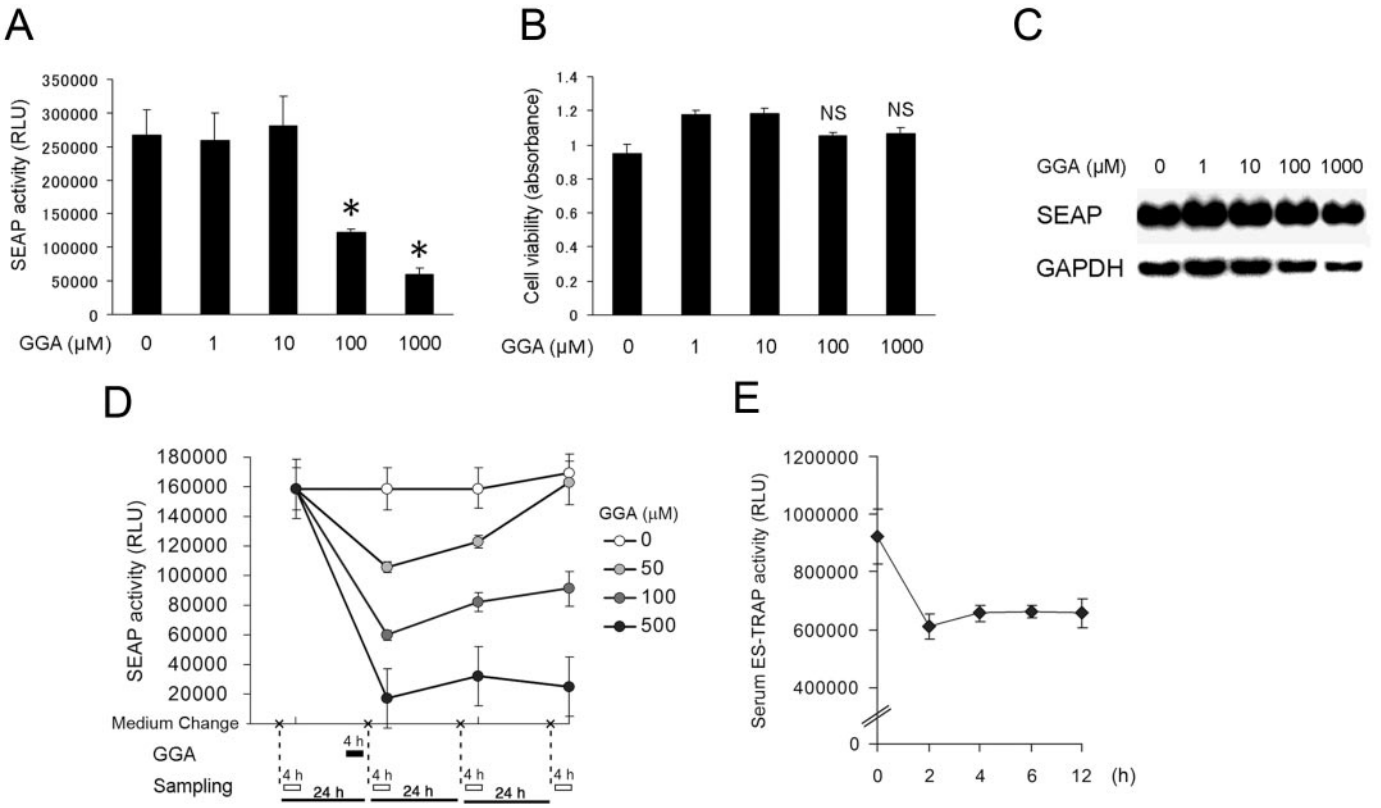


Fig. 2. Induction of ER stress by GGA evidenced by ER stress-responsive alkaline phosphatase (ES-TRAP). A and B, SM43 cells stably expressing SEAP under the control of the simian virus 40 promoter (SM/SV-SEAP cells) were treated with GGA for 2 h at indicated concentrations. The culture media were then replaced with fresh media containing GGA at the same concentrations. After 4-h incubation, culture media and cells were subjected to ES-TRAP assay (A) and formazan assay (B), respectively. Data are expressed as means ± S.E., and asterisks indicate statistically significant differences ($p < 0.05$). N.S., not statistically significant. RLU, relative light unit. C, SM/SV-SEAP cells were treated with GGA for 8 h at indicated concentrations, and then expression of SEAP was examined by Northern blot analysis. D, SM/SV-SEAP cells were treated with 50 to 500 μM GGA for 4 h. After the exposure, culture media were replaced every 24 h with fresh medium without GGA. Activity of SEAP in media after the initial 4-h incubations was evaluated. E, ES-TRAP mice (25 g b.wt.) were orally administered with 50 mg of GGA. Serum was collected periodically from the tail vein, and activity of SEAP was evaluated by chemiluminescent assay.

a sensitive and selective exogenous indicator of ER stress, ES-TRAP (Hiramatsu et al., 2006). The ES-TRAP assay is based on the facts that 1) activity of SEAP constitutively produced by transfected cells is rapidly, sensitively, and selectively down-regulated by ER stress independently of its transcriptional regulation; 2) the magnitude of the decrease in SEAP is proportional to the extent of ER stress; and 3) the decrease in SEAP activity is caused by abnormal post-translational modification, accelerated degradation, and reduced secretion of SEAP protein (Hiramatsu et al., 2006). SM/SV-SEAP mesangial cells constitutively expressing SEAP were treated with serial concentrations of GGA for 4 h, and culture media were subjected to chemiluminescent assay. Consistent with the result shown in Fig. 1A, GGA reduced SEAP activity dose-dependently at concentrations $\geq$100 μM (Fig. 2A). Formazan assay showed that, under this experimental setting (4-h exposure), the number of viable cells was not affected by GGA at any concentrations tested (Fig. 2B). To confirm the down-regulation of SEAP activity by GGA was independent of transcriptional suppression, expression of SEAP was examined by Northern blot analysis. In contrast to the down-regulation of SEAP activity, the level of SEAP mRNA was unaffected by GGA (Fig. 2C).

To examine whether the induction of ER stress by GGA is reversible, SM/SV-SEAP cells were exposed to GGA for 4 h at different concentrations. After the exposure, culture media were replaced every 24 h with fresh medium without GGA. Activity of SEAP in media during the initial 4-h incubations was evaluated. When the cells were exposed to 50 μM GGA, the level of SEAP activity was significantly reduced to 67% (versus initial level 100%), and it was completely recovered to the initial level after 48 h. Higher concentrations of GGA caused more intense suppression of SEAP, and the recovery was only partial (Fig. 2D).

We examined whether ER stress is also induced in vivo after oral administration with GGA. ER stress reporter mice

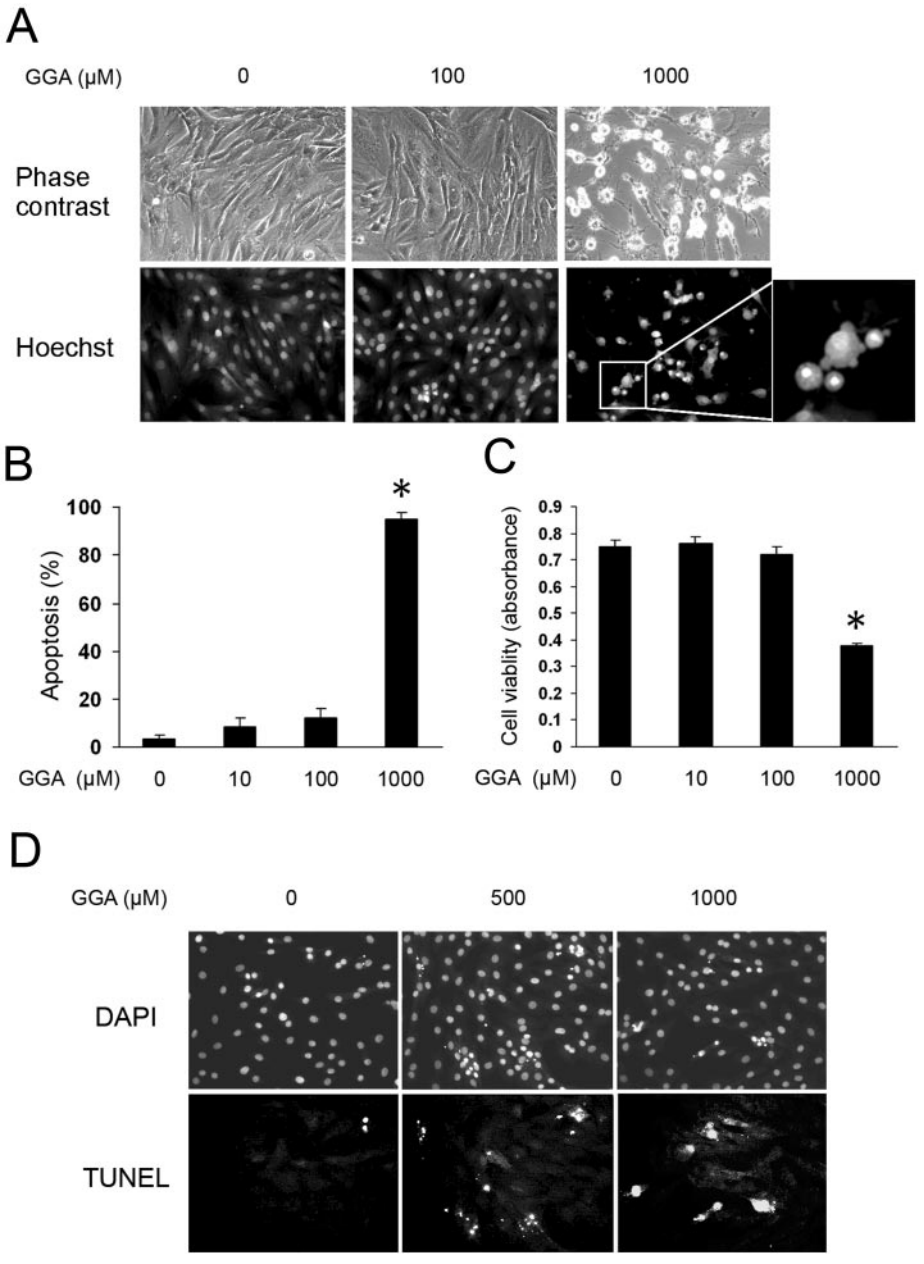


Fig. 3. Induction of apoptosis by GGA. A to C, SM43 cells were treated with 100 μM or 1 mM GGA for 20 h, and then they were subjected to phase-contrast microscopy (top) and Hoechst staining (bottom) in A. Quantitative analysis of apoptosis evaluated by Hoechst staining and formazan assay is shown in B and C, respectively. Data are expressed as means $\pm$ S.E., and asterisks indicate statistically significant differences ($p < 0.05$). Assays were performed in quadruplicate. D, cells were treated with GGA for 4 h, and then they were subjected to TUNEL assay (bottom). The nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) (top).

(ES-TRAP mice) (Hiramatsu et al., 2007) were orally administered with GGA using feeding needles, and serum was collected periodically from the tail vein. As shown in Fig. 2E, activity of serum SEAP was rapidly down-regulated after administration with GGA, suggesting induction of ER stress in vivo by GGA.

Induction of Apoptosis by GGA. ER stress is a trigger to induce apoptosis in various cell types. We examined whether GGA has the potential to induce apoptosis in mesangial cells. SM43 cells were exposed to GGA for 24 h, and then they were subjected to microscopic analyses. Phase-contrast microscopy and Hoechst staining showed that the cells exposed to 1 mM GGA exhibited shrinkage of the cytoplasm and nuclear condensation typical of apoptosis (Fig. 3A). Quantitative analysis revealed that 1 mM GGA, but not 100 μM GGA, caused dramatic apoptosis (Fig. 3B). Reduction in the number of viable cells was also evidenced by formazan assay (Fig. 3C). To further confirm that apoptosis was indeed induced by GGA, cells exposed to GGA were fixed, and then they were subjected to TUNEL assay. Fluorescent microscopy showed that the number of apoptotic cells increased by GGA in a dose-dependent manner (Fig. 3D).

Role of ER Stress in GGA-Induced Apoptosis. In rodents, ER stress induces apoptosis through activation of an ER resident caspase, caspase-12 (Nakagawa et al., 2000). To examine whether the induction of apoptosis by GGA was mediated by ER stress, we created SM/C12DN cells that stably express a dominant-negative mutant of caspase-12 (Fig. 4A). The signal observed in SM/Neo cells (mock-transfected control) is expression of endogenous caspase-12. SM/Neo and SM/C12DN cells were treated with GGA for 9 h, and microscopic analysis was performed. Hoechst staining showed that induction of apoptosis observed in SM/Neo cells was significantly suppressed in SM/C12DN cells (Fig. 4B, left). The percentages of apoptotic cells were 69.8 $\pm$ 3.5 in SM/Neo versus 42.3 $\pm$ 1.7 in SM/C12DN (means $\pm$ S.E.; $p < 0.05$) (Fig. 4B, right). To further confirm the crucial role of ER stress in the induction of apoptosis by GGA, we used another inhibitor of caspase-12, MAGE-3. MAGE-3 binds to both the p10 caspase-12 fragment and procaspase-12, but not to other caspases, in mammalian cells. Overexpression of MAGE-3 renders cells resistant to apoptosis by suppression of caspase-12 activation, and the cytoprotective effect of MAGE-3 is specific to the ER stress-induced apoptotic path-

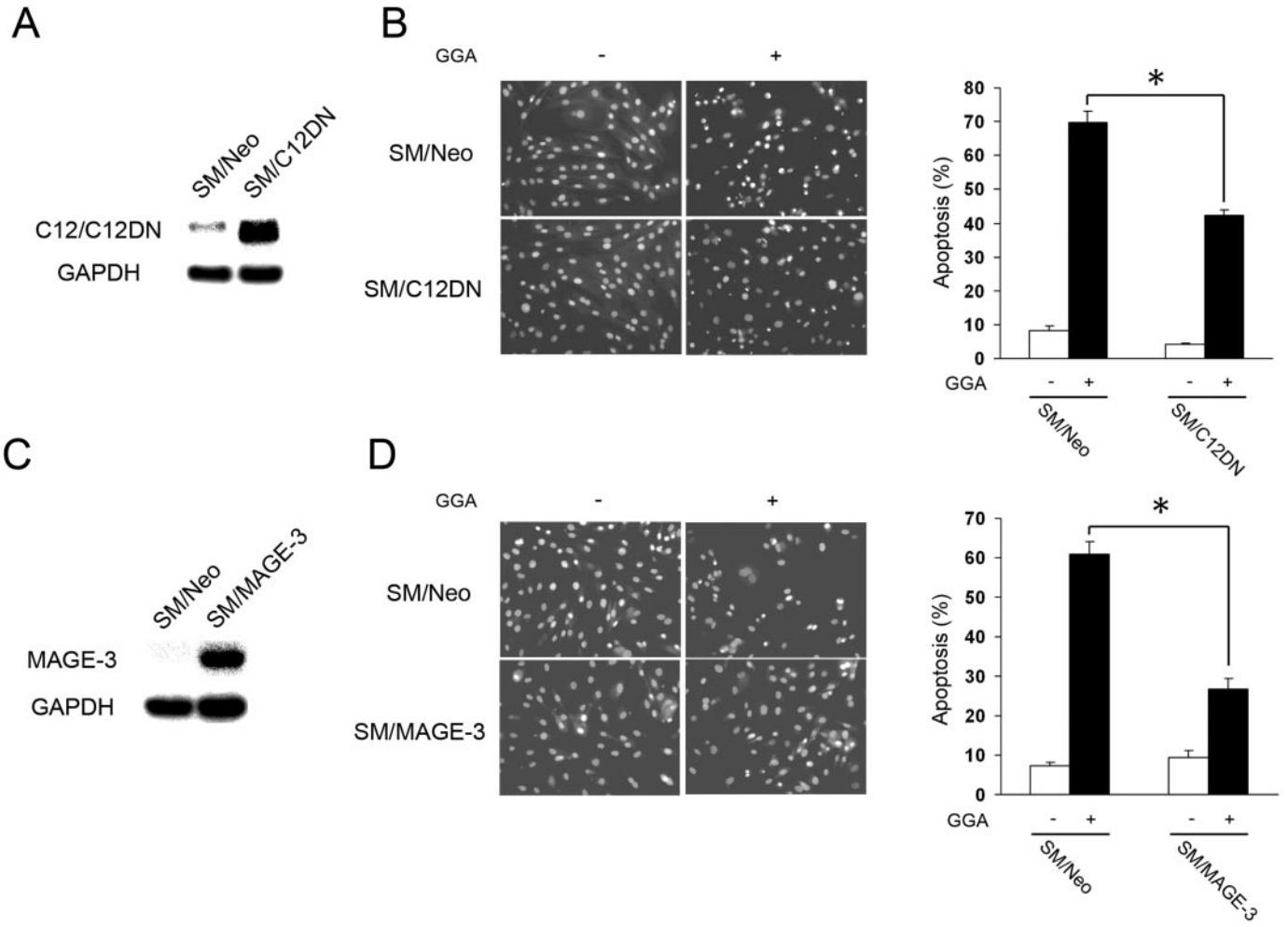


Fig. 4. Role of ER stress in GGA-induced apoptosis. A and C, SM43 cells were stably transfected with a dominant-negative mutant of caspase-12 (C12DN) or another inhibitor of caspase-12, MAGE-3, and SM/C12DN cells and SM/MAGE-3 cells were established. Expression of the transgenes was confirmed by Northern blot analysis. SM/Neo is a mock-transfected cell expressing neo alone. B and D, SM/Neo, SM/C12DN, and SM/MAGE-3 cells were treated with (+) or without (−) 1 mM GGA for 9 h and subjected to Hoechst staining (left). Quantitative analysis of apoptosis was shown in the graphs (right). Data are expressed as means $\pm$ S.E., and asterisks indicate statistically significant differences ($p < 0.05$).

way (Morishima et al., 2002). We created SM/MAGE-3 cells that stably express MAGE-3 (Fig. 4C). SM/Neo and SM/MAGE-3 cells were treated with GGA, and microscopic analysis was performed. Consistent with the results from SM/C12DN cells, induction of apoptosis was significantly suppressed in SM/MAGE-3 cells (Fig. 4D, left). The percentages of apoptotic cells were 61.0 ± 3.0 in SM/Neo versus 26.7 ± 2.8 in SM/MAGE-3 ($p < 0.05$) (Fig. 4D, right).

UPR Triggered by GGA. In general, ER stress triggers UPR initiated by IRE1, ATF6, and PERK. To investigate whether GGA activates the IRE1-XBP1 pathway, we used ERAI (Iwawaki et al., 2004). SM43 cells were stably transfected with a gene encoding Venus, a variant of green fluorescent protein, downstream of a partial sequence of XBP1, including the 26-nt ER stress-specific intron. Under unstressed conditions, the mRNA of the transgene is not spliced, and its translation is terminated at the stop codon near the joint between the XBP1 and Venus genes. In contrast, under ER stress, the 26-nt intron is spliced out by IRE1, leading to a frameshift of the mRNA and production of the XBP1-Venus fusion protein. Established SM/XBP1-Venus cells were treated with GGA for 6 h, further incubated for 7 h in the absence of GGA, and then subjected to fluorescence microscopy. Thapsigargin, a known inducer of ER stress, was used as a positive control. As shown in Fig. 5A, treatment of the cells with GGA induced a fluorogenic response in a dose-dependent manner, suggesting that the IRE1-XBP1 pathway was activated by GGA.

We next examined whether the ATF6 pathway is activated by GGA. SM43 cells were transiently transfected with a cDNA encoding FLAG-tagged ATF6, and then they were treated with GGA for 1 or 3 h. As a positive control, a known inducer of ER stress, DTT, was used. Western blot analysis revealed that, like DTT, GGA induced cleavage of ATF6 after the treatment with GGA for 3 h (Fig. 5B), suggesting activation of the ATF6 pathway.

In contrast to the IRE1-XBP1 pathway and the ATF6 pathway, phosphorylation of PERK was not evident after treatment with GGA (Fig. 5C). Furthermore, treatment with salubrinal (an inhibitor of eIF2α dephosphorylation) (Boyce et al. 2005) that reinforces the eIF2α-mediated signaling did not affect induction of GRP78 by GGA (Fig. 5D). These results indicated the lack of involvement of the PERK-eIF2α pathway in GGA-triggered UPR.

Role of the IRE1 Pathway in GGA-Induced Apoptosis. To examine whether the induction of apoptosis by GGA was mediated by the IRE1-XBP1 pathway, we created SM/IRE1βDN cells that stably express a dominant-negative mutant of IRE1β (Fig. 6A). SM/Neo and SM/IRE1βDN cells were treated with GGA for 9 h, and microscopic analyses were performed. Phase-contrast microscopy showed that GGA-triggered apoptosis was suppressed by dominant-negative inhibition of IRE1 (Fig. 6B, left). Quantitative analysis using Hoechst staining showed that the percentages of apoptotic cells were 61.0 ± 3.0 in SM/Neo and 39.5 ± 1.1 in SM/IRE1βDN ($p < 0.05$) (Fig. 6B, right).

In general, IRE1 transduces a proapoptotic signal via activation of the apoptosis signal-regulating kinase (ASK) 1-JNK pathway and via activation of procaspases (Kim et al., 2006). Conversely, IRE1 also transduces an antiapoptotic signal via splicing of XBP1 mRNA and consequent transcriptional induction of ER chaperones (Lee, 2001; Kim et al.,

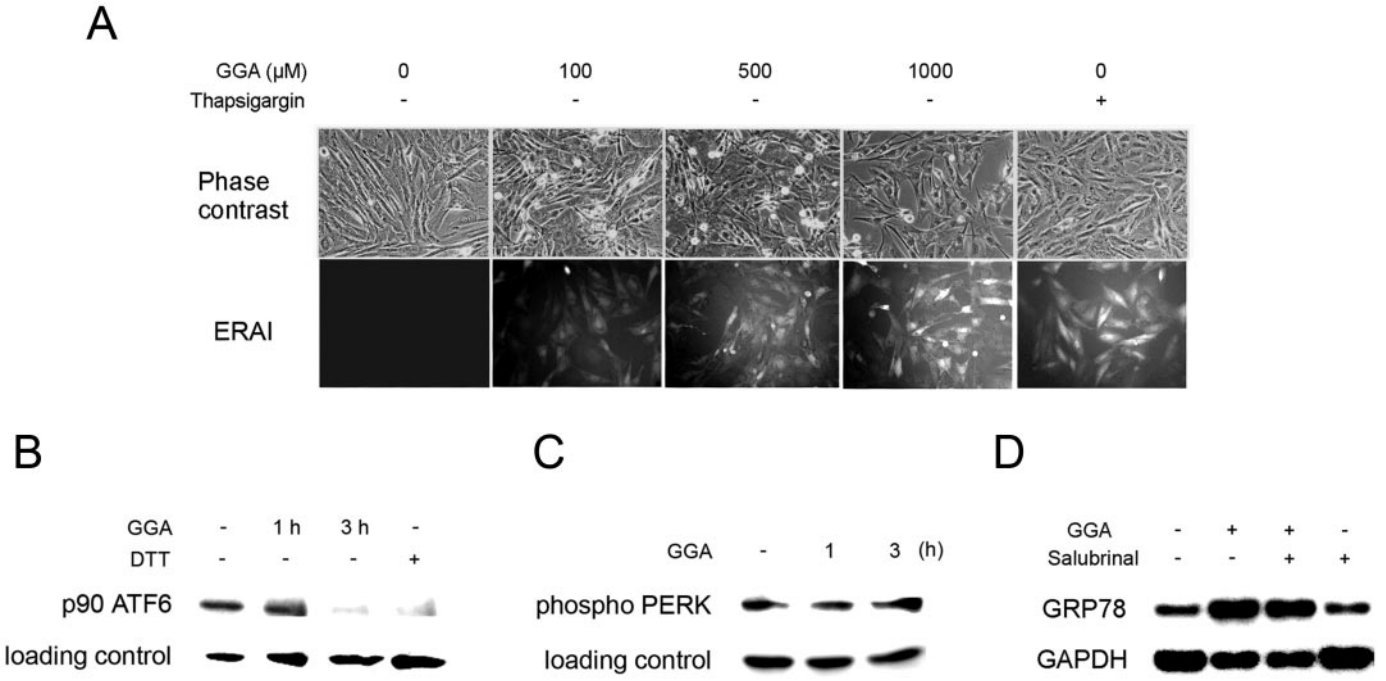
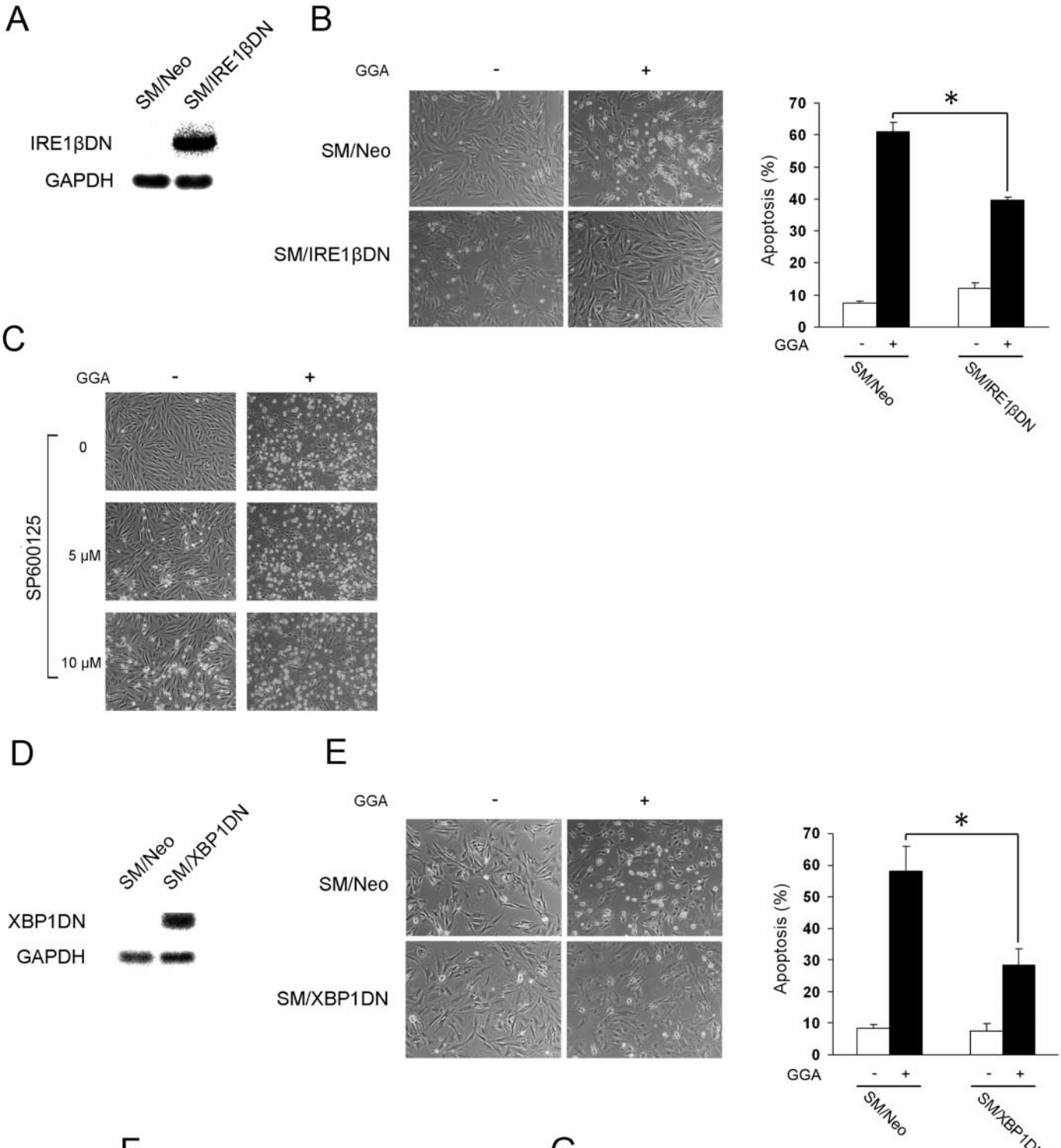


Fig. 5. UPR triggered by GGA. A, SM43 cells were stably transfected with a gene encoding Venus, a variant of green fluorescent protein, downstream of a partial sequence of XBP1, including the 26-nt ER stress-specific intron. Established SM/XBP1-Venus cells were treated with GGA for 6 h at indicated concentrations, further incubated for 7 h in the absence of GGA, and then subjected to phase-contrast microscopy (top) and fluorescence microscopy (ERAI, ER stress-activated indicator; bottom). Thapsigargin (1 μM) was used as a positive control. B, SM43 cells were transiently transfected with a cDNA encoding FLAG-tagged p90ATF6, and then they were treated with 1 mM GGA for 1 or 3 h. As a positive control, treatment with 1 mM DTT for 1 h was used. The cleavage of FLAG-p90ATF6 was evaluated by Western blot analysis. C, cells were treated with GGA for 1 or 3 h, and phosphorylation of PERK was assessed by Western blot analysis. D, SM43 cells were pretreated with 10 μM salubrinal for 30 min, stimulated with 500 μM GGA for 6 h, and then subjected to Northern blot analysis of GRP78.



2006). However, we found that a selective inhibitor of JNK, SP600125, did not attenuate GGA-induced apoptosis (Fig. 6C). We hypothesized that the IRE1-XBP1 pathway, but not IRE1-JNK pathway, may contribute to GGA-triggered apoptosis. To examine this possibility, we created SM/XBP1DN cells that stably express a dominant-negative mutant of XBP1 (Fig. 6D). SM/Neo and SM/XBP1DN cells were treated with GGA, and microscopic analyses were performed. Phase-contrast microscopy revealed that GGA-induced apoptosis was attenuated by dominant-negative inhibition of XBP1 (Fig. 6E, left). The percentages of apoptotic cells evaluated by Hoechst staining were 58.0 ± 8.1 in SM/Neo versus 28.4 ± 5.3 in SM/XBP1DN ($p < 0.05$) (Fig. 6E, right).

CHOP is known as a proapoptotic molecule involved in ER stress-induced apoptosis in various cell types. We found that, after treatment of SM43 cells with GGA, expression of CHOP was rapidly induced within 1 h and that it peaked to maximum at 6 to 8 h (Fig. 6F). To examine mechanisms involved in the IRE1-XBP1-mediated apoptosis by GGA, expression levels of CHOP as well as GRP78 were examined in SM/IRE1βDN and SM/XBP1DN cells. Northern blot analysis revealed that the induction of CHOP by GGA was not attenuated by dominant-negative inhibition of IRE1 or XBP1 (Fig. 6G). Likewise, induction of GRP78 by GGA was not enhanced in SM/IRE1βDN and SM/XBP1DN cells. These results indicated that the GGA-triggered IRE1-XBP1 pathway contributed to apoptosis independently of CHOP and GRP78.

Role of the ATF6 Pathway in GGA-Induced Apoptosis. As shown in Fig. 5B, GGA activated ATF6. In response to ER stress, p90ATF6 transits to the Golgi where it is cleaved by S1P and S2P proteases, yielding an active transcription factor. We examined a role of the ATF6 pathway in GGA-induced apoptosis using a selective inhibitor of S1P/S2P, AEBSF (Okada et al., 2003). Treatment of the cells with AEBSF markedly enhanced GGA-induced apoptosis (Fig. 7A, left). Quantitative analysis using Hoechst staining showed 7.0 ± 0.8% apoptosis by GGA alone and 28.2 ± 3.8% apoptosis in GGA plus AEBSF ($p < 0.05$) (Fig. 7A, right). This result indicated that the ATF6 pathway triggered by GGA participated mainly in the prevention of apoptosis.

Previous reports indicated that both GRP78 and CHOP may be induced via the ATF6 pathway (Lee, 2001; Ma et al., 2002). We therefore examined effects of AEBSF on the induction of these genes by GGA. Northern blot analysis revealed that expression of GRP78 by GGA was markedly inhibited by AEBSF (Fig. 7B, top row). It is noteworthy that induction of CHOP was also attenuated by the treatment with AEBSF (Fig. 7B, middle row), indicating that activation of this pathway was responsible for the GGA-triggered induction of not only GRP78 but also CHOP.

Up-regulated GRP78 promotes protein folding in the ER, and it protect cells from ER stress-induced apoptosis (Lee, 2001). To examine whether the induction of GRP78 was indeed responsible for the antiapoptotic action of the ATF6 pathway, SM43 cells were stably transfected with a gene encoding GRP78, and SM/GRP78 cells were established (Fig. 7C). SM/Neo and SM/GRP78 cells were then treated with GGA, and microscopic analyses were performed. Phase-contrast microscopy showed that GGA-induced apoptosis was attenuated by overexpression of GRP78. The percentages of apoptotic cells evaluated by Hoechst staining were 69.8 ± 3.5 in SM/Neo versus 42.4 ± 3.9 in SM/GRP78 ($p < 0.05$) (Fig. 7D).

The ATF6 pathway triggered not only GRP78 expression but also expression of CHOP. To investigate involvement of CHOP in the coordination of cellular fate by GGA, SM43 cells were transiently cotransfected with empty vector or a plasmid encoding a dominant-negative mutant of CHOP (CHOP-DN) together with an enhanced green fluorescent protein gene. After the transfection, cells were stimulated with GGA, and then they were subjected to fluorescence microscopy to evaluate the percentages of fluorescence-positive round cells. As shown in Fig. 7E, dominant-negative inhibition of CHOP significantly attenuated GGA-induced apoptosis. The percentages of apoptotic cells were 41.0 ± 0.7 in 500 μM-treated, mock-transfected cells versus 21.5 ± 2.9 in 500 μM-treated, CHOP-DN-transfected cells; and 56.8 ± 4.1 in 1 mM-treated, mock-transfected cells versus 36.8 ± 3.4 in 1 mM-treated, CHOP-DN-transfected cells ($p < 0.05$). These results suggested that GGA triggered both the ATF6-GRP78 antiapoptotic pathway and the ATF6-CHOP proapoptotic pathway and that the potential of the former predominated over the potential of the latter.

Discussion

GGA has been used as a selective inducer of HSP70 in biomedical research. However, there is no solid evidence that GGA does not affect other HSP family molecules. In the present report, we disclosed that, in rat mesangial cells, GGA markedly up-regulated GRP78, a member of a different subclass of the HSP70 family, without affecting the level of HSP70. This effect was not specific to the particular cell type, but it was observed generally in various cell types originated from different species. Together with the fact that, in gastric mucosal cells, GGA induced not only HSP70 but also HSP60 and HSP90 (Hirakawa et al., 1996), our results raised some objection to the current notion that GGA is a "selective" inducer of HSP70. Indeed, some previous reports indicated that GGA may protect cells from cytotoxic stimuli independently of HSP70. For example, Aron et al. (2001) reported that GGA protected monocytes from cigarette smoke-triggered cellular damage, whereas it did not significantly affect either basal or cigarette smoke-induced HSP70 expression. Under some situations, therefore, cytoprotective action of

Fig. 6. Role of the IRE1 pathway in GGA-induced apoptosis. A and B, SM43 cells were stably transfected with a dominant-negative mutant of IRE1β, and SM/IRE1βDN cells were established. Northern blot analysis of the transgene is shown in A. SM/Neo and SM/IRE1βDN cells were treated with or without 1 mM GGA for 9 h, and microscopic analyses were performed (B, left). Quantitative analysis of apoptosis using Hoechst staining is shown (B, right). Data are expressed as means ± S.E., and an asterisk indicates a statistically significant difference ($p < 0.05$). Assays were performed in quadruplicate. C, SM43 cells were pretreated with 5 to 10 μM SP600125 for 30 min, stimulated with 500 μM GGA for 15 h, and then subjected to microscopic analysis. D and E, SM43 cells were stably transfected with a dominant-negative mutant of XBP1, and SM/XBP1DN cells were established. Northern blot analysis of the transgene is shown (D). SM/Neo and SM/XBP1DN cells were treated with or without GGA, and then they were subjected to phase-contrast microscopy (E, left). Quantitative analysis of apoptosis is shown (E, right). F, SM43 cells were exposed to 1 mM GGA for indicated times, and then they were subjected to Northern blot analysis of GRP78 and CHOP. G, SM/Neo, SM/IRE1βDN, and SM/XBP1DN cells were treated with or without 1 mM GGA for 4 h, and then expression of GRP78 and CHOP was evaluated.

GGA may be due to induction of other HSP family members, including GRP78.

In the majority of previous reports, induction of HSP70 was evaluated at the protein level. There is little evidence showing that GGA can induce HSP70 at the transcriptional level. Only few reports provided evidence for up-regulation of HSP70 mRNA by GGA (Hirakawa et al., 1996). The lack of HSP70 RNA induction by GGA in various cells, therefore, raises a possibility that, in many cell types, GGA may not cause transcriptional induction of HSP70 and that the increase in the level of HSP70 protein observed in previous reports could be caused through other mechanisms (e.g., via reduction in protein degradation).

GRP78 is the most popular endogenous indicator of ER stress. Indeed, we provided the following evidence showing that GGA induced ER stress. First, GGA suppressed activity of ES-TRAP, the exogenous marker of ER stress we reported recently (Hiramatsu et al., 2006). Second, GGA triggered UPR, including activation of the IRE1-XBP1 pathway and the ATF6 pathway. Third, GGA induced not only GRP78 but also another endogenous indicator of ER stress, CHOP. However, the mechanism by which GGA induced ER stress and UPR is unclear. One possibility is inhibition of protein isoprenylation by GGA. Protein isoprenylation such as geranylgeranylation and farnesylation is a post-translational modification essential for membrane localization and activity of various proteins, including GTP-binding proteins (Casey, 1992). A previous study showed that GGA inhibited isoprenylation of small GTP-binding proteins, Rap1 and Ras, in human leukemia cells (Okada et al., 1999). Disturbed protein isoprenylation caused by GGA could generate unfolded/misfolded proteins and thereby trigger ER stress.

ER stress causes apoptotic cell death through several mechanisms (Kim et al., 2006). One mechanism is via caspase-12, which is activated selectively by ER stress. A previous report showed that activation of caspase-12 is linked to the IRE1 pathway, because IRE1 recruits tumor necrosis factor receptor-associated factor (TRAF) 2 that interacts with caspase-12, resulting in formation of the IRE1/TRAF2/caspase-12 complex and subsequent activation of

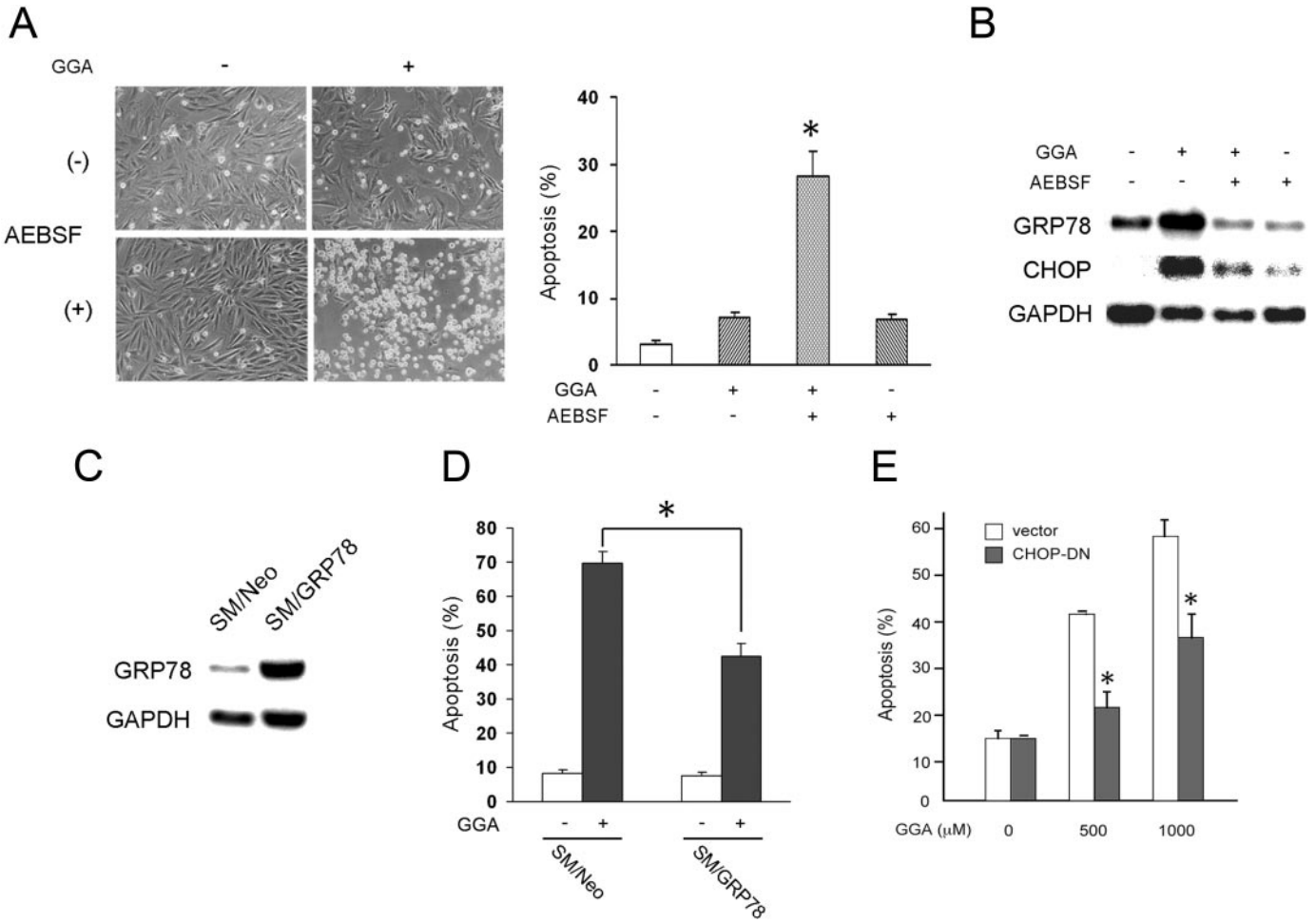


Fig. 7. Role of the activating transcription factor 6 pathway in GGA-induced apoptosis. A, SM43 cells were pretreated with 500 μM AEBSF for 0.5 h, and then they were treated with 500 μM GGA for 6 h in the presence or absence of AEBSF. After the treatment, cells were subjected to phase-contrast microscopy (left) and Hoechst staining to evaluate apoptosis quantitatively (right). B, SM43 cells were treated with GGA for 6 h in the presence (+) or absence (−) of 500 μM AEBSF, and expression of GRP78 and CHOP was examined by Northern blot analysis. C, SM43 cells were stably transfected with a gene encoding GRP78, and SM/GRP78 cells were established. D, SM/Neo cells and SM/GRP78 cells were treated for 9 h with or without GGA and subjected to Hoechst staining to evaluate apoptosis. E, SM43 cells were transiently cotransfected with empty vector or a plasmid encoding CHOP-DN together with an enhanced green fluorescent protein gene. After the transfection, cells were stimulated with 500 μM to 1 mM GGA for 9 h, and then they were subjected to fluorescence microscopy to evaluate the percentages of fluorescence-positive round cells versus total fluorescence-positive cells. Assays were performed in quadruplicate. Data are expressed as means ± S.E., and asterisks indicate statistically significant differences ($p < 0.05$).

caspase-12 (Yoneda et al., 2001). In the present report, we showed that IRE1 was activated by GGA, which was evidenced by cleavage of XBP1 mRNA by IRE1. Dominant-negative inhibition of IRE1 as well as inhibition of caspase-12 significantly attenuated GGA-induced apoptosis, suggesting involvement of the IRE1-caspase-12 pathway in the apoptotic process. However, unexpectedly, we also found that dominant-negative inhibition of XBP1 attenuated GGA-induced apoptosis. XBP1 is a transcription factor activated by UPR and induces various molecular chaperones in the ER (Lee et al., 2003b). Thus, XBP1 has been considered an antiapoptotic molecule. For example, XBP1 is essential for survival and growth of hepatocytes during development (Reimold et al., 2000). In contrast to this current concept, our results disclosed the proapoptotic aspect of XBP1. The proapoptotic potential of XBP1 is consistent with our recent report showing that XBP1 plays a crucial role in the induction of apoptosis in LLC-PK1 cells exposed to cadmium (Yokouchi et al., 2007). Proapoptotic targets downstream of XBP1 have not been identified, but we showed that neither GRP78, CHOP, nor JNK was the possible candidate.

Another important proapoptotic mechanism mediated by IRE1 is via the ASK1-JNK pathway. Activation of JNK occurs after recruitment of TRAF2 by IRE1 in response to ER stress. Subsequently, ASK1 is activated, leading to phosphorylation of JNK and consequent apoptosis (Kim et al., 2006). ASK1-deficient cells are resistant to ER stress-induced JNK activation and apoptosis (Kadowaki et al., 2005), and Bcl-2, which is phosphorylated and inactivated by ASK1 and JNK, might be a downstream target responsible for induction of apoptosis (Kim et al., 2006). In the present study, however, we did not observe activation of JNK in GGA-exposed SM43 cells (our unpublished observation). Furthermore, selective inhibition of JNK by SP600125 did not attenuate GGA-induced apoptosis. These results suggested lack of involvement of the ASK1-JNK pathway in mediating IRE1-transduced proapoptotic signaling in GGA-exposed cells.

CHOP is the first molecule identified to be induced by UPR and to mediate ER stress-induced apoptosis. Overexpression of CHOP induces apoptosis, and CHOP-deficient cells are resistant to ER stress-initiated cell death (Zinszner et al., 1998). In the present report, we showed that CHOP was induced by GGA. However, this induction was not mediated by the IRE1-XBP1 pathway, the major UPR triggered by GGA, because dominant-negative inhibition of XBP1 did not affect induction of CHOP by GGA. It is noteworthy that induction of CHOP as well as GRP78 by GGA was attenuated by inhibition of the ATF6 pathway, the antiapoptotic pathway triggered by GGA. Thus, GGA triggered both the ATF6-GRP78 antiapoptotic pathway and the ATF6-CHOP proapoptotic pathway, and the potential of the former predominated over the potential of the latter.

GRP78 is composed of the ATPase domain, the peptide-binding domain, and a C-terminal domain with unknown function (King et al., 2001). GRP78 binds to the unfolded proteins through the peptide-binding domain and uses the energy from hydrolyzing ATP to promote proper folding and to prevent aggregation (Kleizen and Braakman, 2004). GRP78 also possesses the capacity to bind Ca^{2+}, which supports maintenance of calcium homeostasis in the ER (Lee, 2001). Furthermore, GRP78 serves as a master modulator for the UPR network by binding to the ER stress sensors, including PERK, IRE1, and ATF6 and consequently inhibiting their activation (Bertolotti et al., 2000). Several previous investigations showed the antiapoptotic property of GRP78 (Lee, 2001). Consistent with those previous findings, we also showed that GRP78 counterbalanced the proapoptotic processes triggered by GGA. We further showed that the induction of GRP78 by GGA was largely dependent on the ATF6 pathway, but not by the IRE1-XBP1 pathway. Taken together, these results suggested that GGA has the potential for regulating apoptosis via particular UPR branches independently of JNK and HSP70.

GGA has the ability to protect various cells from apoptosis triggered by a wide range of stimuli, including ethanol, reactive oxygen species, proteasome inhibitors, and nonsteroidal anti-inflammatory drugs and ischemia (Hirakawa et al., 1996; Ikeyama et al., 2001; Kikuchi et al., 2002; Nishida et al., 2006). It is worthwhile to note that all of these agents are potential inducers of ER stress. Although the cytoprotective effects of GGA have been ascribed to its ability to induce HSP70, GRP78 and other ER chaperones induced by GGA could, at least in part, contribute to its cytoprotective action. In addition to the antiapoptotic effect, some previous reports showed that GGA has the potential to induce apoptosis in malignant cells (Okada et al., 1999). Our present data suggest a possibility that the proapoptotic effect of GGA is ascribed to induction of ER stress.

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