for accumulation of Ca^2 in the ER) may also be involved in this process (Johnson et al., 2002). The mechanism how increase in the intracellular Ca^2 level induces ER stress response is unclear at present. One possibility mechanism Ca^2 -dependent protease is involved in this process. NO known to induce ER stress response. It was recently su

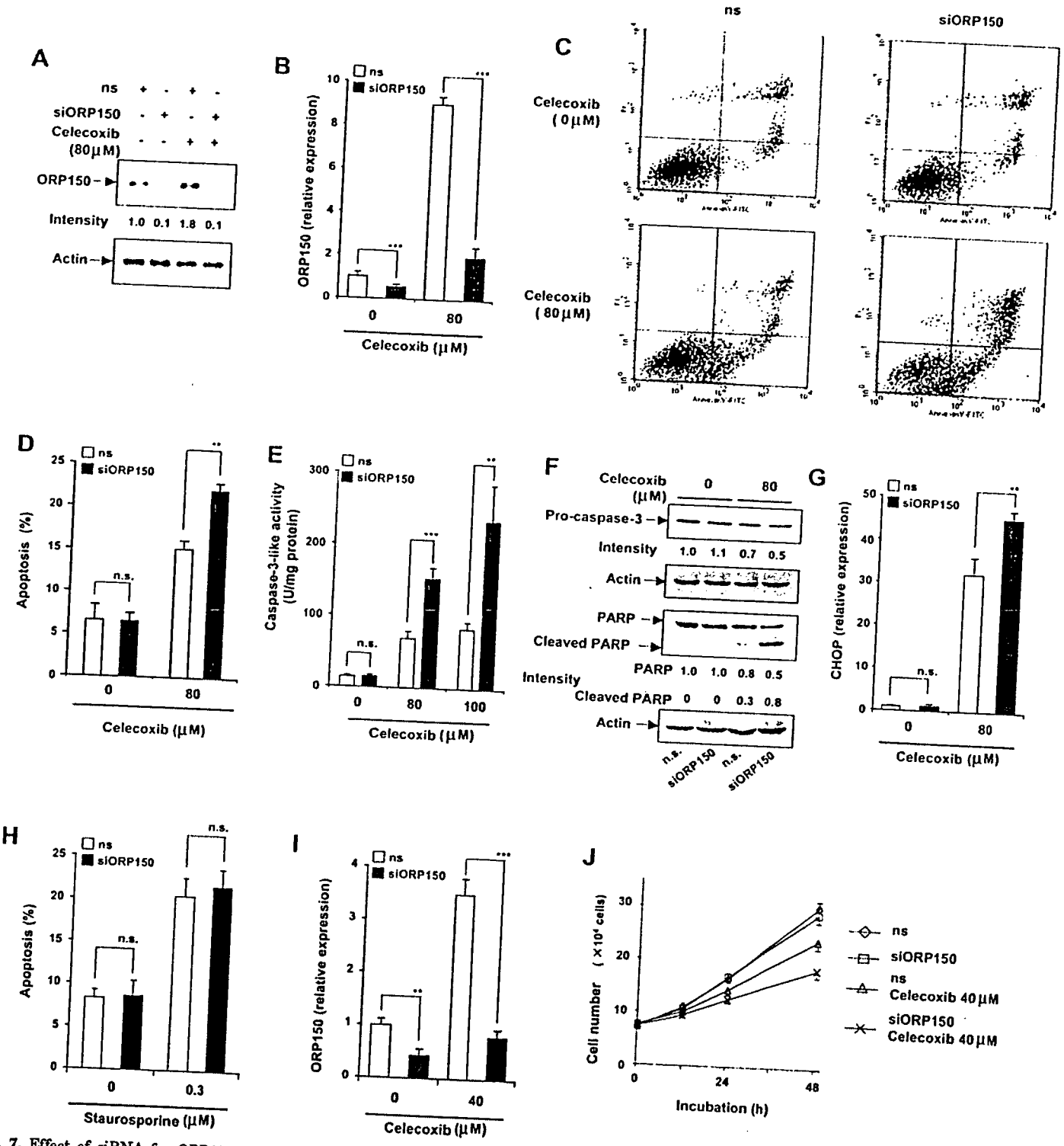

Fig. 7. Effect of *si*RNA for ORP150 on celecoxib-induced apoptosis. AGS cells were transfected with 5 g of siRNA for ORP150 (siORP150) or nonsilencing *si*RNA (ns) (A–J). After 48 h (A–H), cells were incubated with or without indicated concentrations of celecoxib (A–G) or staurosporine (H) for 6 h (A–F and H) or 3 h (G). The levels of ORP150 protein (A), ORP150 mRNA (B), and CHOP mRNA (G) were estimated by immunoblotting or real-time RT-PCR experiments as described in the legends of Figs. 1 and 2. Apoptosis was monitored as described in the legend of Fig. 6. After 24 h (I and J), cells were incubated with or without 40 M celecoxib for indicated periods and cell numbers were determined by direct cell counting (J). The level of ORP150 mRNA after 24-h incubation was estimated by RT-PCR experiments (I). Values shown are mean S.D. (*n* 3). , *P* 0.001; , *P* 0.01.

gested that this ER stress response is mediated by increase in the intracellular Ca^2 level and Ca^2 -dependent activation of site-1 protease involved in cleavage of p90-ATF6 into p50-ATF6 (Xu et al., 2004). This pathway may be involved in celecoxib-dependent activation of ATF6 and resulting induction of ER stress response. Another possibility is involvement of reactive oxygen species (ROS). It is known that ROS inhibited sarcoplasmic/endoplasmic reticulum Ca^2 ATPase (Downey, 1990; Suzuki and Ford, 1991), as is the case of thapsigargin, an inducer of ER stress response. It was recently reported that a Ca^2 ionophore A23187 enhances production of ROS (Przygodzki et al., 2005). Therefore, the increase in the intracellular Ca^2 level may induce ER stress response through stimulation of ROS production.

Although it was previously shown that the expression of ORP150 in cells renders them resistant to apoptosis induced by hypoxia, glutamate, and -amino-3-hydroxy-5-methylisoxazole-propionate (Ozawa et al., 1999; Kitao et al., 2001, 2004; Tamatani et al., 2001; Asahi et al., 2002), this is the first demonstration that the expression of ORP150 protects cancer cells from apoptosis induced by chemotherapy and chemoprevention drugs. Stimulation of ORP150 expression inhibited celecoxib-induced apoptosis, whereas its inhibition had the opposite effect. In contrast, staurosporine, a chemotherapy drug that lacks ORP150-inducing activity, had no effect, suggesting that the up-regulation of ORP150 induced by celecoxib decreases its potential as a chemotherapy and chemoprevention drug through inhibition of apoptosis. We also suggested that the up-regulation of ORP150 induced by celecoxib suppresses celecoxib-dependent growth inhibition of tumor cells, which may also decreases its potential as a chemotherapy and chemoprevention drug. Although siRNA for ORP150 almost completely inhibited the expression of ORP150, this siRNA caused a modest increase in apoptosis (Fig. 7). This may be due to that not only ORP150 but also GRP78 is involved in inhibition of celecoxib-induced apoptosis (Tsutsumi et al., 2006). Given that the expression of ORP150 has been shown to suppress glutamate-dependent increases in intracellular Ca^2 levels in cultured neurons (Kitao et al., 2001) and that the Ca^2 -dependent up-regulation of CHOP is involved in NSAID-induced apoptosis (Tsutsumi et al., 2004; Tanaka et al., 2005), the antiapoptotic effect of ORP150 may be mediated through changes in intracellular Ca^2 levels.

Solid tumors usually exist under conditions of glucose starvation and hypoxia, which causes induction of the ER stress response. Moreover, ORP150 was reported to be up-regulated in various clinically isolated tumors and cancer cell lines (Miyagi et al., 2002; Tsukamoto et al., 1998). Furthermore, stronger expression of ORP150 in bladder cancer was reported to reflect a more advanced stage of the disease (Asahi et al., 2002), with the suppression of ORP150 expression by antisense RNA causing inhibition of tumor formation in vivo (Miyagi et al., 2002). Results in this study suggest that not only celecoxib-induced ORP150 but also constitutively overproduced ORP150 in tumors may render them resistant to chemotherapy regimes involving celecoxib or other chemotherapy drugs with ER stress response-inducing activity. We consider that the antiapoptotic activity of ORP150 may partially explain the close relationship between ORP150 expression and tumor progression. Another mechanism that may underpin this relationship seems to be mediated by vascular endothelial growth factor (VEGF), which is representative of the angiogenic factors. Several previous studies have shown that VEGF is deeply involved in tumor progression (Ferrara et al., 1996; Machein et al., 1999). Overexpression of ORP150 stimulates secretion of VEGF and therefore seems to play an important role in tumor-mediated angiogenesis (Ozawa et al., 2001). Based on the evidence outlined above, we propose that an inhibitor of ORP150 (and chemicals that inhibit both ORP150 and GRP78 may be more beneficial) may offer considerable clinical benefit as a chemotherapy and chemoprevention drug.

References

Asahi H, Koshida K, Hori O, Ogawa S, and Namiki M (2002) Immunohistochemical detection of the 150-kDa oxygen-regulated protein in bladder cancer. *BJU Int* 90:462–466.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.

Downey JM (1990) Free radicals and their involvement during long-term myocardial ischemia and reperfusion. *Annu Rev Physiol* 52:487–504.

Drummond IA, Lee AS, Resendez E Jr, and Steinhardt RA (1987) Depletion of intracellular calcium stores by calcium ionophore A23187 induces the genes for glucose-regulated proteins in hamster fibroblasts. *J Biol Chem* 262:12801–12805.

Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, and DuBois RN (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 107:1183–1188.

Elder DJ, Halton DE, Hague A, and Paraskeva C (1997) Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin Cancer Res* 3:1679–1683.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, and Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature (Lond)* 380:439–442.

Gupta RA and Dubois RN (2001) Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer* 1:11–21.

Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI, and Rigas B (1996) Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol* 52:237–245.

Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, and Mizushima T (2003) Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. *J Biol Chem* 278:12752–12758.

Johnson AJ, Hsu AL, Lin HP, Song X, and Chen CS (2002) The cyclo-oxygenase-2 inhibitor celecoxib perturbs intracellular calcium by inhibiting endoplasmic reticulum Ca2 -ATPases: a plausible link with its anti-tumour effect and cardiovascular risks. *Biochem J* 366:831–837.

Kaneda S, Yura T, and Yanagi H (2000) Production of three distinct mRNAs of 150 kDa oxygen-regulated protein (ORP150) by alternative promoters: preferential induction of one species under stress conditions. *J Biochem (Tokyo)* 128:529–538.

Kaufman RJ (2002) Orchestrating the unfolded protein response in health and disease. *J Clin Investig* 110:1389–1398.

Kismet K, Akay MT, Abbasoglu O, and Ercan A (2004) Celecoxib: a potent cyclooxygenase-2 inhibitor in cancer prevention. *Cancer Detect Prev* 28:127–142.

Kitao Y, Hashimoto K, Matsuyama T, Iso H, Tamatani T, Hori O, Stern DM, Kano M, Ozawa K, and Ogawa S (2004) ORP150/HSP12A regulates Purkinje cell survival: a role for endoplasmic reticulum stress in cerebellar development. *J Neurosci* 24:1486–1496.

Kitao Y, Ozawa K, Miyazaki M, Tamatani M, Kobayashi T, Yanagi H, Okabe M, Ikawa M, Yamashima T, Stern DM, et al. (2001) Expression of the endoplasmic reticulum molecular chaperone (ORP150) rescues hippocampal neurons from glutamate toxicity. *J Clin Investig* 108:1439–1450.

Kuwabara K, Matsumoto M, Ikeda J, Hori O, Ogawa S, Maeda Y, Kitagawa K, Imuta N, Kinoshita T, Stern DM, et al. (1996) Purification and characterization of a novel stress protein, the 150-kDa oxygen-regulated protein (ORP150), from cultured rat astrocytes and its expression in ischemic mouse brain. *J Biol Chem* 271:5025–5032.

Luo S, Baumeister P, Yang S, Abcouwer SF, and Lee AS (2003) Induction of Grp78/BiP by translational block: activation of the Grp78 promoter by ATF4 through and upstream ATF/CRE site independent of the endoplasmic reticulum stress elements. *J Biol Chem* 278:37375–37385.

Machein MR, Risau W, and Plate KH (1999) Antiangiogenic gene therapy in a rat glioma model using a dominant-negative vascular endothelial growth factor receptor 2. *Hum Gene Ther* 10:1117–1128.

Matsushita K, Matsuyama T, Nishimura H, Takaoka T, Kuwabara K, Tsukamoto Y, Sugita M, and Ogawa S (1998) Marked, sustained expression of a novel 150-kDa oxygen-regulated stress protein, in severely ischemic mouse neurons. *Brain Res Mol Brain Res* 60:98–106.

Mima S, Tsutsumi S, Ushijima H, Takeda M, Fukuda I, Yokomizo K, Suzuki K, Sano K, Nakanishi T, Tomisato W, et al. (2005) Induction of claudin-4 by nonsteroidal anti-inflammatory drugs and its contribution to their chemopreventive effect. *Cancer Res* 65:1868–1876.

Miyagi T, Hori O, Koshida K, Egawa M, Kato H, Kitagawa Y, Ozawa K, Ogawa S, and Namiki M (2002) Antitumor effect of reduction of 150-kDa oxygen-regulated protein expression on human prostate cancer cells. *Int J Urol* 9:577–585.

Ozawa K, Kuwabara K, Tamatani M, Takatsuji K, Tsukamoto Y, Kaneda S, Yanagi H, Stern DM, Eguchi Y, Tsujimoto Y, et al. (1999) 150-kDa oxygen-regulated protein (ORP150) suppresses hypoxia-induced apoptotic cell death. *J Biol Chem* 274:6397–6404.

Ozawa K, Miyazaki M, Matsuhisa M, Takano K, Nakatani Y, Hatazaki M, Tamatani T, Yamagata K, Miyagawa J, Kitao Y, et al. (2005) The endoplasmic reticulum chaperone improves insulin resistance in type 2 diabetes. *Diabetes* 54:657–663.

Ozawa K, Tsukamoto Y, Hori O, Kitao Y, Yanagi H, Stern DM, and Ogawa S (2001) Regulation of tumor angiogenesis by oxygen-regulated protein 150, an inducible endoplasmic reticulum chaperone. *Cancer Res* 61:4206–4213.

Przygodzki T, Sokal A, and Bryszewska M (2005) Calcium ionophore A23187 action on cardiac myocytes is accompanied by enhanced production of reactive oxygen species. *Biochim Biophys Acta* 1740:481–488.

Ristimaki A, Honkanen N, Jankala H, Sipponen P, and Harkonen M (1997) Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res* 57:1276–1280.

Ron D (2002) Translational control in the endoplasmic reticulum stress response. *J Clin Investig* 110:1383–1388.

Saukkonen K, Nieminen O, van Rees B, Vilkki S, Harkonen M, Juhola M, Mecklin JP, Sipponen P, and Ristimaki A (2001) Expression of cyclooxygenase-2 in dysplasia of the stomach and in intestinal-type gastric adenocarcinoma. *Clin Cancer Res* 7:1923–1931.

Smalley WE, Ray WA, Daugherty JR, and Griffin MR (1995) Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. *Am J Epidemiol* 141:539–545.

Suzuki YJ and Ford GD (1991) Inhibition of Ca^2 -ATPase of vascular smooth muscle sarcoplasmic reticulum by reactive oxygen intermediates. *Am J Physiol* 261: H568–H574.

Tamatani M, Matsuyama T, Yamaguchi A, Mitsuda N, Tsukamoto Y, Taniguchi M, Che YH, Ozawa K, Hori O, Nishimura H, et al. (2001) ORP150 protects against hypoxia/ischemia-induced neuronal death. *Nat Med* 7:317–323.

Tanaka K, Tomisato W, Hoshino T, Ishihara T, Namba T, Aburaya M, Katsu T, Suzuki K, Tsutsumi S, and Mizushima T (2005) Involvement of intracellular Ca2 levels in nonsteroidal anti-inflammatory drug-induced apoptosis. *J Biol Chem* 280:31059–31067.

Tomisato W, Tanaka K, Katsu T, Kakuta H, Sasaki K, Tsutsumi S, Hoshino T, Aburaya M, Li D, Tsuchiya T, et al. (2004) Membrane permeabilization by nonsteroidal anti-inflammatory drugs. *Biochem Biophys Res Commun* 323:1032–1039.

Tsukamoto Y, Kuwabara K, Hirota S, Ikeda J, Stern D, Yanagi H, Matsumoto M, Ogawa S, and Kitamura Y (1996) 150-kD oxygen-regulated protein is expressed in human atherosclerotic plaques and allows mononuclear phagocytes to withstand cellular stress on exposure to hypoxia and modified low density lipoprotein. *J Cli Investig* 98:1930–1941.

Tsukamoto Y, Kuwabara K, Hirota S, Kawano K, Yoshikawa K, Ozawa K, Kobayasl T, Yanagi H, Stern DM, Tohyama M, et al. (1998) Expression of the 150-kl oxygen-regulated protein in human breast cancer. *Lab Investig* 78:699–706.

Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ, Takenaka H Tsuchiya T, Mori M, and Mizushima T (2004) Endoplasmic reticulum stres response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis *Cell Death Differ* 11:1009–1016.

Tsutsumi S, Namba T, Tanaka KI, Arai Y, Ishihara T, Aburaya M, Mima S, Hoshin T, and Mizushima T (2006) Celecoxib upregulates endoplasmic reticulum chaper ones that inhibit celecoxib-induced apoptosis in human gastric cells. *Oncogene* 25:1018–1029.

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.

Wang WH, Huang JQ, Zheng GF, Lam SK, Karlberg J, and Wong BC (2003) Non-steroidal anti-inflammatory drug use and the risk of gastric cancer: a systematic review and meta-analysis. *J Natl Cancer Inst* 95:1784–1791.

Wooden SK, Li LJ, Navarro D, Qadri I, Pereira L, and Lee AS (1991) Transactivation of the grp78 promoter by malfolded proteins, glycosylation block, and calcium ionophore is mediated through a proximal region containing a CCAAT motif which interacts with CTF/NF-I. *Mol Cell Biol* 11:5612–5623.

Xu W, Liu L, Charles IG, and Moncada S (2004) Nitric oxide induces coupling of mitochondrial signalling with the endoplasmic reticulum stress response. *Nat Cell Biol* 6:1129–1134.

Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M, and Mori K (2000) ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Mol Cell Biol* 20:6755–6767.

Zhang X, Morham SG, Langenbach R, and Young DA (1999) Malignant transformation and antineoplastic actions of nonsteroidal antiinflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts. *J Exp Med* 190:451–459.

Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, and Ron D (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev* 12:982–995.

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

0026-895X/07/7104-985–993$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 71:985–993, 2007

Vol. 71, No. 4
33282/3185217
Printed in U.S.A.

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_2 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_2, 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_2 were obtained from Sigma (St. Louis, MO). PGE_2 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_2 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_2 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'-ggagcuggagcaggugugudTdT-3' and 5'-acacaccugcuccagcuccdTdT-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'-acgugacacguucggagaadTdT-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). 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. 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 Scheffe'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 $P < 0.05$.

TABLE 1
Primers

Gene Name	Sequence
hsp25	
Forward	5'-cctcttccctatcccctgag-3'
Reverse	5'-ttggctccagactgttcaga-3'
hsp60	
Forward	5'-cgttgccaataacacaaacg-3'
Reverse	5'-cttcaggggttgtcacaggt-3'
hsp70	
Forward	5'-tggtgctgacgaagatgaag-3'
Reverse	5'-aggtcgaagatgagcacgtt-3'
hsp90α	
Forward	5'-aaaggcagaggctgacaaga-3'
Reverse	5'-aggggaggcatttcttcagt-3'
hsp90β	
Forward	5'-gcggcaaagacaagaaaaag-3'
Reverse	5'-gaagtggtcctcccagtcat-3'

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

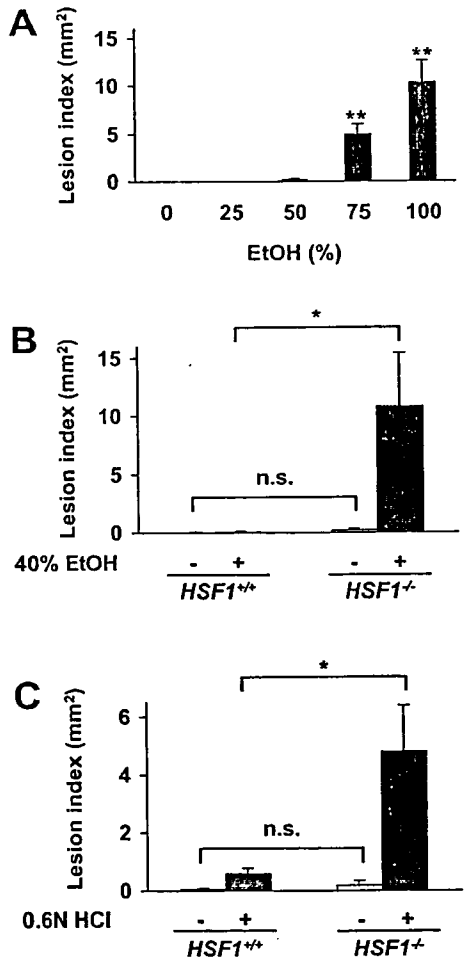

Fig. 1. Production of gastric lesions in wild-type and HSF1-null mice. Wild-type (A–C) and HSF1-null mice (B–C) were orally administered the indicated doses of ethanol (A and B) or hydrochloric acid (C). After 4 h, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n = 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 displayed the strongest HSF1-dependent up-regulation induced by ethanol (Fig. 2A).

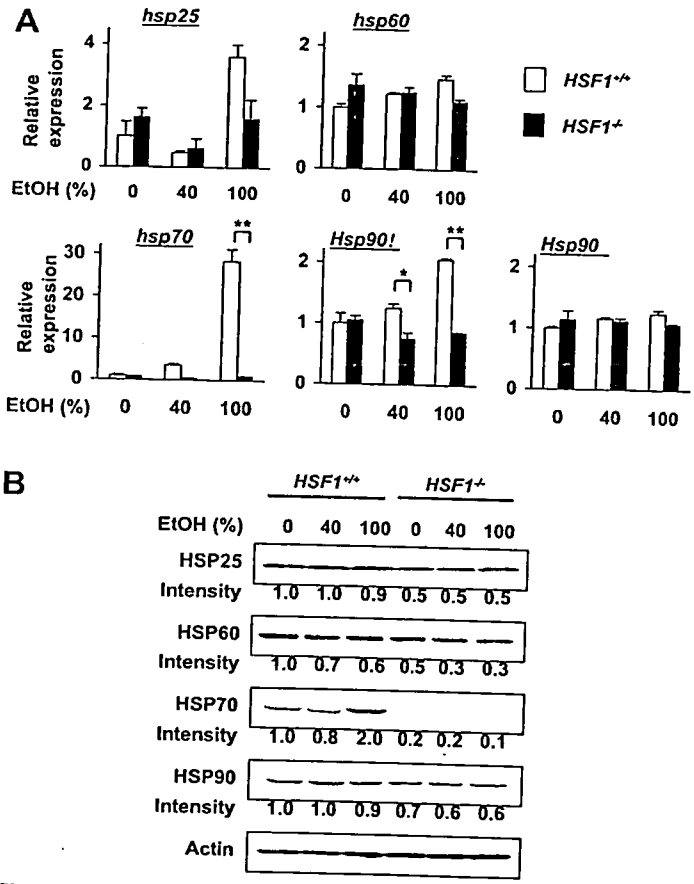

Fig. 2. HSF1-dependent up-regulation of gastric mucosal HSPs induced by ethanol. Wild-type and HSF1-null mice were orally administered the indicated doses of ethanol (A and B). A, after 4 h, the gastric mucosa was removed, and total RNA was extracted. Samples were subjected to real-time RT-PCR using a specific primer for each gene. Values normalized to the glyceraldehyde-3-phosphate dehydrogenase gene and expressed relative to the control sample (i.e., wild-type mice not exposed to ethanol) are given as the mean ± 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.

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_2) 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-

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

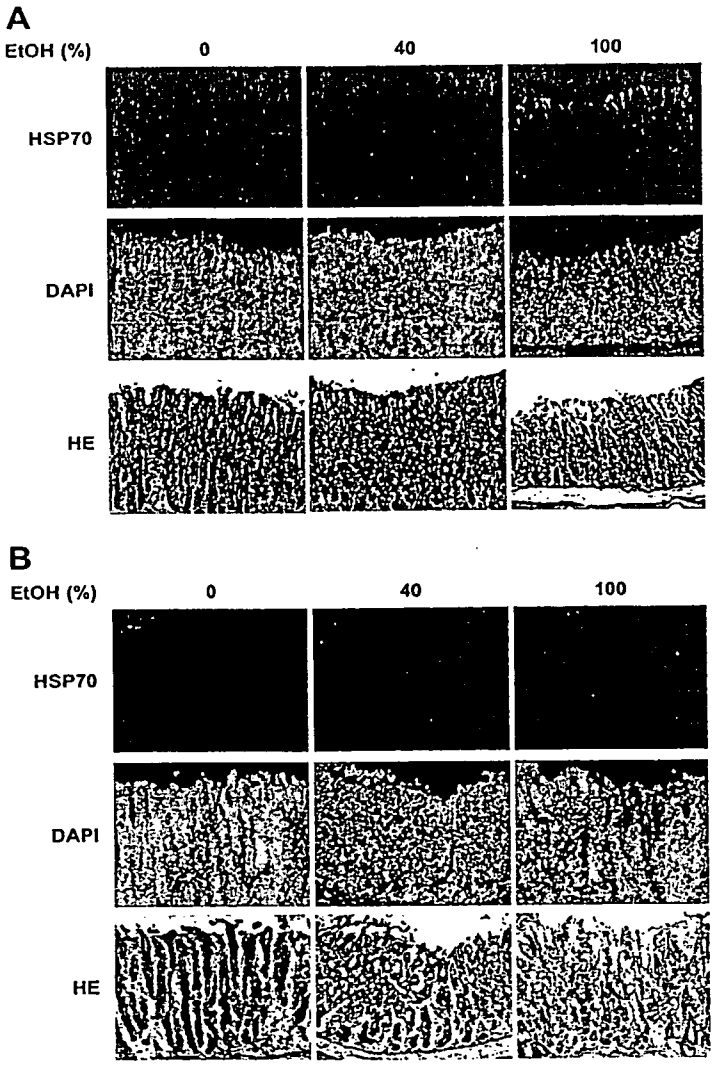

Fig. 3. Ethanol-induced HSF1-dependent up-regulation of HSP70 in gastric mucosa. Wild-type (A) and HSF1-null mice (B) were orally administered the indicated doses of ethanol. After 4 h, sections of gastric tissues were prepared and subjected to histological examination (HE) and immunohistochemical analysis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

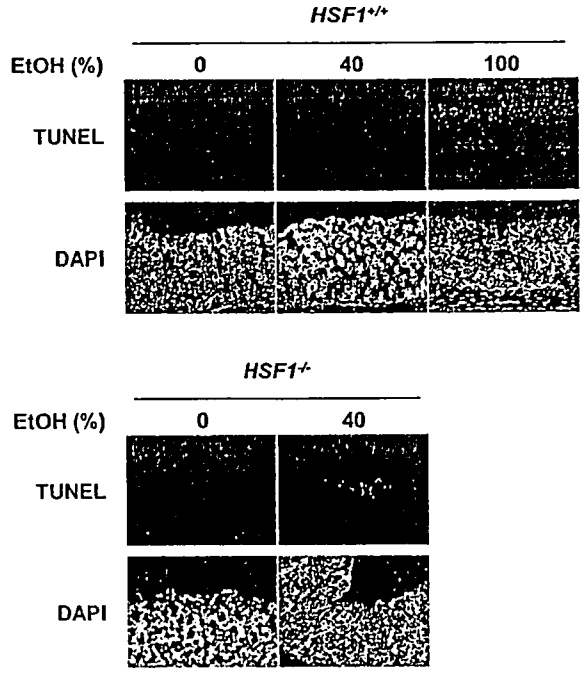

Fig. 4. Induction of apoptosis by ethanol in gastric mucosa. Wild-type and HSF1-null mice were orally administered the indicated doses of ethanol. After 4 h, sections of gastric tissues were prepared and subjected to TUNEL assay.

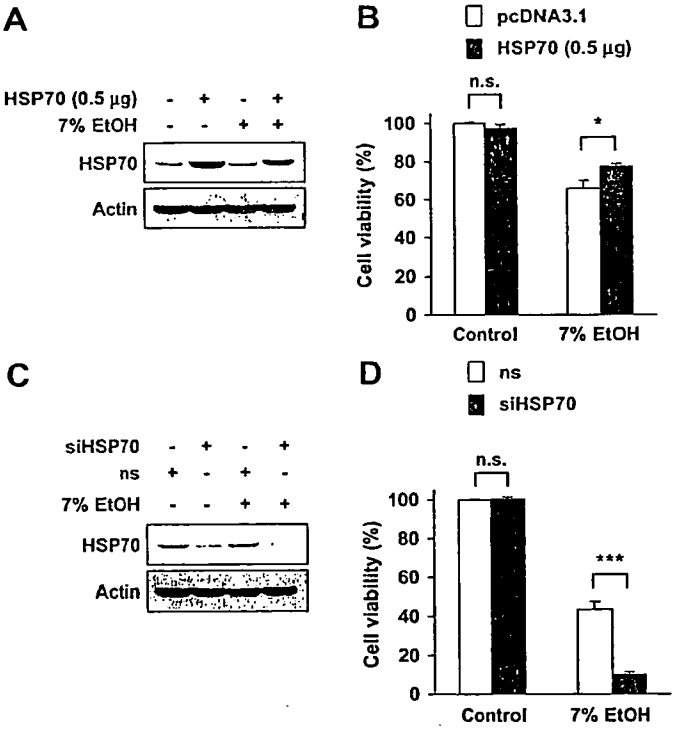

Fig. 5. Effect of increase or decrease in the expression of HSP70 on ethanol-induced cell death. AGS cells were transfected with plasmid with the *hsp70* gene (A and B) or siRNA for the *hsp70* gene (siHSP70) or nonsilencing siRNA (ns) (C and D). After 24 h, cells were incubated with or without 7% ethanol 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 ± 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_2, 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_2 is a major defensive factor for the gastric mucosa, exerting a protective effect against various irritants by decreasing gastric acid secretion and increasing 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_2. To confirm this, we examined the effect of ethanol on gastric mucosal PGE_2 levels in wild-type and HSF1-null mice. As shown in Fig. 6D, ethanol administration did not affect the level of PGE_2 in either group of animals. Furthermore, there was no significant difference in PGE_2 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_2 production.

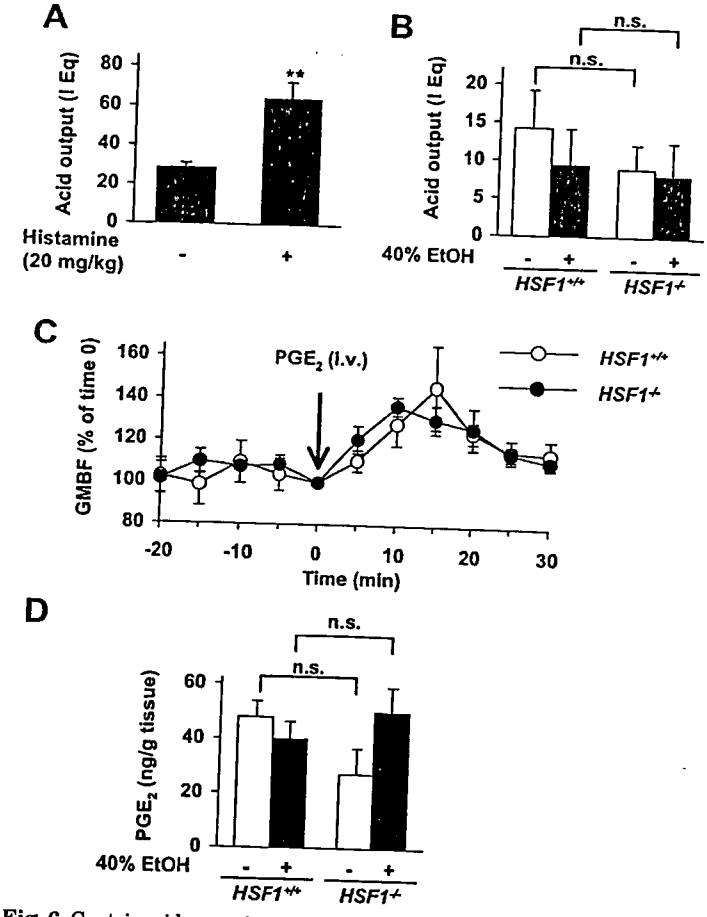

Fig. 6. Gastric acid secretion, GMBF, and PGE_2 levels in HSF1-null mice. A, the pylorus-ligated wild-type mice were administered 20 mg/kg histamine s.c.. B, wild-type and HSF1-null mice were orally administered 40% ethanol, and 2 h later, the pylorus was ligated. A further 2 h after the pylorus ligation, 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_2 (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_2 level was determined by ELISA (D) (n = 3–4). n.s., not significant.

Antiulcer 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-

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

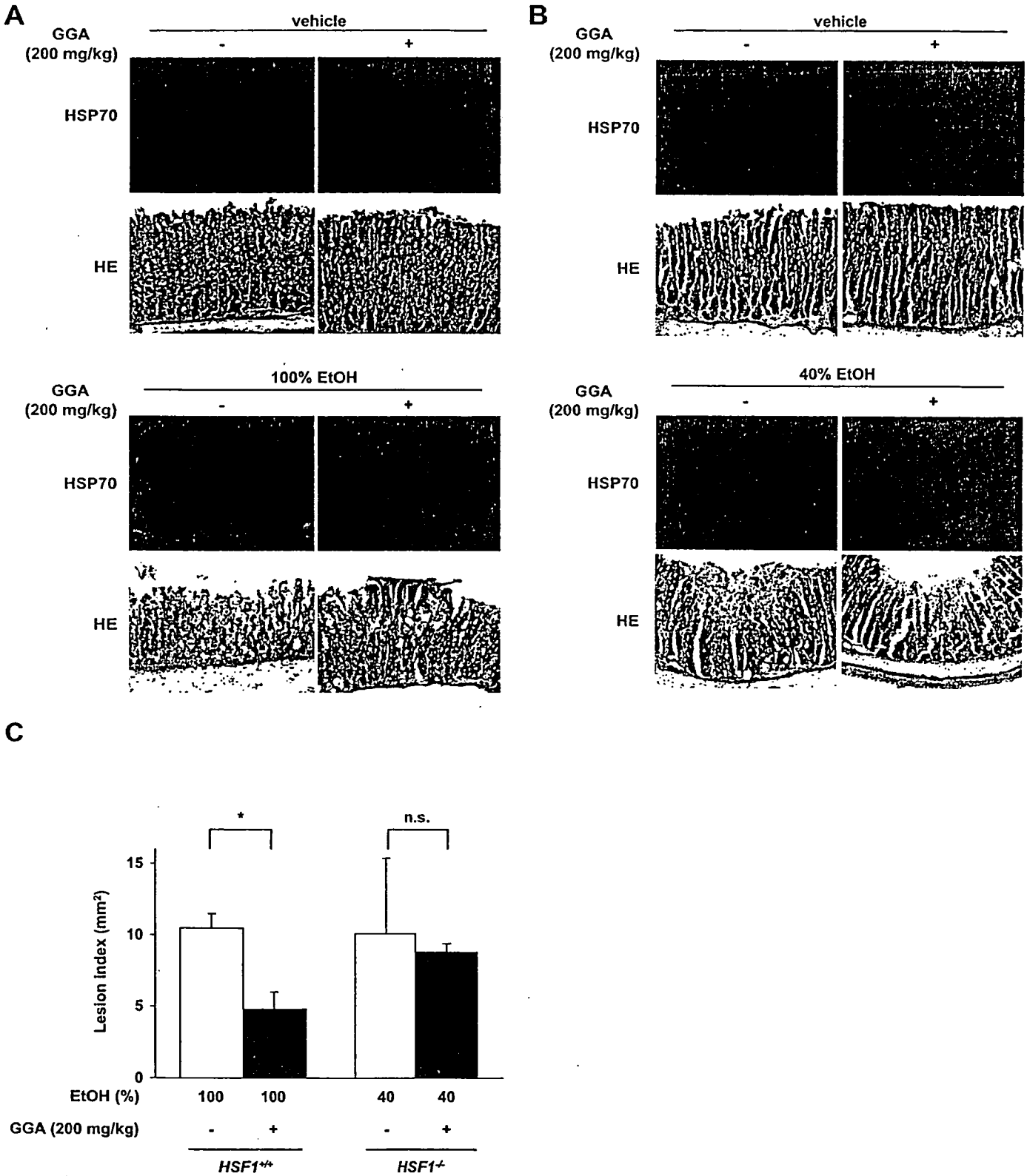

Fig. 7. Effect of ethanol and/or GGA on expression of HSP70 and production of gastric lesions. Wild-type (A and C) and HSF1-null (B and C) mice were orally preadministered 200 mg/kg GGA (10 ml/kg as emulsion with 5% gum arabic) 1 h after which they were orally administered with the indicated doses of 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 ± 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_2 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 α (TNF)-α, 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-α, stimulate the production of gastric lesions, it remains possible that the development of such lesions in HSF1-null mice involves elevated levels of TNF-α. 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 E2 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 IJ (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 G, 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 prostanoid 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 IJ (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

Inflammopharmacology 15 (2007) 67–73
0925-4692/07/020067-7
DOI 10.1007/s10787-006-1560-2

Inflammopharmacology

Review

Various stress proteins protect gastric mucosal cells against non-steroidal anti-inflammatory drugs

T. Mizushima

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan, Tel: ++81-96-371-4323, e-mail: mizu@gpo.kumamoto-u.ac.jp

Received 12 July 2006; accepted 1 September 2006

Abstract. Gastric mucosal cell death induced by non-steroidal anti-inflammatory drugs (NSAIDs) is suggested to be involved in NSAID-induced gastric lesions. Therefore, cellular factors that suppress this cell death are important for protection of the gastric mucosa from NSAIDs. When cells are exposed to various stressors, including NSAIDs, they induce a number of proteins, so-called stress proteins, in order to protect themselves against such stressors. Stress proteins contain cytosolic molecular chaperons (such as heat shock proteins), endoplasmic reticulum molecular chaperons (such as glucose-regulated proteins) and heme oxygenase-1. We recently showed that (i) these stress proteins are up-regulated by NSAIDs both *in vitro* and *in vivo*; (ii) these up-regulation make gastric mucosal cells resistant to NSAIDs *in vitro*; (iii) these up-regulation protects the gastric mucosa from NSAID-induced gastric lesions *in vivo*. In this review, I summarize these results and propose that non-toxic inducers of these stress proteins are therapeutically beneficial as anti-ulcer drugs.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a useful family of therapeutics, accounting for nearly 5% of all prescribed medications (Smalley et al., 1995). The anti-inflammatory actions of NSAIDs are mediated through their inhibitory effects on cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the other hand, NSAID use is associated with gastrointestinal complications (Hawkey, 2000), with about 15–30% of chronic users of NSAIDs suffering from gastrointestinal ulcers and bleeding (Barrier and Hirschowitz, 1989; Fries et al., 1989).

Although PGs have a strong protective effect on gastrointestinal mucosa, the inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side-effects of NSAIDs (Lichtenberger, 2001). We have recently demonstrated that NSAIDs induce apoptosis in primary cultures of gastric mucosal cells in a manner independent of COX inhibition (Tanaka et al., 2005; Tomisato et al., 2001 & 2004a; Tsutsumi et al., 2004). As for 2001 the molecular mechanism governing this apoptosis, we recently proposed that permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca^{2+} influx which in turn induces production of the C/EBP homologous transcription factor (CHOP), and activates calpain, a Ca^{2+}-dependent cysteine protease, both of which have apoptosis-inducing ability (Tanaka et al., 2005). Furthermore, we suggested that both COX-inhibition and NSAID-induced cell death (such as apoptosis) in gastric mucosa are required for production of NSAID-induced gastric lesions *in vivo* (Tomisato et al., 2004b). Cellular factors that suppress NSAID-induced apoptosis are therefore important for protection of gastric mucosa from NSAID-induced gastric lesions.

When cells are exposed to various stressors, including NSAIDs, they induce a number of proteins, so-called stress proteins, in order to protect themselves against such stressors. Molecular chaperons are representative stress proteins. Their up-regulation in cells confers resistance to various stressors as the chaperons re-fold or degrade denatured proteins produced by stressors (Mathew and Morimoto, 1998). Molecular chaperones can be divided into cytosolic molecular chaperons (such as heat shock proteins (HSPs)) and endoplasmic reticulum (ER) molecular chaperons (such as glucose-regulated proteins (GRPs)). Heme oxygenase-1 (HO-1) is another type of stress protein. Not only its substrate, heme, but also various stressors such as oxidative stressors, ultraviolet irradiation, inflammatory cytokines and heavy metals, have been reported to induce HO-1 production (Maines, 1997; Ponka, 1999; Tenhunen et al., 1969). HO-1 degrades heme to carbon monoxide (CO), free iron and biliverdin. Biliverdin is subsequently converted into bilirubin by biliverdin reductase (Maines, 1997; Ponka, 1999; Tenhunen et al., 1969). Bilirubin and biliverdin are potent antioxidants and CO has anti-apoptotic activity. Therefore, up-regulation of HO-1 in cells makes cells resistant to apoptosis induced

by various stressors (Brouard et al., 2000; Maines, 1997; Tenhunen et al., 1969).

Based on these results, we consider a possibility that various stress proteins are up-regulated by NSAIDs and this up-regulation contributes to suppress NSAID-induced apoptosis and NSAID-induced gastric lesions.

Experimental procedures

Gastric Damage Assay – Gastric damage assays were performed as described previously (Tomisato et al., 2004b). Rats, which had been fasted for 24 h, were intraperitoneally injected with SnMP (dissolved in 0.1 N NaOH, adjusted to pH7.6 with HCl). One hour later, indomethacin in 1 % methylcellulose was orally administered. Three hours after administration, the rats were sacrificed by decapitation under light anesthesia with ethyl ether and the stomachs were removed and scored for hemorrhagic damage by an observer unaware of the treatment the rats had received. Calculation of the scores involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index.

Cell Culture, Transfection and Cell Viability Assay – Gastric mucosal cells were isolated from guinea pig fundic glands, as described previously (Hirakawa et al., 1996; Tomisato et al., 2002). Isolated gastric mucosal cells were cultured for 12 h in RPMI 1640 containing 0.3 % v/v FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in type-I collagen-coated plastic culture plates in 5 % CO_2/95 % air at 37 °C. After removing non-adherent cells by washing with RPMI 1640, cells that were attached to the plate at approximately 50 % confluence were used. Guinea pig gastric mucosal cells prepared under these conditions have been previously characterized, with the majority (about 90 %) of such cells being identified as pit cells (Hirakawa et al., 1996; Tomisato et al., 2002).

Human gastric carcinoma cells (AGS) were cultured in RPMI1640 medium supplemented with 10 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in 5 % CO_2/95 % air at 37 °C. Unless otherwise noted, cells (0.8×10^4 cells per well in 24-well plates, 4×10^4 cells per well in 6-well plates, 6×10^5 cells in 100-mm plates) were cultured for 24 h and then used in the experiments. Transfection of cells with plasmid was carried out using Lipofectamine (TM2000) according to the manufacturer's instructions. Transfected cells were used for experiments after a 24 h recovery period. Transfection efficiency was determined in parallel plates by transfection of cells with the pEGFP-C1 control vector. Transfection efficiency was more than 80 % in all experiments.

NSAIDs were dissolved in DMSO or Na_2CO_3 (for indomethacin only) and control experiments (without NSAIDs) were performed in the presence of the same concentrations of DMSO or Na_2CO_3. Cells were exposed to NSAIDs by changing the medium. Cell viability was determined by the MTT method.

Immuno-blotting Analysis – Whole cell extracts and nuclear extracts were prepared as described previously (Schreiber et al., 1989; Tsutsumi et al., 2002). The protein concentration of samples was determined by the Bradford method. Samples were applied to 8 % (HSP72 and GRP78), 10 % (lamin B, Nrf2, p38 MAPK and actin) or 12 % (HO-1) polyacrylamide SDS gels, subjected to electrophoresis, and proteins then immuno-blotted with appropriate antibodies.

Histological and Immunohistochemical Analysis – Gastric tissue samples were fixed in 4 % buffered paraformaldehyde, embedded in O.C.T. 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 microscopy (Olympus IX70).
For immunohistochemical analysis, sections were blocked with 2.5 % goat serum for 10 min and then incubated for 12 h with antibody against HO-1 (1:500 dilution) in the presence of 2.5 % BSA, and finally incubated for 1 h with Alexa Fluor 488 goat anti-mouse immunoglobulin G. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX70).

TdT-mediated dUTP-biotin End Labeling (TUNEL) Assay – Gastric tissue samples were fixed in 4 % buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were first incubated with protenase K (10 µg/ml) for 15 min at 37 °C, then with TdTase 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).

Statistical Analysis – All values are expressed as the mean ± standard deviation (S.D). One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison 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 and discussion

NSAIDs Up-regulate Various Stress Proteins – Up-regulation of various stress proteins was examined in primary cultures of guinea pig gastric mucosal cells. This type of cell has been used as an *in vitro* model for physiological and pathological studies of gastric mucosa because various characteristic features of gastric mucosal cells *in vivo* (such as vigorous secretion of mucin) are reproduced in this system (Hirakawa et al., 1996). As shown in Figure 1A, treatment of cells with indomethacin up-regulated HO-1 very rapidly (within 3 h of the addition of indomethacin) and transiently (HO-1 levels returned to pre-treatment levels 24 h after the addition) (Aburaya et al., 2006). Indomethacin also up-regulates other stress proteins (HSP72 and GRP78) (Fig. 1A). The results in Figure 1A show that up-regulation of HO-1 by indomethacin occurs prior to that of HSP72 and GRP78. Figure 1B shows the effects of different concentrations of indomethacin on HO-1 up-regulation. Up-regulation of HO-1 was just apparent at 25–50 µM indomethacin and was distinct at 200–400 µM indomethacin. These concentrations of indomethacin did not affect cell viability

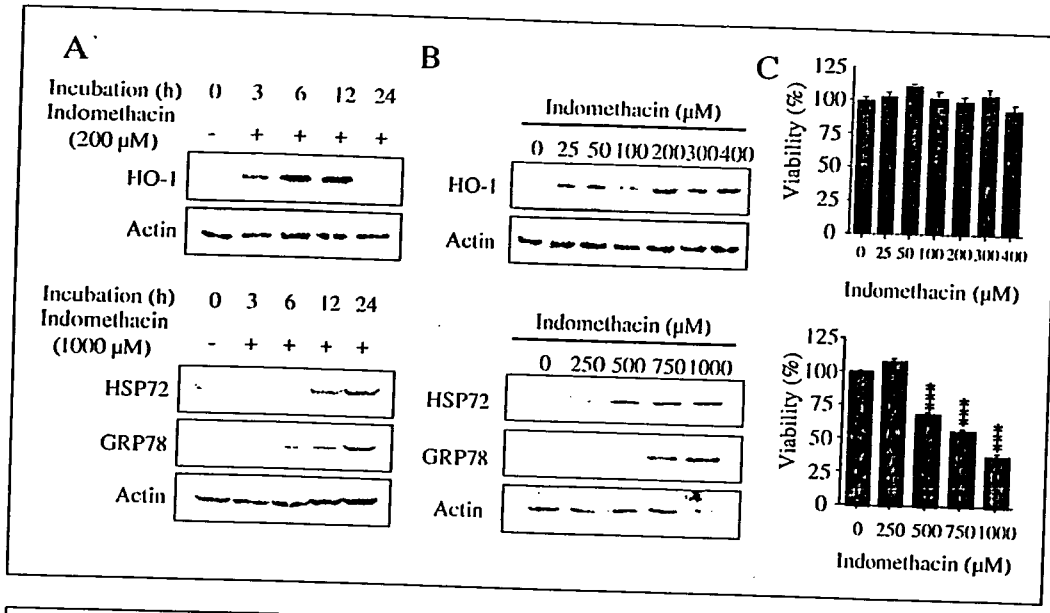

Fig. 1. Up-regulation of HO-1 by indomethacin in gastric mucosal cells in primary culture (Aburaya et al., 2006). Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentrations of indomethacin for the indicated periods (A), 6 h (HO-1 in B and C) or 24 h (HSP72 and GRP78 in B and C). Whole cell extracts were prepared and analyzed by immuno-blotting with an antibody against HO-1, HSP72, GRP78 or actin. The band intensity was determined and expressed relative to the control (A, B). Cell viability was determined by the MTT method. Values shown are relative to the control (in the absence of indomethacin) and are given as the mean ±S.D. (n = 3). ***$P < 0.001$ (C).

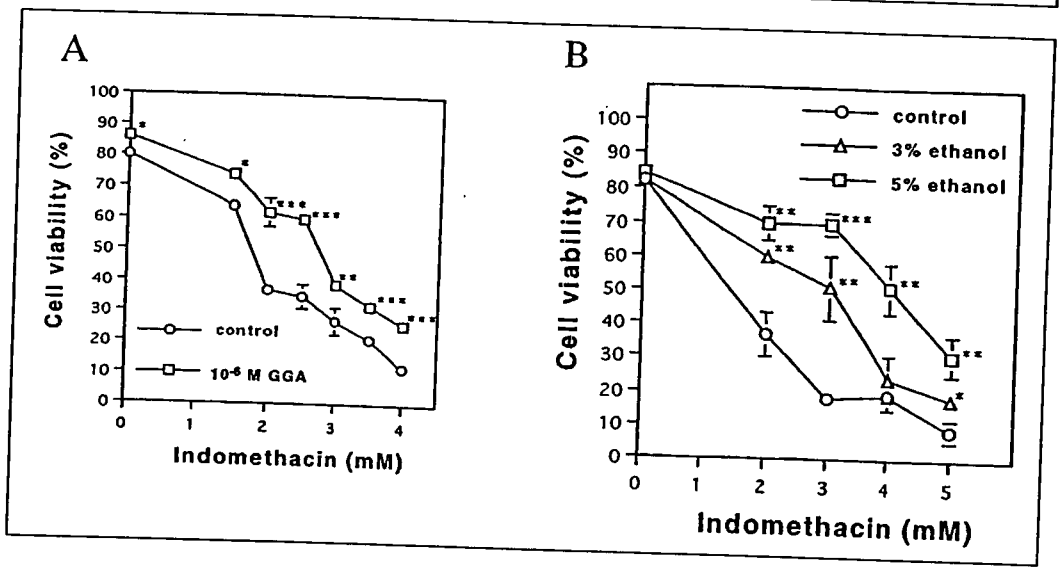

Fig. 2. Protection of cultured gastric mucosal cells from indomethacin by GGA or ethanol (Tomisato et al., 2000). Gastric mucosal cells prepared from guinea pigs were incubated in the presence or absence of 10^{-6} M GGA (A) or indicated concentrations of ethanol (B) for 2 h. Indomethacin was directly added to the culture medium to give the indicated final concentrations and incubation was continued for 1 h. Cell viability was determined by the trypan blue exclusion test. Values are mean ±S.D. (n = 3). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

(Fig. 1C), showing that up-regulation of HO-1 by indomethacin is not the result of indomethacin-induced cell damage. On the other hand, up-regulation of HSP72 and GRP78 required much higher concentrations of indomethacin (Fig. 1B); in other words, up-regulation of these proteins occurs simultaneously with cell damage (Fig. 1C).

COX exists as two subtypes, COX-1 and COX-2, for which celecoxib and flurbiprofen are COX-2-selective in their action. We showed that all NSAIDs tested increased cellular these stress proteins, irrespective of their COX-2 specificity. IC_{50} values for COX-inhibition of each NSAID are not related to the concentration required for up-regulation of these stress proteins. Furthermore, the addition of excess amounts of PGE_2 to the culture medium did not attenuate the indomethacin-induced up-regulation of these stress proteins. Therefore, it seems that NSAIDs up-regulate HO-1 independently of COX-inhibition (Aburaya et al., 2006; Tsutsumi et al., 2004; Tsutsumi et al., 2006).

Contribution of HSP Up-regulation by NSAIDs to Protection of Gastric Mucosal Cells – Geranylgeranylacetone (GGA) is a unique antiulcer drug that effectively protects the gastric mucosa from various stresses, including NSAIDs *in vivo* (Murakami et al., 1981). The action of GGA on the gastric mucosa does not depend on endogenous PGs *in vivo* (Bilski et al., 1987; Terano et al., 1986) and thus, the mechanism of GGA-dependent cytoprotection against NSAIDs has remained to be elucidated. Recently, GGA was shown to directly stimulate the transcription of HSP genes in cultured gastric mucosal cells and in the gastric mucosa, and the protective effects of GGA against gastric mucosal cell damage caused by ethanol were suggested to be at least in part due to this novel action (Hirakawa et al., 1996). Since HSPs are thought to protect cells from various stresses, it is reasonable to assume that induction of HSPs by GGA protects gastric mucosal cells from various stresses other than ethanol, such as NSAIDs *in vitro*, which may partly explain how GGA protects the gastric mucosa from NSAIDs *in vivo*.

Prepared gastric mucosal cells from guinea pig were incubated with indomethacin for 1 h, and cell viability was monitored by the trypan blue exclusion test. As shown in Figure 1, indomethacin significantly decreased the cell viability

in a dose dependent-manner. When cells were pretreated with 10^{-6} M GGA for 2h, the viability of cells exposed to each dosage of indomethacin significantly increased, and the IC_{50} value of the cytotoxic effect of indomethacin increased to about 3mM (Fig. 2A).

It was previously demonstrated that GGA induces HSPs through transiently activating heat-shock factor I (Hirakawa et al., 1996). HSPs protect cells from various stresses, thus, HSPs are candidates for the proteins that are involved in the cytoprotective effect of GGA against indomethacin. If such is indeed the case, induction of HSPs by other means may protect cells from the cytotoxic effect of indomethacin as GGA did. To test this idea, we examined the effect of pretreatment of cells with low concentrations of ethanol, which was previously shown to induce HSPs in guinea pig gastric mucosal cells. Cultured gastric mucosal cells were pre-incubated for 2h with 3 or 5% ethanol. After removing ethanol-containing medium, cells were treated with various concentrations of indomethacin. As shown in Figure 2B, the ethanol pretreatment made cells significantly resistant to indomethacin; pre-exposure to 5% ethanol was more effective than pre-exposure to 3% ethanol. These results support the idea that the cytoprotective effect of GGA against indomethacin may be mediated at least partly by induction of HSPs. Based on these results we propose that GGA, as a non-toxic inducer of HSPs would be of potential therapeutic benefit for avoiding the gastric mucosal injury induced by NSAIDs.

Contribution of GRP78 Up-regulation by NSAIDs to Protection of Gastric Mucosal Cells – Previous reports showed that over-expression of GRP78 in cells suppresses apoptosis induced by topoisomerase inhibitors and ER stressors (Morris et al., 1997; Reddy et al., 2003). Therefore, it is possible that NSAID-induced GRP78 protects gastric mucosal cells from NSAID-induced apoptosis. In order to test this possibility, we examined the effect of over-expression of GRP78 on

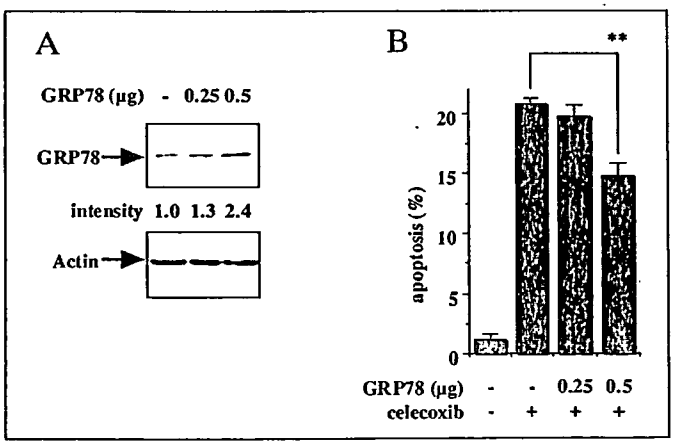

Fig. 3. Effect of GRP78 over-expression on celecoxib-induced apoptosis (Tsutsumi et al., 2006). AGS cells were transfected with the indicated amount of plasmid for the over-expression of GRP78 and pcDNA3.1 vector (total DNA amounts were fixed at 4μg). After 48h, cells were incubated with or without100μM celecoxib for 6h (B). The level of GRP78 protein was estimated by immuno-blotting. Apoptotic cell numbers were determined by FACS (B). Values shown are mean ±S.D. (n = 3). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

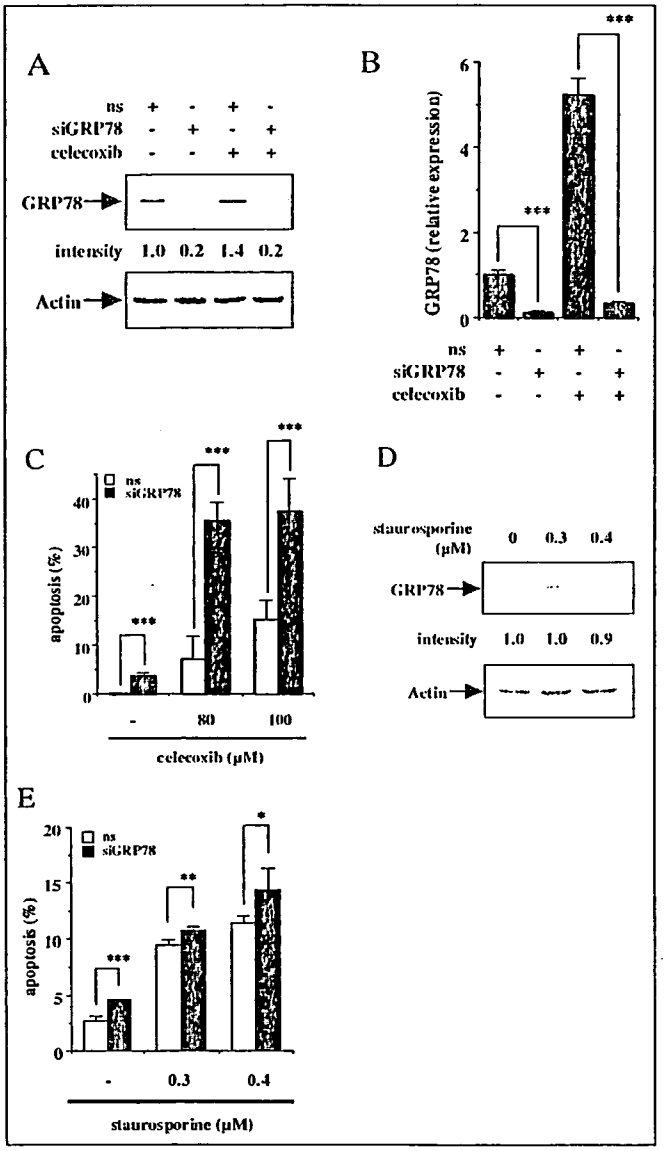

Fig. 4. Effect of GRP78 siRNA on celecoxib-induced apoptosis (Tsutsumi et al., 2006). AGS cells were transfected with 5μg of siRNA for GRP78 (siGRP78) or non-silencing (ns) siRNA. After 48h, cells were incubated with or without 80μM celecoxib (A, B), indicated concentrations of celecoxib (C) or indicated concentrations of staurosporine (D, E) for 6h. The levels of GRP78 protein (A, E) and GRP78 mRNA (B) were estimated by immuno-blotting or real-time RT-PCR experiments. Apoptotic cell numbers were determined by FACS (C, D). Values shown are mean ±S.D. (n = 3). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

apoptosis induced by celecoxib, a COX-2-selective NSAID (Tsutsumi et al., 2006). Transfection of pcDNA3.1 containing the GRP78 gene caused both an increase in the level of GRP78 in cells and partial suppression of celecoxib-induced apoptosis in a manner that depended on the dose of transfected DNA (Fig. 3A and B). We confirmed that over-expression of GRP78 did not affect the spontaneous apoptosis (apoptosis in the absence of celecoxib) (data not shown). These results suggest that the celecoxib-induced increase in GRP78 expression protects cells from celecoxib-induced apoptosis.

The siRNA technique was used to further confirm that celecoxib-induced GRP78 protects cells from celecoxib-

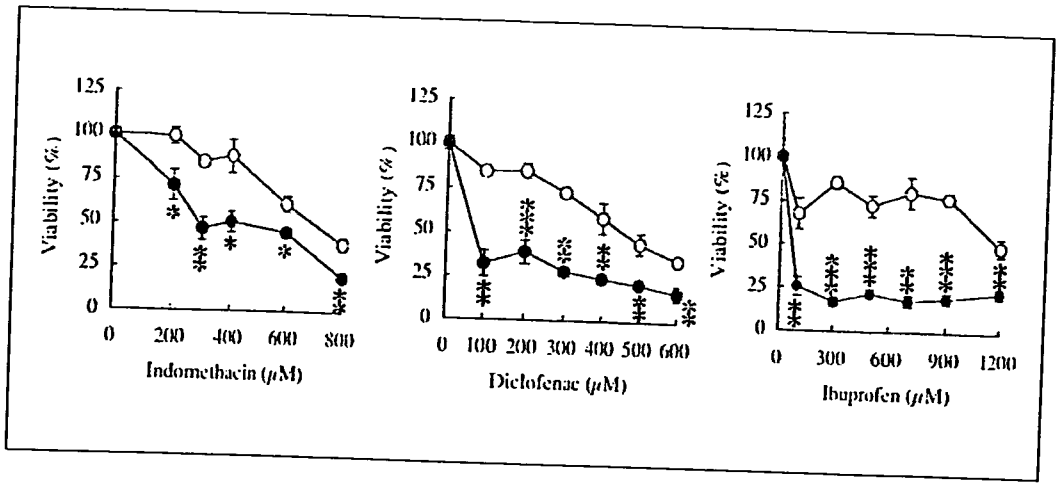

Fig. 5. Effect of SnMP on NSAID-induced apoptosis *in vitro* (Aburaya et al., 2006). Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentrations of indomethacin, diclofenac or ibuprofen in the presence (closed circle or absence (open squares) of 50 μM SnMP for 16 h. Cell viability was determined using the MTT method and shown are relative to the control (in the absence of both NSAIDs and SnMP). Values are given as mean ±S.D. (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

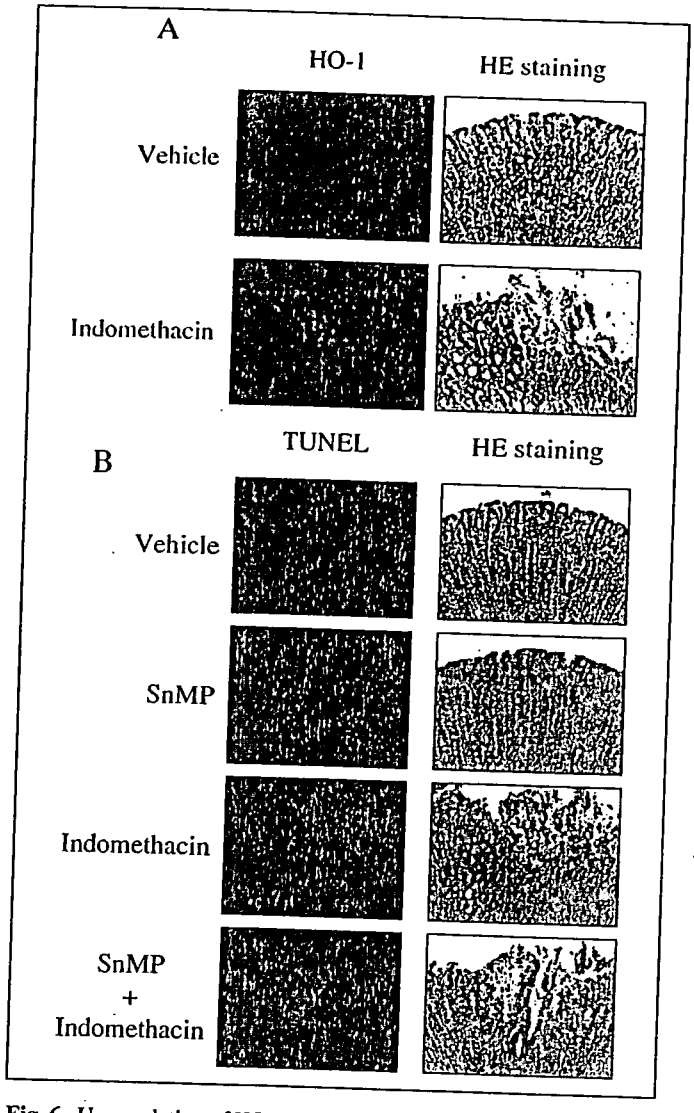

Fig. 6. Up-regulation of HO-1 and induction of apoptosis by indomethacin at gastric mucosa *in vivo* (Aburaya et al., 2006). Rats were intraperitoneally pre-administered with 1 μmol/kg SnMP or vehicle 1 h before the administration of indomethacin (B). Rats were orally administered with 10 mg/kg indomethacin (A, B). After 4 h, sections of gastric tissues were prepared and subjected to histological examination (HE staining) and immunohistochemical analysis with an antibody against HO-1 (A) or TUNEL assay (B).

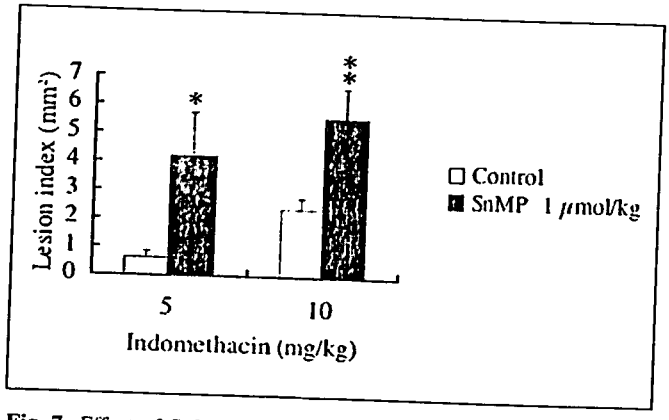

Fig. 7. Effect of SnMP on NSAIDs-induced gastric lesions (Aburaya et al., 2006). Rats were intraperitoneally administered with 1 μmol/kg SnMP or vehicle. After 1 h, animals were administered orally with the indicated doses of indomethacin. After 3 h, the stomach was removed. The stomach was scored for hemorrhagic damage. AValues are given as mean ±S.D. (n = 3–6). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

induced apoptosis. Transfection of siRNA for GRP78 decreased the expression of GRP78 protein (Fig. 4A) and GRP78 mRNA (Fig. 4B), both in the presence and absence of celecoxib and also stimulated celecoxib-induced apoptosis (Fig. 4C). These results strongly suggest that celecoxib-induced GRP78 protects cells from apoptosis in the presence of celecoxib.

We also tried to examine the effect of siRNA for GRP78 on apoptosis induced by a chemotherapy drug. Staurosporine without the ability to induce ER stress response. As shown in Figure 4E, staurosporine, a chemotherapy drug, did not up-regulate GRP78 at concentrations that are enough to induce apoptosis (Fig. 4D). As shown in Figure 4D, transfection of siRNA for GRP78 slightly stimulated apoptosis induced by staurosporine. These results suggested that the stimulatory effect of siRNA for GRP78 on apoptosis is apparent for apoptosis induced by chemotherapy drugs that induce ER stress response.

Contribution of HO-1 Up-regulation by NSAIDs to Protection of Gastric Mucosal Cells In Vitro and In Vivo – Since up-regulation of HO-1 in cells protects cells against various stressors (Brouard et al., 2000; Maines, 1997), it is possi-

ble that up-regulation of HO-1 by NSAIDs protects gastric mucosal cells against NSAIDs. To test this idea, we examined the effect of an inhibitor of HO on NSAID-induced cell death *in vitro*. SnMP is a representative inhibitor of HO, which inhibits the enzymatic activity of HO by acting as a substrate analogue (Valaes et al., 1994). As shown in Figure 5, SnMP stimulated cell death in the presence of various concentrations of NSAIDs (indomethacin, diclofenac and ibuprofen), lowering the concentrations of NSAIDs required for induction of cell death. Cell death, as highlighted in Figure 5, appears to be mediated by apoptosis given that we observed NSAID-dependent activation of caspase-3 under the same experimental conditions as in Figure 5 (data not shown). On this basis, the results in Figure 5 show that SnMP stimulates NSAID-induced apoptosis and, therefore, suggest that up-regulation of HO-1 by NSAIDs contributes to protection of gastric mucosal cells from NSAID-induced apoptosis.

To address the *in vivo* relevance of the *in vitro* result (HO-1 up-regulation by NSAIDs), we tested whether orally administered NSAIDs up-regulate HO-1 in the gastric mucosa of rats. Oral administration of 10 mg/kg indomethacin produced gastric lesions in rats (see Fig. 7) as described previously (Tomisato et al., 2004b). Sections were prepared from the gastric tissues of these rats and were subjected to histological and immunohistochemical analysis. HE staining showed the presence of lesions in the gastric mucosa of indomethacin-administered rats but not in that from vehicle-administered rats (Fig. 6A). Furthermore, immunohistochemical analysis with an antibody against HO-1 showed that HO-1 is up-regulated in the gastric mucosa of indomethacin-administered rats relative to that from vehicle-administered rats (Fig. 6A).

We also examined effect of indomethacin on the level of apoptosis at gastric mucosa that was monitored by TUNEL assay. Accompanying the production of gastric lesions, an increase in TUNEL-positive cells (apoptotic cells) was observed with the indomethacin administration (Fig. 6B). Furthermore, pre-administration of SnMP stimulates indomethacin-induced apoptosis whereas this pre-administration did not induce apoptosis without subsequent indomethacin administration (Fig. 6B). These results suggest that up-regulation of HO-1 by indomethacin contributes to protection of gastric mucosal cells from NSAID-induced apoptosis also *in vivo*.

To examine the role of this NSAID-dependent HO-1 up-regulation in gastric mucosa, we examined the effect of SnMP on NSAID-induced gastric lesions in rats. As shown in Figure 7, pre-administration of SnMP (1 μmol/kg, intraperitoneally) stimulated the production of gastric lesions following oral administration of indomethacin. This administration of SnMP did not produce gastric lesions unless it was followed by the oral administration of indomethacin (data not shown). These results strongly suggest that the indomethacin-induced up-regulation of HO-1 in gastric mucosa contributes to the protection of gastric mucosa from the formation of indomethacin-induced gastric lesions.

Conclusion – NSAIDs up-regulates various stress proteins such as cytosolic chaperons (such as HSP72), ER chaperons (such as GRP78) and HO-1. These up-regulation protects the gastric mucosa from NSAID-induced gastric lesions through inhibition of NSAID-induced cell death. Therefore, non-toxic inducers of these stress proteins should be therapeutically beneficial as anti-ulcer drugs.

References

Aburaya, M., Tanaka, K., Hoshino, T. *et al.* (2006). Heme oxygenase-1 protects gastric mucosal cells against non-steroidal anti-inflammatory drugs. *J. Biol. Chem.*, **281**, 33422–33432.

Barrier, C. H., Hirschowitz, B. I. (1989). Controversies in the detection and management of nonsteroidal antiinflammatory drug-induced side effects of the upper gastrointestinal tract. *Arthritis Rheum.* **32**, 926–932.

Bilski, J., Sarosiek, J., Murty, V. L. *et al.* (1987). Enhancement of the lipid content and physical properties of gastric mucus by geranylgeranylacetone. *Biochem. Pharmacol.* **36**, 4059–4065.

Brouard, S., Otterbein, L. E., Anrather, J. *et al.* (2000). Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J. Exp. Med.* **192**, 1015–1026.

Fries, J. F., Miller, S. R., Spitz, P. W. *et al.* (1989). Toward an epidemiology of gastropathy associated with nonsteroidal antiinflammatory drug use. *Gastroenterology* **96**, 647–655.

Hawkey, C. J. (2000). Nonsteroidal anti-inflammatory drug gastropathy. *Gastroenterology* **119**, 521–535.

Hirakawa, T., Rokutan, K., Nikawa, T. *et al.* (1996). Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* **111**, 345–357.

Lichtenberger, L. M. (2001). Where is the evidence that cyclooxygenase inhibition is the primary cause of nonsteroidal anti-inflammatory drug (NSAID)-induced gastrointestinal injury? Topical injury revisited. *Biochem. Pharmacol.* **61**, 631–637.

Maines, M. D. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol.* **37**, 517–554.

Mathew, A., Morimoto, R. I. (1998). Role of the heat-shock response in the life and death of proteins. *Ann. NY Acad. Sci.* **851**, 99–111.

Morris, J. A., Dorner, A. J., Edwards, C. A. *et al.* (1997). Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. *J. Biol. Chem.* **272**, 4327–4334.

Murakami, M., Oketani, K., Fujisaki, H. *et al.* (1981). Antiulcer effect of geranylgeranylacetone, a new acyclic polyisoprenoid on experimentally induced gastric and duodenal ulcers in rats. *Arzneimittelforschung* **31**, 799–804.

Ponka, P. (1999). Cell biology of heme, *Am. J. Med. Sci.* **318**, 241–256.

Reddy, R. K., Mao, C., Baumeister, P. *et al.* (2003). Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. *J. Biol. Chem.* **278**, 20915–20924.

Schreiber, E., Matthias, P., Muller, M. M. *et al.* (1989). Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic. Acids Res.* **17**, 6419.

Smalley, W. E., Ray, W. A., Daugherty, J. R. *et al.* (1995). Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. *Am. J. Epidemiol.* **141**, 539–545.

Tanaka, K., Tomisato, W., Hoshino, T. *et al.* (2005). Involvement of Intracellular Ca2+ Levels in Nonsteroidal Anti-inflammatory Drug-induced Apoptosis. *J. Biol. Chem.* **280**, 31059–31067.

Tenhunen, R., Marver, H. S., Schmid, R. (1969). Microsomal heme oxygenase. Characterization of the enzyme. *J. Biol. Chem.* **244**, 6388–6394.

Terano, A., Hiraishi, H., Ota, S. *et al.* (1986). Geranylgeranylacetone, a novel anti-ulcer drug, stimulates mucus synthesis and secretion in rat gastric cultured cells. *Digestion* **33**, 206–210.

Tomisato, W., Hoshino, T., Tsutsumi, S. *et al.* (2002). Maturation-associated increase in sensitivity of cultured guinea pig gastric pit cells to hydrogen peroxide. *Dig. Dis. Sci.* **47**, 2125–2133.

Tomisato, W., Takahashi, N., Komoto, C. *et al.* (2000). Geranylgeranylacetone protects cultured guinea pig gastric mucosal cells from indomethacin. *Dig. Dis. Sci.* **45**, 1674–1679.

Tomisato, W., Tanaka, K., Katsu, T. *et al.* (2004a). Membrane permeabilization by non-steroidal anti-inflammatory drugs. *Biochem. Biophys. Res. Commun.* **323**, 1032–1039.

Tomisato, W., Tsutsumi, S., Hoshino, T. *et al.* (2004b). Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. *Biochem. Pharmacol.* **67**, 575–585.

Tomisato, W., Tsutsumi, S., Rokutan, K. *et al.* (2001). NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281**, G1092–1100.

Tsutsumi, S., Gotoh, T., Tomisato, W. *et al.* (2004). Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. *Cell Death Differ.* **11**, 1009–1016.

Tsutsumi, S., Namba, T., Tanaka, K. I. *et al.* (2006). Celecoxib upregulates endoplasmic reticulum chaperones that inhibit celecoxib-induced apoptosis in human gastric cells. *Oncogene* **25**, 1018–1029.

Tsutsumi, S., Tomisato, W., Takano, T. *et al.* (2002). Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. *Biochim. Biophys. Acta* **1589**, 168–180.

Valaes, T., Petmezaki, S., Henschke, C. *et al.* (1994). Control of jaundice in preterm newborns by an inhibitor of bilirubin production: studies with tin-mesoporphyrin. *Pédiatrics* **93**, 1–11.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 355 (2007) 543–548
BBRC
www.elsevier.com/locate/ybbrc

Transcriptional activation of ATF6 by endoplasmic reticulum stressors

Takushi Namba, Tomoaki Ishihara, Ken-ichiro Tanaka, Tatsuya Hoshino, Tohru Mizushima *

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan

Received 25 January 2007
Available online 8 February 2007

Abstract

Previous studies have shown that modification of activating transcription factor 6 (ATF6) protein is important for the endoplasmic reticulum (ER) stress response; ER stressors stimulate the degradation of ATF6 by Site-1 protease (S1P) and Site-2 protease (S2P) into p50-ATF6, which acts as a transcription factor. In the current study, we found that all of the ER stressors tested (such as thapsigargin) up-regulate *ATF6* mRNA expression. As thapsigargin did not a ect the stability of the *ATF6* mRNA, it was concluded that this up-regulation is due to transcriptional activation of *ATF6*. An inhibitor of S1P suppressed this up-regulation of *ATF6* mRNA expression and putative ATF6-binding elements in the promoter of *ATF6* were identified, suggesting that p50-ATF6 positively regulates the gene expression of *ATF6*. Since cells over-expressing ATF6 showed an enhanced ER stress response, we propose that up-regulation of *ATF6* mRNA expression is involved in enhancing the ER stress response.

Keywords: Endoplasmic reticulum; ATF6; ER stress response; S1P; Transcriptional activation

Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) induces the ER stress response, otherwise known as the unfolded protein response (UPR). The ER stress response can be induced not only by alterations in physiological conditions such as glucose starvation and hypoxia but also by exogenous factors such as drugs, including tunicamycin (an inhibitor of protein glycosylation in ER) and thapsigargin (an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [1–4]. We recently reported that non-steroidal anti-inflammatory drugs (NSAIDs), such as celecoxib, also induce the ER stress response and this induction plays an important role in the formation of NSAID-induced gastric ulcers and in the anti-tumor activity of NSAIDs [5–7].

In the mammalian ER stress response, three types of ER transmembrane proteins are important for sensing ER stressors and inducing the response: protein kinase and site-specific endoribonuclease (IRE1), protein kinase R-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [8–10]. The mammalian ER stress response can be separated into four steps: attenuation of global translation to avoid further accumulation of unfolded or misfolded proteins; induction of ER chaperones, such as glucose-regulated protein 78 (GRP78) and other folding enzymes, to refold unfolded or misfolded proteins; degradation of unfolded or misfolded proteins by endoplasmic reticulum associated degradation (ERAD); and up-regulation of C/EBP homologous transcription factor (CHOP) for induction of apoptosis [1,2,4,11,12]. Of these steps, ATF6 is thought to be mainly involved in ER stressor-dependent induction of ER chaperones and other folding enzymes [4,13].

ATF6 (p90-ATF6, full length ATF6) is located in the ER membrane and, in the absence of ER stressors, is maintained in an inactive form by binding to GRP78 [14]. Unfolded or misfolded proteins generated by ER stressors dissociate GRP78 from ATF6, resulting in translocation of ATF6 to the Golgi apparatus where it is cleaved into p50-ATF6 by the Golgi-resident proteases, Site-1 protease (S1P) and Site-2 protease (S2P). The p50-ATF6 then translocates into the nucleus where it specifically activates

* Corresponding author. Fax: +81 96 371 4323.
E-mail address: mizu@gpo.kumamoto-u.ac.jp (T. Mizushima).

0006-291X/$ - see front matter
doi:10.1016/j.bbrc.2007.02.004