

# Genetic Evidence for a Protective Role of Heat Shock Factor 1 against Irritant-Induced Gastric Lesions

Ken-ichiro Tanaka, Shinji Tsutsumi, Yasuhiro Arai, Tatsuya Hoshino, Keitarou Suzuki, Eiichi Takaki, Takaaki Ito, Koji Takeuchi, Akira Nakai, and Tohru Mizushima

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan (K.-I.T., S.T., Y.A., T.H., K.S., T.I., T.M.); Department of Biochemistry and Molecular Biology, Yamaguchi University School of Medicine, Ube, Japan (E.T., A.N.); and Department of Pharmacology and Experimental Therapeutics, Kyoto Pharmaceutical University, Kyoto, Japan (K.T.)

Received December 7, 2006; accepted December 22, 2006

## ABSTRACT

Gastric lesions result from an imbalance between aggressive and defensive factors. Indirect lines of evidence suggest that heat shock proteins (HSPs) induced by various aggressive factors provide a major protective mechanism. In this study, we compared gastric ulcerogenic response in wild-type mice and in those lacking heat shock factor 1 (HSF1), a transcription factor for *hsp* genes. The severity of gastric lesions induced by ethanol or hydrochloric acid was worsened in HSF1-null mice. Immunoblotting, real-time reverse transcription-polymerase chain reaction, immunohistochemical analysis, and terminal deoxynucleotidyl transferase dUTP nick-end labeling assay revealed that the ethanol administration up-regulated gastric mucosal HSPs, in particular HSP70, in an HSF1-dependent manner, and more apoptotic cells were observed in the gastric mucosa of HSF1-null mice than in wild-type mice. In contrast,

other parameters governing the gastric ulcerogenic response, including gastric acid secretion, gastric mucosal blood flow, and prostaglandin E<sub>2</sub> levels, were not significantly affected by the absence of the *hsf1* gene. Geranylgeranylacetone (GGA), a clinically used antiulcer drug with HSP-inducing activity, suppressed ethanol-induced gastric lesions in wild-type mice but not in heat shock factor 1 (HSF1)-null mice. The results suggest that the aggravation of irritant-induced gastric lesions in HSF1-null mice is due to their inability to up-regulate HSPs, leading to apoptosis. It is also suggested that the HSP-inducing activity of GGA contributes to the drug's antiulcer activity. This study provides direct genetic evidence that HSPs, after their HSF1-dependent up-regulation, confer gastric protection against the irritant-induced lesions.

The balance between aggressive and defensive factors determines development of gastric lesions, with either a relative increase in aggressive insults or a relative decrease in protective factors, resulting in lesions. The gastric mucosa is challenged by a variety of both endogenous and exogenous irritants (aggressive factors), including ethanol, gastric acid,

pepsin, reactive oxygen species, nonsteroidal anti-inflammatory drugs (NSAIDs) and *Helicobacter pylori*. These irritants damage the mucosal cells, inducing cell death, which leads to the formation of gastric lesions (Holzer, 1998). To protect the gastric mucosa, a complex defense system, which includes the production of surface mucus and bicarbonate and the regulation of gastric mucosal blood flow (GMBF), has evolved. Prostaglandins (PGs), in particular PGE<sub>2</sub>, enhance these protective mechanisms and are therefore believed to comprise a major gastric mucosal defensive factor (Miller, 1983).

Heat shock proteins (HSPs) have also attracted considerable attention as another major defensive factor. When cells are exposed to stressors, a number of so-called stress proteins are induced to confer protection against such stressors. HSPs

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan and by Japan Science and Technology Agency, the Daiwa Securities Health Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Suzuken Memorial Foundation, and the Japan Research Foundation for Clinical Pharmacology.

K.T. and S.T. contributed equally to this work.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
doi:10.1124/mol.106.033282.

**ABBREVIATIONS:** NSAID, nonsteroidal anti-inflammatory drug; DAPI, 4', 6-diamidino-2-phenylindole dihydrochloride; GGA, geranylgeranylacetone; GMBF, gastric mucosal blood flow; HE, hematoxylin and eosin; HSF, heat shock factor; HSP, heat shock protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OCT, optimal cutting temperature; PG, prostaglandin; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling; HSF1, heat shock factor 1; ELISA, enzyme-linked immunosorbent assay; siRNA, short interfering RNA; AGS, human gastric carcinoma; iNOS, inducible nitric-oxide synthase; RT-PCR, reverse transcription-polymerase chain reaction.

are representative of these stress proteins, and their cellular up-regulation, especially that of HSP70, provides resistance as they refold or degrade denatured proteins produced by the stressors (Gething and Sambrook, 1992; Jaattela et al., 1998; Kiang and Tsokos, 1998; Mathew and Morimoto, 1998; Jaattela, 1999; Beere et al., 2000; Saleh et al., 2000; Ravagnan et al., 2001). It has been reported not only that various gastric irritants, including ethanol, up-regulate HSPs, but also that artificial up-regulation of HSPs confers resistance to these irritants in cultured gastric mucosal cells (Nakamura et al., 1991; Hirakawa et al., 1996; Mizushima et al., 1999; Saika et al., 2000; Tomisato et al., 2000, 2001). Similar up-regulation of HSPs by gastric irritants has also been recorded in vivo, in addition to which whole-body heat treatment has been shown to suppress gastric irritant-induced lesions (Zeniya et al., 1995; Otani et al., 1997; Itoh and Noguchi, 2000; Saika et al., 2000). Although these findings strongly indicate that HSPs are protective, very little direct evidence exists, and to date, no in vivo study has been conducted to demonstrate that inhibition of HSPs results in a phenotype susceptible to irritant-induced gastric lesions.

It is interesting that geranylgeranylacetone (GGA), a leading antiulcer drug on the Japanese market, has been reported to be a nontoxic HSP-inducer, up-regulating various HSPs not only in cultured gastric mucosal cells at concentrations that do not affect cell viability but also in various tissues, including the gastric mucosa in vivo (Hirakawa et al., 1996; Ooie et al., 2001; Katsuno et al., 2005; Yasuda et al., 2005). We have reported previously that preinduction of HSPs by GGA protects cultured gastric mucosal cells from cell death induced by various irritants, including ethanol, hydrochloric acid, hydrogen peroxide, and NSAIDs (Mizushima et al., 1999; Tomisato et al., 2000, 2001; Takano et al., 2002). These previous results suggest that the antiulcer effect of GGA is due to its HSP-inducing activity. However, because GGA mediates various other gastroprotective mechanisms, such as an increase in GMBF, stimulation of surface mucus production, and direct protection of gastric mucosal cell membranes (Terano et al., 1986; Kunisaki and Sugiyama, 1992; Ushijima et al., 2005), it remains unclear whether up-regulation of HSPs represents GGA's major mode of antiulcer activity.

The up-regulation of HSPs by various stressors, including heat shock, is regulated at the transcription level by a consensus *cis*-element (heat shock element) and a transcription factor (HSF1) that specifically binds to heat shock element located on the upstream region of *hsp* genes (Morimoto, 1998). The essential role of HSF1 in the up-regulation of HSPs, conferring cytoprotection against stressors, was demonstrated by the observation that disruption of the activity of HSF1 leads to the loss of stressor-induced HSP up-regulation and the emergence of cells that are sensitive to apoptosis (McMillan et al., 1998; Morimoto, 1998). Furthermore, analysis of HSF1-null mice revealed that up-regulation of HSPs is involved in various physiological and pathological phenomena, suggesting that HSF1-null mice provide a powerful tool for examination of the role of HSPs in vivo (Xiao et al., 1999; Christians et al., 2000; Yan et al., 2002). In this study, we used the HSF1-null mouse model to obtain direct genetic evidence for the contribution of HSPs to the protection of the gastric mucosa. We also investigated whether up-regulation of HSPs by GGA contributes to its antiulcer activity.

## Materials and Methods

**Chemicals and Animals.** Paraformaldehyde, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), and PGE<sub>2</sub> were obtained from Sigma (St. Louis, MO). PGE<sub>2</sub> ELISA kit was from Cayman Chemical (Ann Arbor, MI). Antibodies against HSP25, HSP60, HSP70, HSP90, or actin were purchased from StressGen (San Diego, CA) or Santa Cruz Biotechnology (Santa Cruz, CA). Optimal cutting temperature (OCT) compound was from Sakura FineTek (Zoeterwoude, the Netherlands). Mayer's hematoxylin, 1% eosin alcohol solution, and malinol were from MUTO Pure Chemicals (Tokyo, Japan). Terminal deoxynucleotidyl transferase was obtained from TOYOBO (Osaka, Japan). Biotin 14-ATP, Alexa Fluor 488 (or 594) goat antirabbit immunoglobulin G, and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen (Carlsbad, CA). VECTASHIELD was from Vector Laboratories (Burlingame, CA). 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) was from Dojindo (Kumamoto, Japan). The RNeasy kit was obtained from QIAGEN (Valencia, CA), the first-strand cDNA synthesis kit was from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and SYBR GREEN PCR Master Mix was from ABI (Foster City, CA). HSF1-null and wild-type mice (ICR) were prepared as described previously (Inouye et al., 2004), and both mice of 10 to 12 weeks of age and 25 to 30 g were used in experiments. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, MD) and were approved by the Animal Care Committee of Kumamoto University.

**Gastric Damage Assay.** Gastric ulcerogenic response was examined as described previously (Tomisato et al., 2004), with some modifications. Mice, which had been fasted for 24 h, were orally administered either ethanol or hydrochloric acid (5 ml/kg). Four hours later, the animals were sacrificed with an overdose of ether, after which their stomachs were removed and scored for hemorrhagic damage by an observer unaware of the treatment they had received. Calculation of the scores involved measuring the area of all lesions in square millimeters and summing the values to give an overall gastric lesion index. Gastric mucosal PGE<sub>2</sub> levels were determined by ELISA as described previously (Futaki et al., 1993).

**Cell Culture, Overexpression, and siRNA Targeting of HSP70.** Human gastric carcinoma (AGS) cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO<sub>2</sub> at 37°C. Cells were exposed to ethanol by changing the medium. Cells were cultured for 24 h and were used in experiments. Cell viability was determined by the MTT method. The transfection with pcDNA3.1 containing the *hsp70* gene (Fujimoto et al., 2005) was carried out using Lipofectamine (TM2000) according to the manufacturer's instructions. Cells were used for experiments after an 18-h recovery period.

We used siRNA of 5'-ggagcuggagcaggugudTdT-3' and 5'-acacccugcuccgucddTdT-3' as annealed oligonucleotides for repressing HSP70 expression. AGS cells were transfected with siRNA using RNAiFect transfection reagent according to the manufacturer's instructions. Nonsilencing siRNA (5'-uucuccgaacgugucacgudTdT-3' and 5'-acugacacguucggagaadTdT-3') was used as a negative control.

**Real-Time RT-PCR Analysis.** Total RNA was extracted from the gastric mucosa using an RNeasy kit according to the manufacturer's protocols. Samples (10 µg of RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA (15 ng) was applied to real-time RT-PCR (Prism 7700; Applied Biosystems, Foster City, CA) using SYBR GREEN PCR Master Mix and analyzed with ABI Prism 7700 Sequence Detection software according to the manufacturer's instructions. Real-time cycle conditions were 2 min at 50°C followed by 10 min at 90°C and finally 45 cycles at

95°C for 30 s and 63°C for 60 s. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, the gene of glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard.

Primers were designed using the Primer3 website ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). Primers are listed in Table 1.

**Immunoblotting Analysis.** Total protein was extracted from the gastric mucosa as described previously (Tsutsumi et al., 2002). The protein concentration of the samples was determined by the Bradford method. Samples were applied to 8% (HSP70 and HSP90), 10% (HSP60 and actin), or 12% (HSP25) polyacrylamide SDS gels and subjected to electrophoresis, after which the proteins were immunoblotted with appropriate antibodies.

**Histological and Immunohistochemical Analysis.** Gastric tissue samples were fixed in 4% buffered paraformaldehyde, embedded in OCT compound, and cryosectioned. Sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution for histological examination [hematoxylin and eosin (HE) staining]. Samples were mounted with malinol and inspected using an Olympus IX70 microscope (Olympus, Tokyo, Japan).

For immunohistochemical analysis, sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with antibody against HSP70 (1:200 dilution) in the presence of 2.5% BSA, and finally incubated for 1 h with Alexa Fluor 488 (or 594) goat antimouse immunoglobulin G in the presence of DAPI (5 µg/ml). Samples were mounted with Vectashield and inspected using fluorescence microscopy (Olympus IX70).

**TdT-Mediated dUTP-Biotin End Labeling Assay.** Gastric tissue samples were fixed in 4% buffered paraformaldehyde, embedded in OCT compound, and cryosectioned. Sections were incubated first with proteinase K (10 µg/ml) for 15 min at 37°C, then with terminal deoxynucleotidyl transferase and biotin 14-ATP for 1 h at 37°C, and finally with Alexa Fluor 488 conjugated with streptavidin for 1 h. Samples were mounted with Vectashield and inspected using fluorescence microscopy (Olympus IX70).

**Measurement of Gastric Acid Secretion and GMBF.** Gastric acid secretion was measured as described previously (Filaretova et al., 2002), with some modifications. Under ether anesthesia, the abdomen was opened, and the pylorus was ligated. After drug administration, mice were sacrificed, and their stomachs were removed. The gastric contents were collected and titrated with 10 mM NaOH to pH 7.0 using an automatic titrator (TITRONIC basis; Schott, Roseville, CA).

GMBF was measured as described elsewhere (Takeuchi et al., 2003), with some modifications. Under urethane-anesthetized conditions, the stomach was exposed and mounted in an ex vivo chamber.

TABLE 1  
Primers

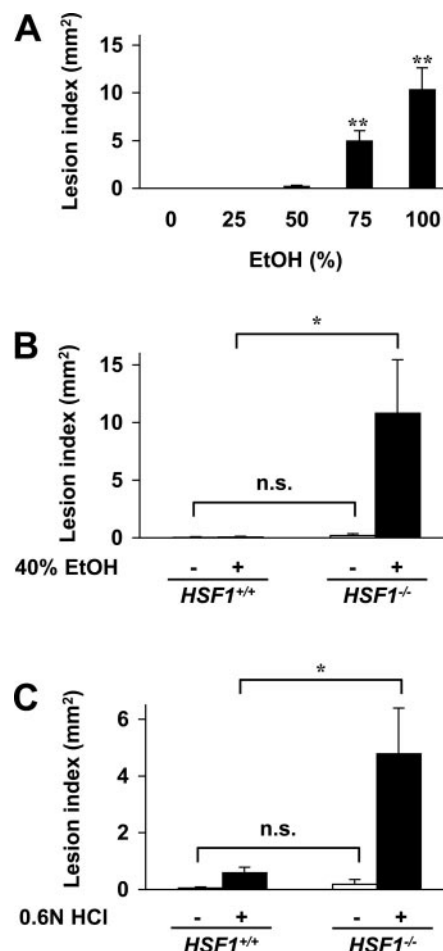
Gene Name	Sequence
<i>hsp25</i>	
Forward	5'-cctcttccctatccctgag-3'
Reverse	5'-ttggctccagactgttcaga-3'
<i>hsp60</i>	
Forward	5'-cggttgccaataacacaaacg-3'
Reverse	5'-cttcagggtgtgtcacaggt-3'
<i>hsp70</i>	
Forward	5'-tggtgctgacgaagatgaag-3'
Reverse	5'-aggtcgaagatgagcacgtt-3'
<i>hsp90α</i>	
Forward	5'-aaaggcagaggctgacaaga-3'
Reverse	5'-aggggaggcatttcttcagt-3'
<i>hsp90β</i>	
Forward	5'-gcggcaagacaagaaaaag-3'
Reverse	5'-gaagtgttctccagctcat-3'

GMBF was measured with a laser Doppler flowmeter (ALF-21; Advance, Tokyo, Japan).

**Statistical Analysis.** All values are expressed as the mean ± standard error (S.E.M.). Two-way analysis of variance followed by Scheffé's multiple comparison test or Tukey test was used for evaluation of differences between groups. The Student's *t* test for unpaired results was used for the evaluation of differences between two groups. Differences were considered to be significant for values of  $P < 0.05$ .

## Results

**Enhanced Gastric Ulcerogenic Response.** The development of gastric lesions after oral administration of ethanol was compared between wild-type and HSF1-null mice. Exposure to ethanol produced gastric lesions in a dose-dependent manner (Fig. 1A). As shown in Fig. 1B, intragastric administration of 40% ethanol resulted in significant gastric lesions in HSF1-null mice but not in wild-type mice. Lack of the *hsf1* gene did not significantly affect the background level (without ethanol administration) of production of gastric lesions (Fig. 1B). These results show that HSF1 plays an important role in protecting the gastric mucosa from ethanol-induced lesions. We also examined the prevalence of hydrochloric acid-induced gastric lesions in HSF1-null mice, revealing



**Fig. 1.** Production of gastric lesions in wild-type and HSF1-null mice. Wild-type (A–C) and HSF1-null mice (B–C) were orally administered the indicated doses of ethanol (A and B) or hydrochloric acid (C). After 4 h, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. ( $n = 4–6$ ). \*\*,  $P < 0.01$ .



that oral administration of 0.6 N hydrochloric acid produces more severe lesions in HSF1-null mice than in wild-type mice (Fig. 1C). Therefore, the protective effects of HSF1 do not seem to be mediated in response to a specific stressor, such as ethanol.

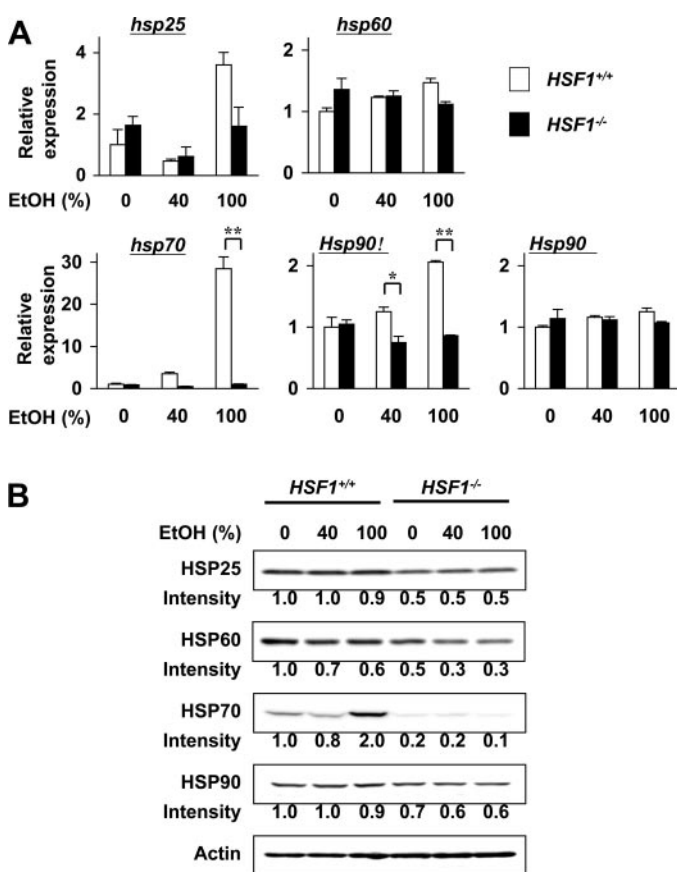
**Ethanol-Induced Up-Regulation of HSPs in Gastric Mucosa.** Given that HSF1 up-regulates the expression of HSPs at the transcriptional level, we examined the effect of ethanol administration on the expression of *hsp* mRNAs and HSPs in the gastric mucosa of HSF1-null mice and wild-type mice. Figure 2A shows the level of various *hsp* mRNAs that were detected by real-time RT-PCR. Lack of the *hsf1* gene did not affect the background level of expression of *hsp* mRNAs, as reported previously (McMillan et al., 1998; Xiao et al., 1999; Inouye et al., 2003). Ethanol administration up-regulated the level of *hsp25*, *hsp70*, and *hsp90α* but not *hsp60* and *hsp90β* mRNA in wild-type mice (Fig. 2A). This up-regulation was not observed in HSF1-null mice (Fig. 2A). Among *hsp25*, *hsp70*, and *hsp90α* mRNAs, the *hsp70* mRNA dis-

played the strongest HSF1-dependent up-regulation induced by ethanol (Fig. 2A).

Figure 2B shows the protein level of various HSPs as assessed by the immunoblotting assay. Unlike the results of the mRNA analysis (Fig. 2A), lack of the *hsf1* gene caused a decrease in the background expression level of HSP25, HSP60, HSP70, and HSP90 (Fig. 2B). This is the first examination of the background expression level of HSPs in stomach of HSF1-null mice, and results were consistent with previous data in other organs and cell species of HSF1-null mice, such as liver, fibroblasts, and dendritic cells (Xiao et al., 1999; Zheng and Li, 2004). Ethanol administration up-regulated the production of only HSP70, a response that was dependent on the function of HSF1 (Fig. 2B). Being different from results in mRNA level (Fig. 2A), the protein level of HSP25 and HSP90α was not up-regulated by the ethanol administration (Fig. 2B) and we have no clear explanation for this discrepancy at present. Based on the results illustrated in Fig. 2, together with those of a previous study suggesting that, among the HSPs, HSP70 plays a major role in cytoprotection (Gething and Sambrook, 1992; Jaattela et al., 1998; Mathew and Morimoto, 1998; Jaattela, 1999; Beere et al., 2000; Saleh et al., 2000; Ravagnan et al., 2001), we subsequently focused on HSP70.

To examine the ethanol-dependent up-regulation of HSP70 in the gastric mucosa in detail, we performed histological and immunohistochemical analyses. Sections were prepared from the gastric tissues of HSF1-null and wild-type mice that had been exposed to ethanol. HE and DAPI staining shows the presence of gastric mucosal lesions in both HSF1-null mice (with both 40 and 100% ethanol administration) and wild-type mice (with only 100% ethanol administration) (Fig. 3), and this is consistent with the results illustrated in Fig. 1B. Furthermore, immunohistochemical analysis with an antibody against HSP70 demonstrated that HSP70 is induced by the administration of 100% ethanol in wild-type mice and that this up-regulation is most apparent in the vicinity of gastric lesions (Fig. 3A), suggesting that HSP70 induced by ethanol plays an important role in development of gastric lesions. In contrast, no significant up-regulation of HSP70 was observed in HSF1-null mice after administration of either 40% or 100% ethanol (Fig. 3B). This pattern of HSP70 expression is consistent with the results illustrated in Fig. 2B. Together, the results outlined in Figs. 2 and 3 show that the induction HSP70 in the gastric mucosa after oral administration of ethanol is dependent on HSF1 function.

**Mechanism for Stimulated Production of Gastric Lesions in HSF1-Null Mice.** To investigate the mechanism governing the severity of production of ethanol-stimulated gastric lesions in HSF1-null mice, we compared various factors that are known to be important for the production of gastric lesions (including the level of apoptosis, gastric acid secretion, GMBF, and the level of PGE<sub>2</sub>) between HSF1-null and wild-type mice. Figure 4 illustrates the level of gastric mucosal apoptosis as determined by TdT-mediated dUTP-biotin end labeling (TUNEL) assay. In wild-type mice, an increase in TUNEL-positive (apoptotic) cells was observed after the administration of 100% but not 40% ethanol, whereas a clear increase in TUNEL-positive cells was observed with 40% ethanol administration in the HSF1-null mice (Fig. 4). Similar level of TUNEL-positive cells was observed after the administration of 100% ethanol in the HSF1-



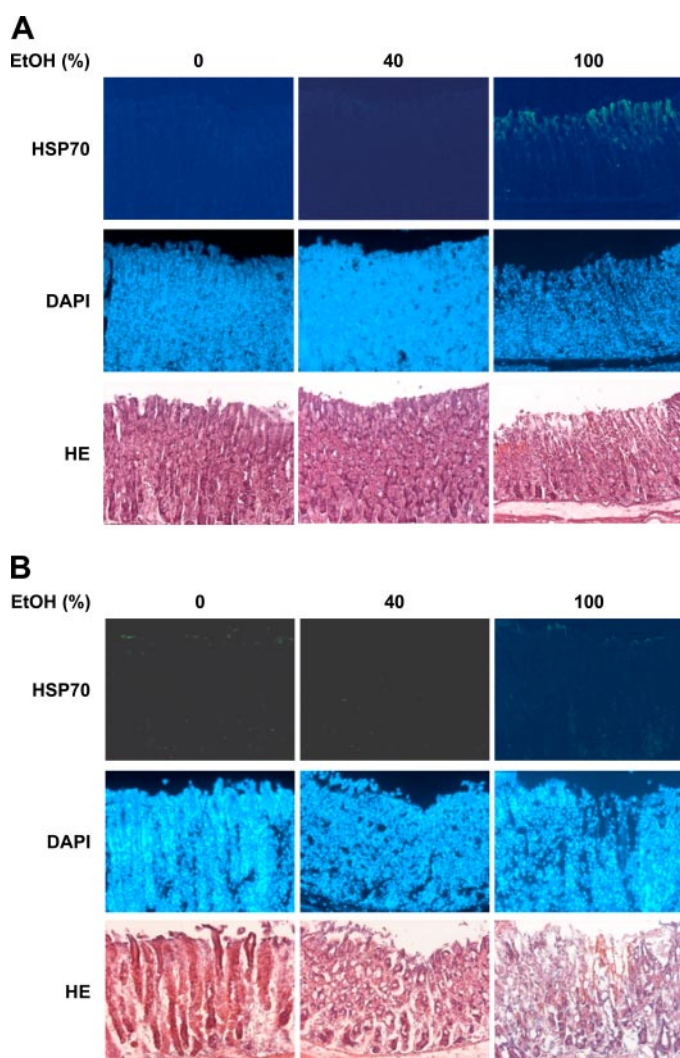
**Fig. 2.** HSF1-dependent up-regulation of gastric mucosal HSPs induced by ethanol. Wild-type and HSF1-null mice were orally administered the indicated doses of ethanol (A and B). A, after 4 h, the gastric mucosa was removed, and total RNA was extracted. Samples were subjected to real-time RT-PCR using a specific primer for each gene. Values normalized to the control sample (i.e., wild-type mice not exposed to ethanol) are given as the mean  $\pm$  S.E.M. ( $n = 3$ ). \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ . B, after 4 h, the gastric mucosa was removed, and protein extracts were prepared and analyzed by immunoblotting with an antibody against HSP25, HSP60, HSP70, HSP90, or actin. The band intensity of each HSP was determined by densitometric scanning, normalized with its respective actin intensity, and the value of the ratio of band intensity between each HSP and the actin is shown under the band.

null mice (data not shown). Lack of the *hsf1* gene did not affect the background level of TUNEL-positive cells (Fig. 4). These results show that induction of apoptosis by ethanol is enhanced in HSF1-null mice compared with wild-type mice; in other words, HSF1 protects gastric mucosal cells from ethanol-induced apoptosis.

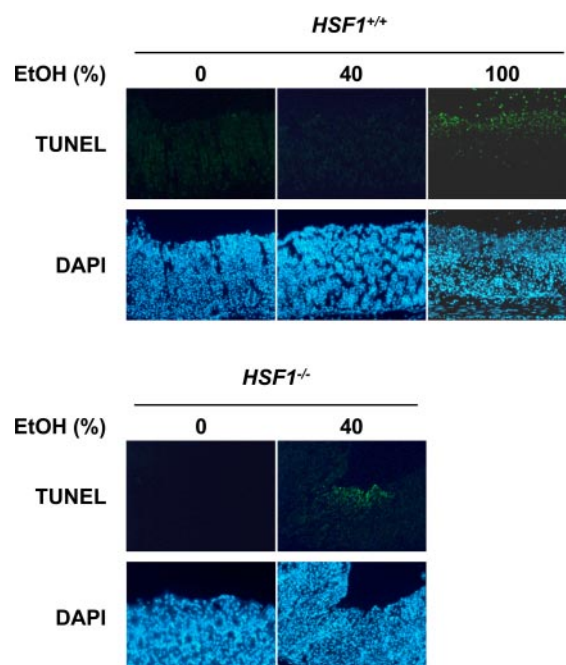
We also examined the role of HSP70 in ethanol-induced cell death in vitro using over-expression plasmid and siRNA for HSP70. Transfection of AGS cells with the plasmid containing *hsp70* gene caused overexpression of HSP70 in both the absence and presence of 7% ethanol (Fig. 5A). This transfection made cells resistant to cell death induced by 7% ethanol (Fig. 5B). On the other hand, transfection of siRNA for the *hsp70* decreased the expression of HSP70 in both the absence and presence of 7% ethanol (Fig. 5C) and made cells sensitive to cell death induced by 7% ethanol (Fig. 5D). These results suggest that HSP70 protects gastric cells against ethanol-induced cell death.

Gastric acid secretion is also an important factor affecting the production of lesions, representing another potential aggressive insult on the gastric mucosa. We therefore examined

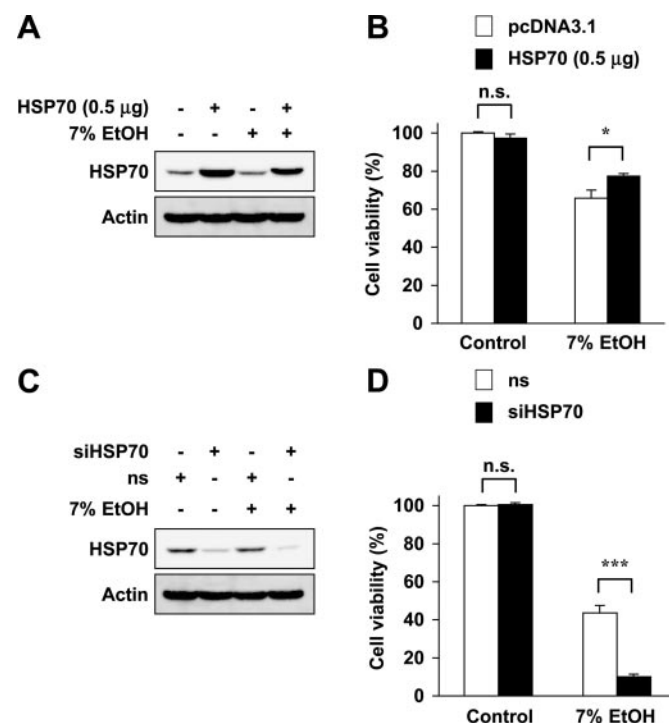
the effect of ethanol on gastric acid secretion in wild-type and HSF1-null mice. As shown in Fig. 6A, gastric acid secretion was increased by the addition of histamine, as described



**Fig. 3.** Ethanol-induced HSF1-dependent up-regulation of HSP70 in gastric mucosa. Wild-type (A) and HSF1-null mice (B) were orally administered the indicated doses of ethanol. After 4 h, sections of gastric tissues were prepared and subjected to histological examination (HE) and immunohistochemical analysis with an antibody against HSP70.



**Fig. 4.** Induction of apoptosis by ethanol in gastric mucosa. Wild-type and HSF1-null mice were orally administered the indicated doses of ethanol. After 4 h, sections of gastric tissues were prepared and subjected to TUNEL assay.



**Fig. 5.** Effect of increase or decrease in the expression of HSP70 on ethanol-induced cell death. AGS cells were transfected with plasmid with the *hsp70* gene (A and B) or siRNA for the *hsp70* gene (siHSP70) or nonsilencing siRNA (ns) (C and D). After 24 h, cells were incubated with or without 7% ethanol for 1 h. The levels of HSP70 and actin were estimated by immunoblotting with an antibody against HSP70 or actin (A and C). Cell viability was determined by MTT method. Values shown are mean  $\pm$  S.D. ( $n = 3$ ). \*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$ . n.s., not significant.



previously (Furutani et al., 2003). Administration of 40% ethanol did not affect gastric acid secretion in either wild-type or HSF1-null mice (Fig. 6B). Similar results were obtained with 100% ethanol (data not shown). Furthermore, both the background level of gastric acid secretion and that recorded after administration of 40% ethanol were not significantly affected by the lack of the *hsf1* gene (Fig. 6B). These results suggest that the stimulation of ethanol-induced gastric lesion production in HSF1-null mice does not involve a change in gastric acid secretion.

GMBF is another important factor in the production of gastric lesions, with a decrease in GMBF having a causative effect. As shown in Fig. 6C, GMBF in wild-type mice was stimulated by the addition of PGE<sub>2</sub>, as described previously (Araki et al., 2000). However, no significant difference in GMBF was recorded between wild-type and HSF1-null mice (Fig. 6C), indicating that HSF1 does not affect GMBF.

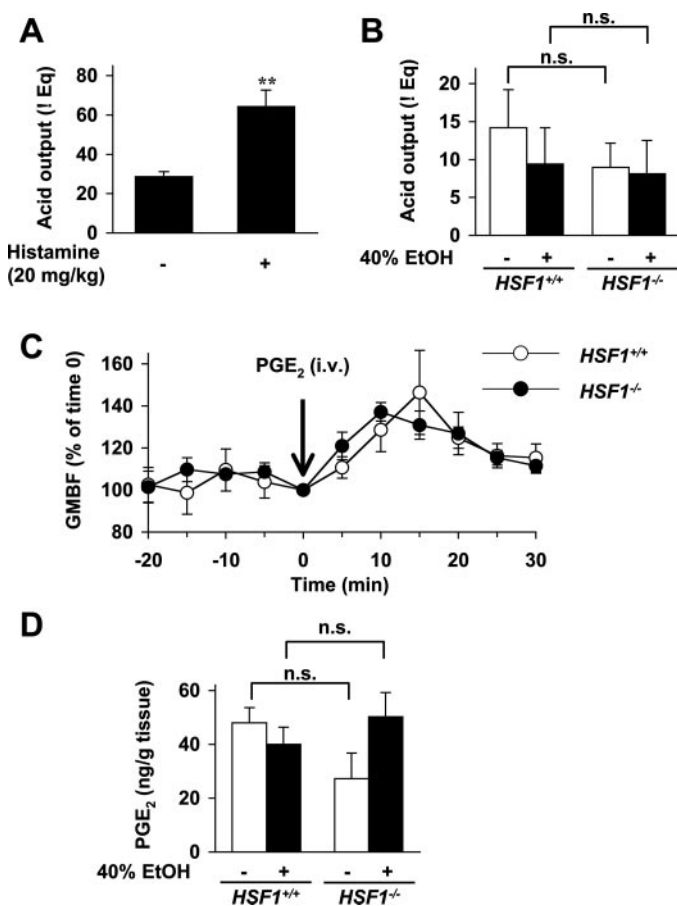
As described above, PGE<sub>2</sub> is a major defensive factor for the gastric mucosa, exerting a protective effect against various irritants by decreasing gastric acid secretion and increas-

ing GMBF, in addition to other mechanisms (Miller, 1983). Therefore, as the results illustrated in Fig. 6, A to C, suggest, it is unlikely that aggravation of ethanol-induced gastric lesions in HSF1-null mice involves PGE<sub>2</sub>. To confirm this, we examined the effect of ethanol on gastric mucosal PGE<sub>2</sub> levels in wild-type and HSF1-null mice. As shown in Fig. 6D, ethanol administration did not affect the level of PGE<sub>2</sub> in either group of animals. Furthermore, there was no significant difference in PGE<sub>2</sub> levels between wild-type and HSF1-null mice in either the presence or absence of ethanol treatment (Fig. 6D). These results support the idea that stimulation of ethanol-induced gastric lesions in HSF1-null is not attributable to the impairment of PGE<sub>2</sub> production.

**Antilucer and HSP-Inducing Activities of GGA in HSF1-Null Mice.** To evaluate the contribution of the HSP-inducing activity of GGA to its antiulcer activity, we investigated the effect of GGA in HSF1-null mice. First, we examined the effect of GGA and/or ethanol on gastric mucosal HSP70 expression in wild-type mice, revealing a potent expression induced by ethanol and a lower level of expression in response to GGA (Fig. 7A). It is interesting that preadministration of GGA enhanced the ethanol-dependent HSP70 response (Fig. 7A). Figure 7C shows the effect of preadministration of GGA on ethanol-produced gastric lesions in wild-type and HSF1-null mice. To obtain similar levels of gastric lesions, 100 and 40% ethanol administration were administered to wild-type and HSF1-null mice, respectively. In fact, 40% ethanol administration in HSF1-null mice caused a comparable lesion score as 100% ethanol administration in wild-type mice (Fig. 7C). Preadministration of GGA significantly suppressed the ethanol-dependent production of gastric lesions in wild-type mice (Fig. 7C), as described previously (Murakami et al., 1981). In contrast, no significant effect was recorded in the HSF1-null mice (Fig. 7C). We confirmed that administration of GGA and/or 40% ethanol did not induce HSP70 (Fig. 7B). This result shows that HSF1 is required for the efficacy of the antiulcer activity of GGA against ethanol. Overall, the results in Fig. 7 suggest that the loss of the protective effect of GGA in HSF1-null mice is due to the lack of expression of HSPs (such as HSP70); in other words, the HSP-inducing activity of GGA contributes to its antiulcer activity.

## Discussion

A number of previous observations have suggested that HSPs and their up-regulation by gastric irritants play an important role in protecting the gastric mucosa against lesion development. Artificial up-regulation of HSPs, especially HSP70, by GGA (a clinically used antiulcer drug) or other methods in cultured gastric mucosal cells confers protection from irritant-induced cell death (Nakamura et al., 1991; Hirakawa et al., 1996; Mizushima et al., 1999; Tomisato et al., 2000, 2001; Takano et al., 2002), whereas exposure to such irritants induces HSP production (Zeniya et al., 1995; Otani et al., 1997; Itoh and Noguchi, 2000; Saika et al., 2000). In this study, we found that HSF1-null mice are more susceptible to irritant-induced gastric lesions, providing direct genetic evidence for the significance of HSPs in ameliorating the outcome of irritant-induced gastric insults. Further genetic evidence in support of this notion has recently been published, revealing that transgenic mice overexpress-

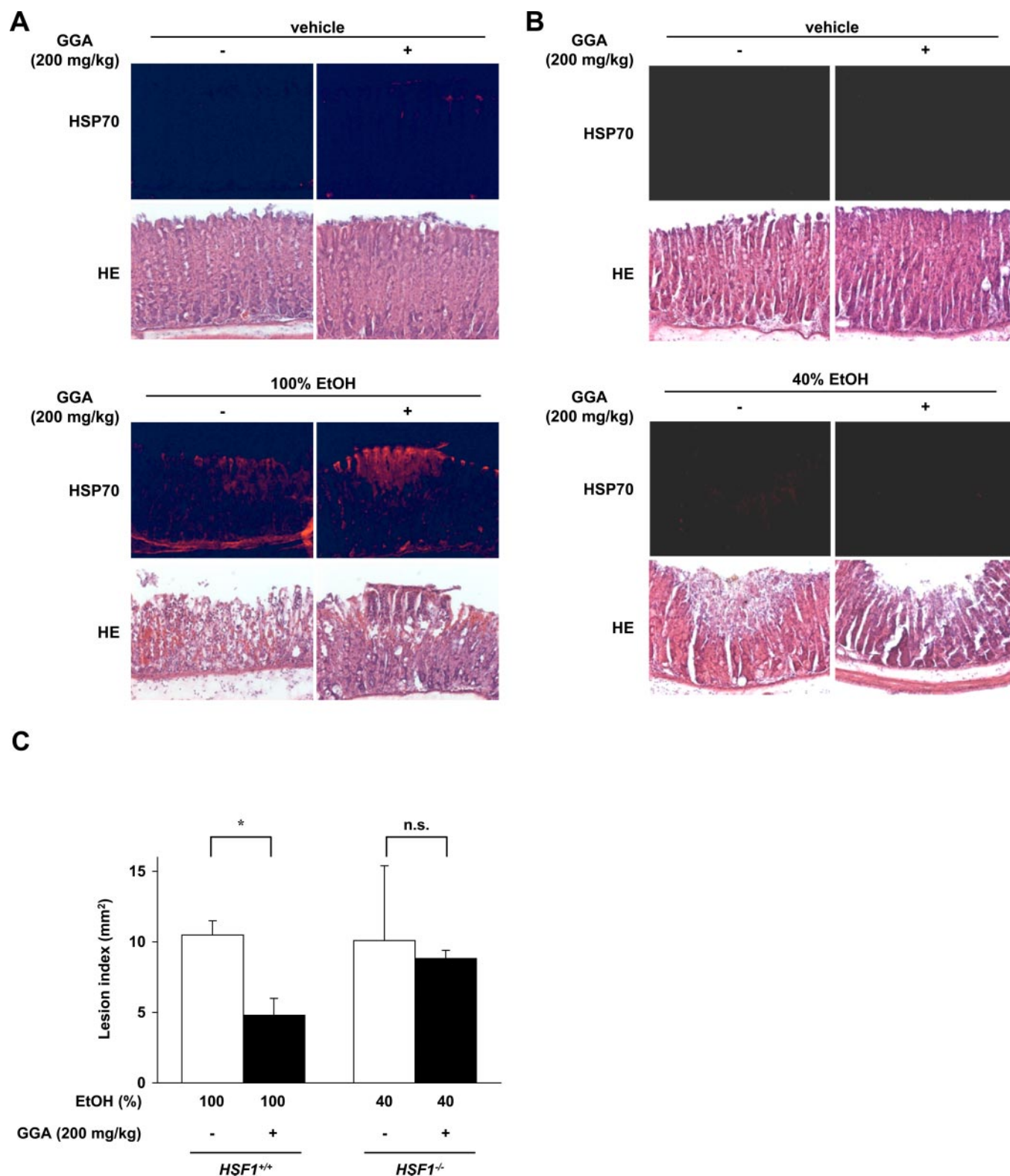


**Fig. 6.** Gastric acid secretion, GMBF, and PGE<sub>2</sub> levels in HSF1-null mice. A, the pylorus-ligated wild-type mice were administered 20 mg/kg histamine s.c.. B, wild-type and HSF1-null mice were orally administered 40% ethanol, and 2 h later, the pylorus was ligated. A further 2 h after the pylorus ligation, the acidity of the gastric contents was measured as described under *Materials and Methods* (A and B) ( $n = 3-4$ ). After exposure of the stomach and GMBF stabilization, PGE<sub>2</sub> (0.03 mg/kg) was administered intravenously via the tail vein of wild-type and HSF1-null mice, and changes in GMBF were monitored (C) ( $n = 6$ ). Wild-type and HSF1-null mice were orally administered 40% ethanol. After 4 h, the gastric mucosal PGE<sub>2</sub> level was determined by ELISA (D) ( $n = 3-4$ ). n.s., not significant.

ing human HSP27 display a phenotype that is resistant to NSAID-induced gastric lesions (Ebert et al., 2005).

Based on our results, we believe that the production of

gastric lesions in HSF1-null mice is due to their inability to express protective HSPs, leading to apoptosis of the gastric mucosal cells. Although oral administration of ethanol led to



**Fig. 7.** Effect of ethanol and/or GGA on expression of HSP70 and production of gastric lesions. Wild-type (A and C) and HSF1-null (B and C) mice were orally preadministered 200 mg/kg GGA (10 ml/kg as emulsion with 5% gum arabic) 1 h after which they were orally administered with the indicated doses of ethanol. A and B, after 4 h, sections of gastric tissues were prepared and subjected to histological examination (HE) and immunohistochemical analysis with an antibody against HSP70. C, after 4 h, the stomach was removed and scored for hemorrhagic damage. Values are mean  $\pm$  S.E.M. ( $n = 3-6$ ). \*,  $P < 0.05$ . n.s., not significant.

the production of gastric lesions, there was a concomitant up-regulation of HSPs (Figs. 2 and 3), with significantly fewer apoptotic cells being recorded in wild-type mice than in HSF1-null mice (Fig. 4). Induction of necrosis by ethanol may also be stimulated in HSF1-null mice, because up-regulation of HSPs made gastric mucosal cells resistant to ethanol-induced necrosis (Tomisato et al., 2001). Other factors that are believed to be involved in the production of gastric lesions, including gastric acid secretion, GMBF, and PGE<sub>2</sub> levels, were similar in both wild-type and HSF1-null mice (Fig. 6). Artificial preinduction of HSPs renders cultured gastric mucosal cells resistant to ethanol-induced apoptosis (Mizushima et al., 1999). Among the various HSPs tested, oral administration of ethanol up-regulated only HSP70 in terms of protein level (Fig. 2B). Furthermore, HSP70 is believed to be the major antiapoptotic HSP; either HSP70 binds to Apaf-1, thereby preventing activation of caspases, or HSP70 suppresses the apoptotic pathway downstream of caspase-3 activation and apoptosis-inducing factor-induced chromatin condensation (Jaattela et al., 1998; Beere et al., 2000; Saleh et al., 2000; Ravagnan et al., 2001). However, despite the apparent significance of HSP70, it should still be noted that loss of the *hsf1* gene also decreased the background level of other HSPs (Fig. 2B), which may play some role in the HSF1-dependent protection of the gastric mucosa. It is also possible that the production of gastric lesions in HSF1-null mice involves other mechanisms, suggested in recent articles (Inouye et al., 2003; Fujimoto et al., 2005). For example, HSF1-null mice display elevated levels of tumor necrosis factor  $\alpha$  (TNF)- $\alpha$ , a proinflammatory cytokine, and are susceptible to increased mortality after endotoxic or inflammatory challenge (Xiao et al., 1999; Wirth et al., 2004). Given that it is well-known that proinflammatory cytokines, including TNF- $\alpha$ , stimulate the production of gastric lesions, it remains possible that the development of such lesions in HSF1-null mice involves elevated levels of TNF- $\alpha$ . In addition, involvement of iNOS is also possible, because it has been shown that iNOS is involved in tissue damage, and overexpression of HSP70 has been shown to inhibit iNOS and ameliorate the damage (Pittet et al., 2002; Kiang, 2004).

GGA has attracted considerable attention as an HSP-inducer, largely because of its clinical value as an antiulcer drug and because it can induce HSPs without affecting cell viability (Hirakawa et al., 1996). GGA has been suggested to play a protective role through HSP induction in a variety of disease states; oral administration of GGA up-regulates HSPs in brain and heart and exerts a protective effect against spinal and bulbar muscular atrophy, cerebral ischemia, and ischemic heart disease (Ooie et al., 2001; Katsuno et al., 2005; Yasuda et al., 2005). However, no previous reports have shown that the HSP-inducing activity of GGA contributes to these clinically beneficial outcomes, including its antiulcer effects. In this study, using immunohistochemical analysis, we have demonstrated that oral administration of GGA alone up-regulates gastric mucosal HSP70 and that preadministration of GGA stimulates the ethanol-induced up-regulation of HSP70. Furthermore, we have revealed that preadministration of GGA suppresses gastric lesions in wild-type mice but not in HSF1-null mice. These results argue strongly in favor of the HSP-inducing activity of GGA contributing to its antiulcer effects, providing the first direct genetic link between the pharmacological behavior of the

drug and the resultant clinical outcome. In summary, this study provides direct genetic evidence suggesting that HSPs, after their HSF1-dependent up-regulation, confer protection against the development of gastric lesions.

## References

- Araki H, Ukawa H, Sugawa Y, Yagi K, Suzuki K, and Takeuchi K (2000) The roles of prostaglandin E receptor subtypes in the cytoprotective action of prostaglandin E<sub>2</sub> in rat stomach. *Aliment Pharmacol Ther* **14** (Suppl 1):116–124.
- Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, Tailor P, Morimoto RI, Cohen GM, and Green DR (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat Cell Biol* **2**:469–475.
- Christians E, Davis AA, Thomas SD, and Benjamin LJ (2000) Maternal effect of Hsf1 on reproductive success. *Nature (Lond)* **407**:693–694.
- Ebert MP, Schafer C, Chen J, Hoffmann J, Gu P, Kubisch C, Carl-McGrath S, Treiber K, Malfertheiner P, and Rocken C (2005) Protective role of heat shock protein 27 in gastric mucosal injury. *J Pathol* **207**:177–184.
- Filaretova L, Tanaka A, Miyazawa T, Kato S, and Takeuchi K (2002) Mechanisms by which endogenous glucocorticoid protects against indomethacin-induced gastric injury in rats. *Am J Physiol* **283**:G1082–G1089.
- Fujimoto M, Takaki E, Hayashi T, Kitaura Y, Tanaka Y, Inouye S, and Nakai A (2005) Active HSF1 significantly suppresses polyglutamine aggregate formation in cellular and mouse models. *J Biol Chem* **280**:34908–34916.
- Furutani K, Aihara T, Nakamura E, Tanaka S, Ichikawa A, Ohtsu H, and Okabe S (2003) Crucial role of histamine for regulation of gastric acid secretion ascertained by histidine decarboxylase-knockout mice. *J Pharmacol Exp Ther* **307**:331–338.
- Futaki N, Arai I, Hamasaka Y, Takahashi S, Higuchi S, and Otomo S (1993) Selective inhibition of NS-398 on prostanoind production in inflamed tissue in rat carrageenan-air-pouch inflammation. *J Pharm Pharmacol* **45**:753–755.
- Gething MJ and Sambrook J (1992) Protein folding in the cell. *Nature (Lond)* **355**:33–45.
- Hirakawa T, Rokutan K, Nikawa T, and Kishi K (1996) Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* **111**:345–357.
- Holzer P (1998) Neural emergency system in the stomach. *Gastroenterology* **114**:823–839.
- Inouye S, Izu H, Takaki E, Suzuki H, Shirai M, Yokota Y, Ichikawa H, Fujimoto M, and Nakai A (2004) Impaired IgG production in mice deficient for heat shock transcription factor 1. *J Biol Chem* **279**:38701–38709.
- Inouye S, Katsuki K, Izu H, Fujimoto M, Sugahara K, Yamada S, Shinkai Y, Oka Y, Katoh Y, and Nakai A (2003) Activation of heat shock genes is not necessary for protection by heat shock transcription factor 1 against cell death due to a single exposure to high temperatures. *Mol Cell Biol* **23**:5882–5895.
- Itoh YH and Noguchi R (2000) Pre-treatment with mild whole-body heating prevents gastric ulcer induced by restraint and water-immersion stress in rats. *Int J Hyperthermia* **16**:183–191.
- Jaattela M (1999) Escaping cell death: survival proteins in cancer. *Exp Cell Res* **248**:30–43.
- Jaattela M, Wissing D, Kokholm K, Kallunki T, and Egeblad M (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *EMBO (Eur Mol Biol Organ) J* **17**:6124–6134.
- Katsuno M, Sang C, Adachi H, Minamiyama M, Waza M, Tanaka F, Doyu M, and Sobue G (2005) Pharmacological induction of heat-shock proteins alleviates polyglutamine-mediated motor neuron disease. *Proc Natl Acad Sci USA* **102**:16801–16806.
- Kiang JG (2004) Inducible heat shock protein 70 kD and inducible nitric oxide synthase in hemorrhage/resuscitation-induced injury. *Cell Res* **14**:450–459.
- Kiang JG and Tsokos GC (1998) Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol Ther* **80**:183–201.
- Kunisaki C and Sugiyama M (1992) Effect of teprenone on acute gastric mucosal lesions induced by cold-restraint stress. *Digestion* **53**:45–53.
- Mathew A and Morimoto RI (1998) Role of the heat-shock response in the life and death of proteins. *Ann NY Acad Sci* **851**:99–111.
- McMillan DR, Xiao X, Shao L, Graves K, and Benjamin LJ (1998) Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *J Biol Chem* **273**:7523–7528.
- Miller TA (1983) Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. *Am J Physiol* **245**:G601–G623.
- Mizushima T, Tsutsumi S, Rokutan K, and Tsuchiya T (1999) Suppression of ethanol-induced apoptotic DNA fragmentation by geranylgeranylacetone in cultured guinea pig gastric mucosal cells. *Dig Dis Sci* **44**:510–514.
- Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev* **12**:3788–3796.
- Murakami M, Oketani K, Fujisaki H, Wakabayashi T, and Ohgo T (1981) Antiulcer effect of geranylgeranylacetone, a new acyclic polyisoprenoid on experimentally induced gastric and duodenal ulcers in rats. *Arzneimittelforschung* **31**:799–804.
- Nakamura K, Rokutan K, Marui N, Aoike A, and Kawai K (1991) Induction of heat shock proteins and their implication in protection against ethanol-induced damage in cultured guinea pig gastric mucosal cells. *Gastroenterology* **101**:161–166.
- Ooie T, Takahashi N, Saikawa T, Nawata T, Arikawa M, Yamanaka K, Hara M, Shimada T, and Sakata T (2001) Single oral dose of geranylgeranylacetone induces heat-shock protein 72 and renders protection against ischemia/reperfusion injury in rat heart. *Circulation* **104**:1837–1843.
- Otani S, Otaka M, Jin M, Okuyama A, Itoh S, Iwabuchi A, Sasahara H, Itoh H, Tashima Y, and Masamune O (1997) Effect of preinduction of heat shock proteins on acetic acid-induced colitis in rats. *Dig Dis Sci* **42**:833–846.



- Pittet JF, Lu LN, Geiser T, Lee H, Matthay MA, and Welch WJ (2002) Stress preconditioning attenuates oxidative injury to the alveolar epithelium of the lung following haemorrhage in rats. *J Physiol* **538**:583–597.
- Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C, et al. (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* **3**:839–843.
- Saika M, Ueyama T, and Senba E (2000) Expression of immediate early genes, HSP70, and COX-2 mRNAs in rat stomach following ethanol ingestion. *Dig Dis Sci* **45**:2455–2462.
- Saleh A, Srinivasula SM, Balkir L, Robbins PD, and Alnemri ES (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat Cell Biol* **2**:476–483.
- Takano T, Tsutsumi S, Tomisato W, Hoshino T, Tsuchiya T, and Mizushima T (2002) Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced apoptosis. *Dig Dis Sci* **47**:1546–1553.
- Takeuchi K, Kato S, Takeeda M, Ogawa Y, Nakashima M, and Matsumoto M (2003) Facilitation by endogenous prostaglandins of capsaicin-induced gastric protection in rodents through EP2 and IP receptors. *J Pharmacol Exp Ther* **304**:1055–1062.
- Terano A, Hiraishi H, Ota S, and Sugimoto T (1986) Geranylgeranylacetone, a novel anti-ulcer drug, stimulates mucus synthesis and secretion in rat gastric cultured cells. *Digestion* **33**:206–210.
- Tomisato W, Takahashi N, Komoto C, Rokutan K, Tsuchiya T, and Mizushima T (2000) Geranylgeranylacetone protects cultured guinea pig gastric mucosal cells from indomethacin. *Dig Dis Sci* **45**:1674–1679.
- Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, Mio M, Tsuchiya T, and Mizushima T (2004) Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. *Biochem Pharmacol* **67**:575–585.
- Tomisato W, Tsutsumi S, Tsuchiya T, and Mizushima T (2001) Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced necrosis by induction of heat-shock proteins. *Biol Pharm Bull* **24**:887–891.
- Tsutsumi S, Tomisato W, Takano T, Rokutan K, Tsuchiya T, and Mizushima T (2002) Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. *Biochim Biophys Acta* **1589**:168–180.
- Ushijima H, Tanaka K, Takeda M, Katsu T, Mima S, and Mizushima T (2005) Geranylgeranylacetone protects membranes against nonsteroidal anti-inflammatory drugs. *Mol Pharmacol* **68**:1156–1161.
- Wirth D, Bureau F, Melotte D, Christians E, and Gustin P (2004) Evidence for a role of heat shock factor 1 in inhibition of NF-kappaB pathway during heat shock response-mediated lung protection. *Am J Physiol* **287**:L953–L961.
- Xiao X, Zuo X, Davis AA, McMillan DR, Curry BB, Richardson JA, and Benjamin IJ (1999) HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *EMBO (Eur Mol Biol Organ) J* **18**:5943–5952.
- Yan LJ, Christians ES, Liu L, Xiao X, Sohal RS, and Benjamin IJ (2002) Mouse heat shock transcription factor 1 deficiency alters cardiac redox homeostasis and increases mitochondrial oxidative damage. *EMBO (Eur Mol Biol Organ) J* **21**:5164–5172.
- Yasuda H, Shichinohe H, Kuroda S, Ishikawa T, and Iwasaki Y (2005) Neuroprotective effect of a heat shock protein inducer, geranylgeranylacetone in permanent focal cerebral ischemia. *Brain Res* **1032**:176–182.
- Zeniya A, Otaka M, Itoh H, Kuwabara T, Fujimori S, Otani S, Tashima Y, and Masamune O (1995) Induction and intracellular localization of a 72-kDa heat shock protein in rat gastric mucosa after water-immersion stress. *J Gastroenterol* **30**:572–577.
- Zheng H and Li Z (2004) Cutting edge: cross-presentation of cell-associated antigens to MHC class I molecule is regulated by a major transcription factor for heat shock proteins. *J Immunol* **173**:5929–5933.

**Address correspondence to:** Dr. Tohru Mizushima, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: mizu@gpo.kumamoto-u.ac.jp