Figure 1

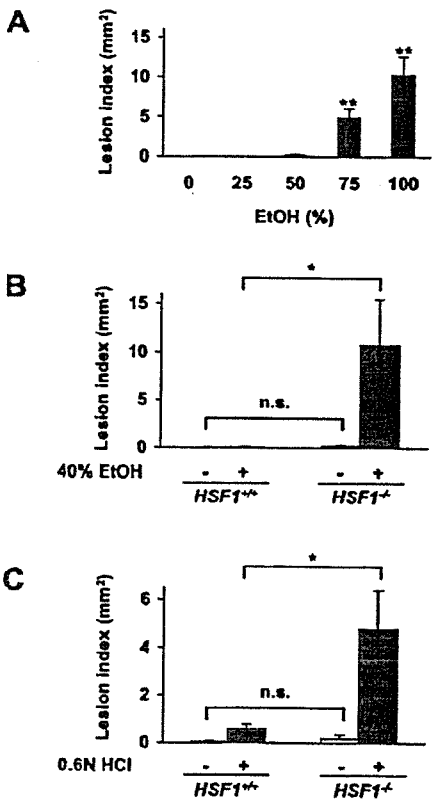

A
Lesion index (mm²)
15
10
5
0
**
**
0
25
50
75
100
EtOH (%)
B
Lesion index (mm²)
15
10
5
0
*
n.s.
40% EtOH
-
+
-
+
HSF1+/+
HSF1-/-
C
Lesion index (mm²)
6
4
2
0
*
n.s.
0.6N HCl
-
+
-
+
HSF1+/+
HSF1-/-

Figure 2

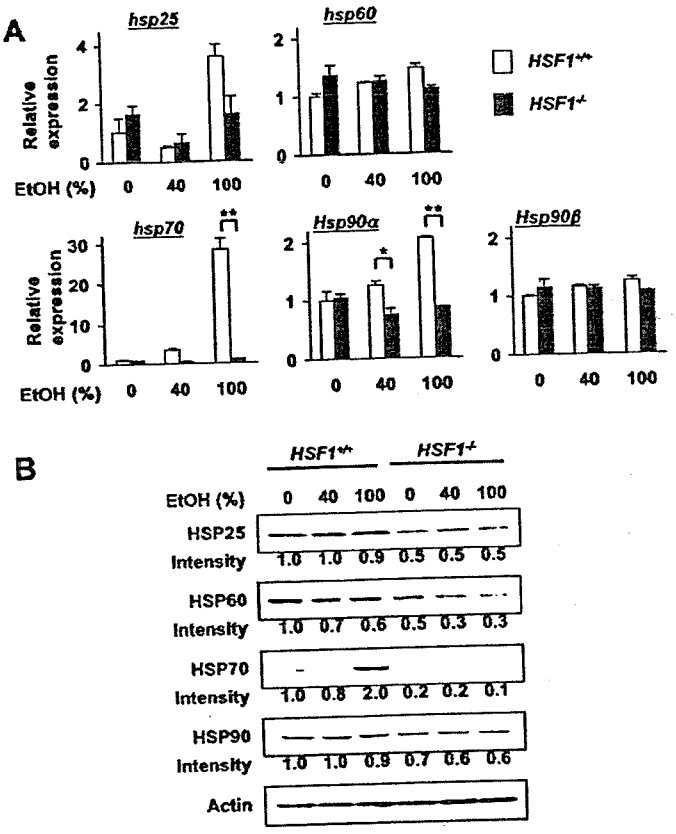

A
hsp25
hsp60
hsp70
Hsp90α
Hsp90β
Relative expression
EtOH (%)
0
40
100
HSF1+/+
HSF1-/-
B
HSF1+/+
HSF1-/-
EtOH (%) 0 40 100 0 40 100
HSP25
Intensity 1.0 1.0 0.9 0.5 0.5 0.5
HSP60
Intensity 1.0 0.7 0.6 0.5 0.3 0.3
HSP70
Intensity 1.0 0.8 2.0 0.2 0.2 0.1
HSP90
Intensity 1.0 1.0 0.9 0.7 0.6 0.6
Actin

Figure 3

A

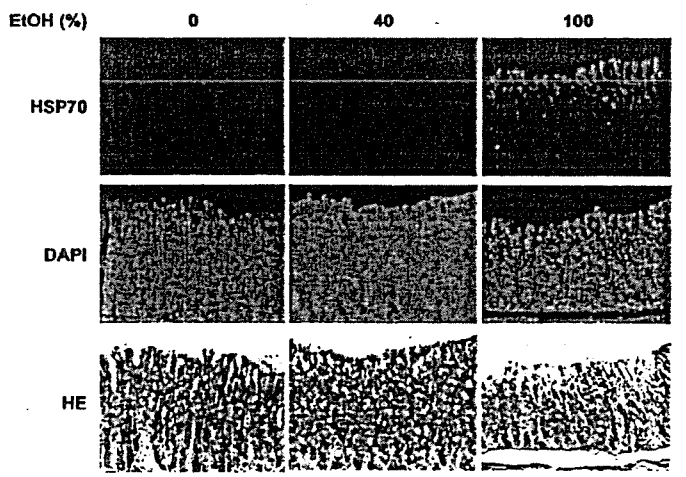


Figure 3

B

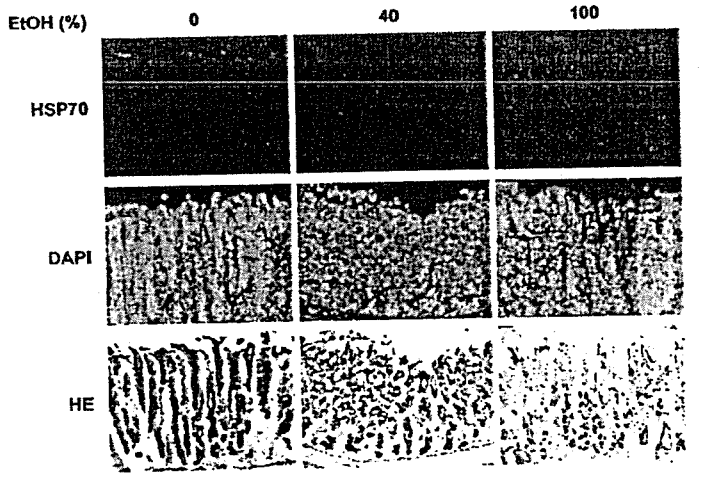


Figure 4

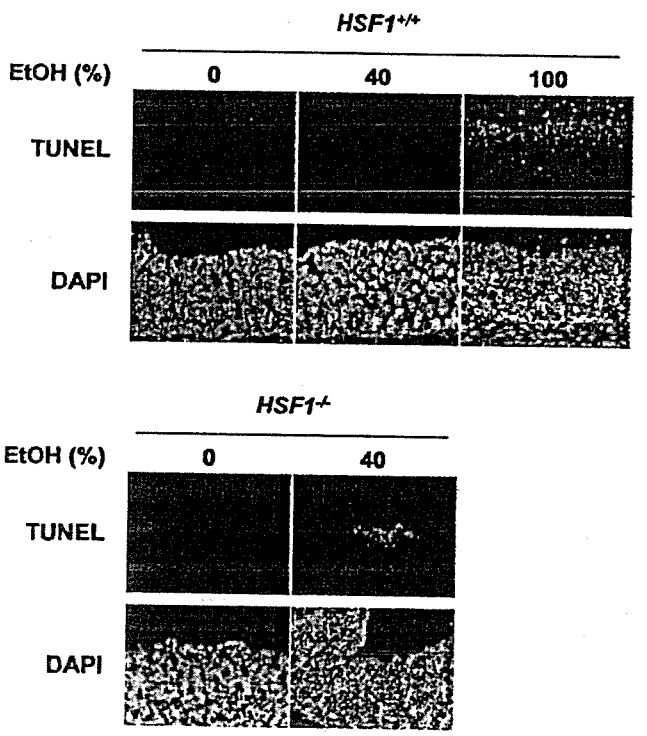


Figure 5

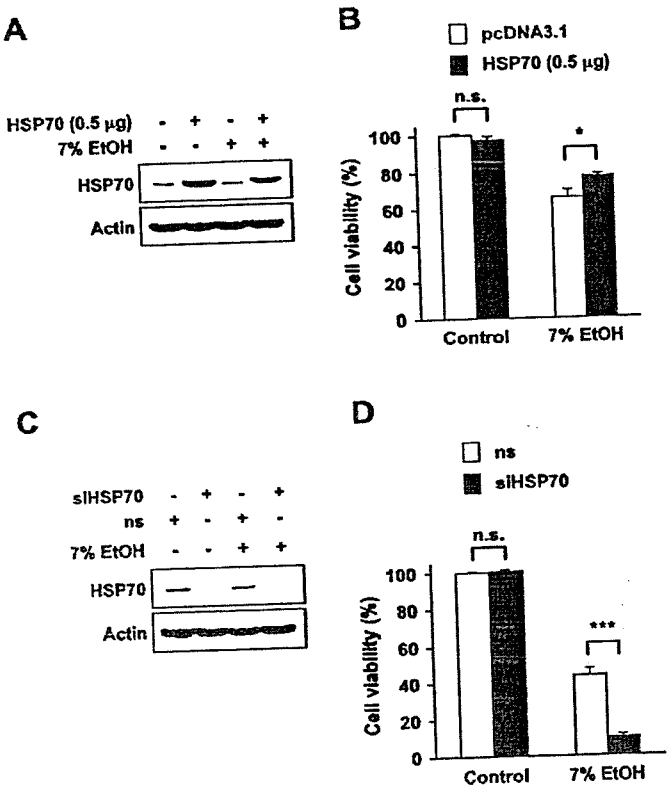

A
HSP70 (0.5 µg) - + - +
7% EtOH - - + +
HSP70
Actin
B
pcDNA3.1
HSP70 (0.5 µg)
n.s.
*
Cell viability (%)
100
80
60
40
20
0
Control
7% EtOH
C
siHSP70 - + - +
ns + - + -
7% EtOH - - + +
HSP70
Actin
D
ns
siHSP70
n.s.
***
Cell viability (%)
100
80
60
40
20
0
Control
7% EtOH

Figure 6

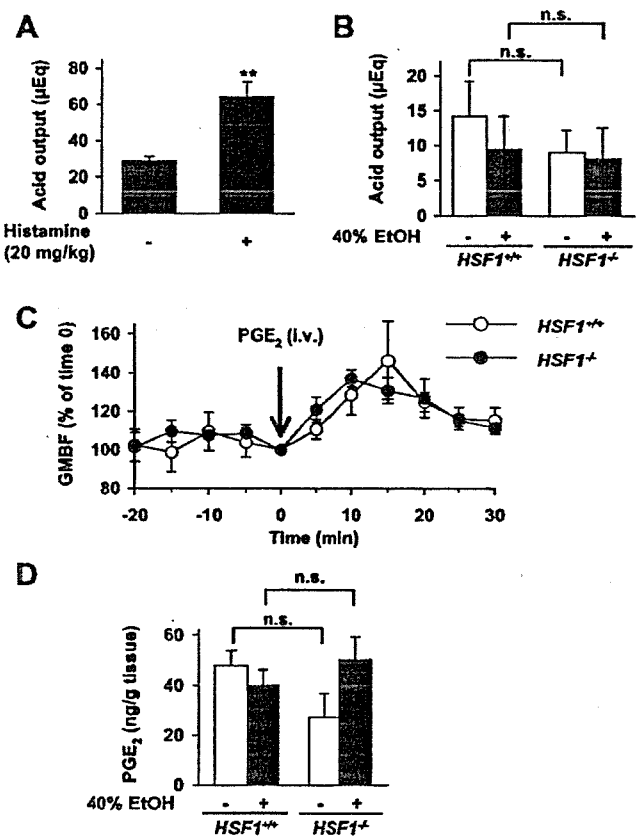

A
Acid output (μEq)
80
60
40
20
0
**
Histamine
(20 mg/kg)
-
+
B
n.s.
n.s.
Acid output (μEq)
20
15
10
5
0
40% EtOH
- + - +
HSF1+/+
HSF1-/-
C
PGE2 (i.v.)
HSF1+/+
HSF1-/-
GMBF (% of time 0)
160
140
120
100
80
-20
-10
0
10
20
30
Time (min)
D
n.s.
n.s.
PGE2 (ng/g tissue)
60
40
20
0
40% EtOH
- + - +
HSF1+/+
HSF1-/-

Figure 7

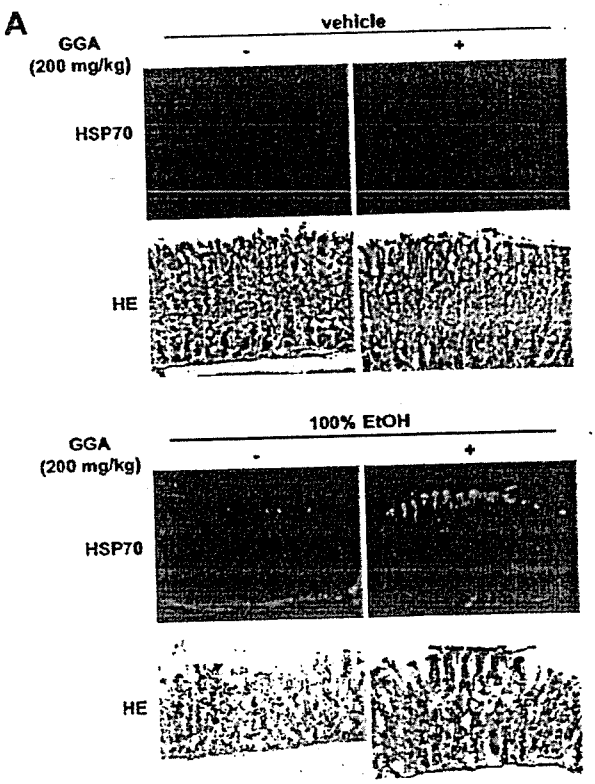


Figure 7

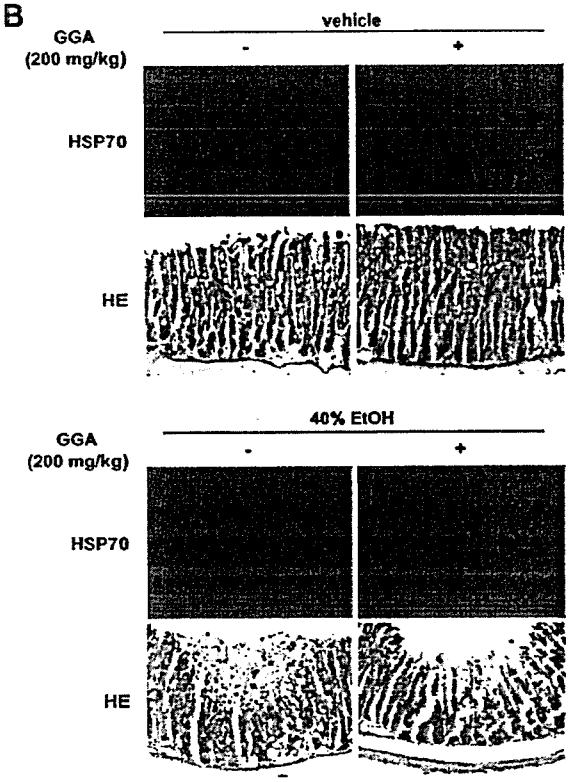
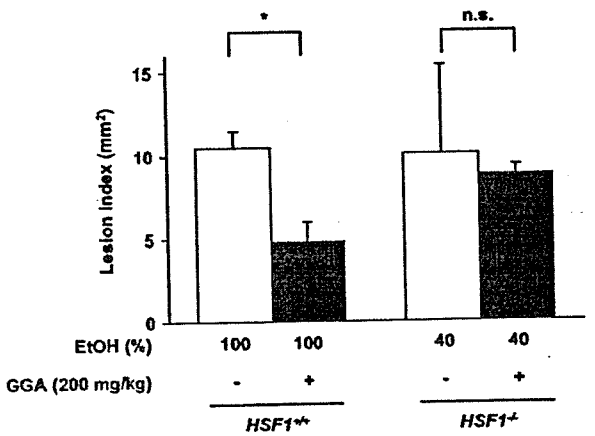


Figure 7

C


*
n.s.
15
10
5
0
Lesion index (mm²)
EtOH (%)
100
100
40
40
GGA (200 mg/kg)
-
+
-
+
HSF1+/+
HSF1-/-

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Inflammopharmacology

Review

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

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Abstract. Gastric mucosal cell death 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 our 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., 2004a; Tomisato et al., 2001; Tsutsumi et al., 2004). As for 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 the suppression of 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 the oral 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 doses 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. 1C), show-

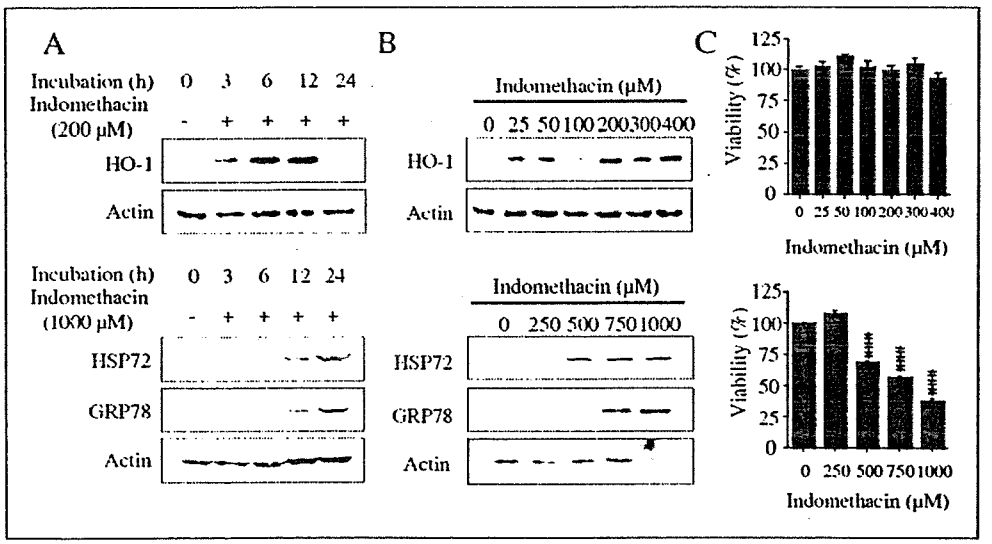

Fig. 1. Up-regulation of HO-1 by indomethacin in gastric mucosal cells in primary culture (Aburaya et al., 2006). Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentrations of indomethacin for indicated periods (A), 6 h (HO-1 in B and C) or 24 h (HSP72 and GRP78 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).

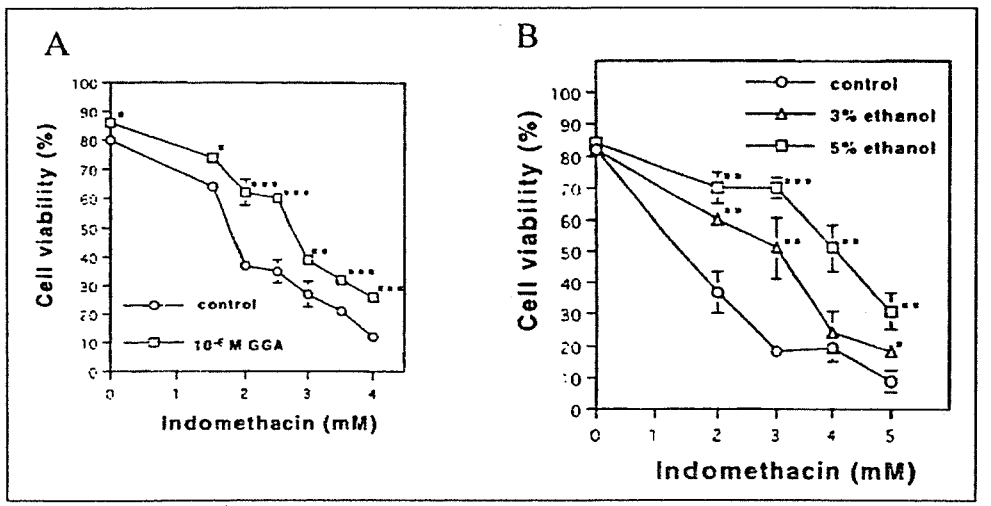

Fig. 2. Protection of cultured gastric mucosal cells from indomethacin by GGA or ethanol (Tomisato et al., 2000). Gastric mucosal cells prepared from guinea pigs were incubated in the presence or absence of 10^{-6} M GGA (A) or indicated concentrations of ethanol (B) for 2 h. Indomethacin was directly 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). ***$P < 0.001$.

ing 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 2 h, 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 3 mM (Fig. 2A).

It was previously demonstrated that GGA induces HSPs through transiently activating heat-shock factor 1 (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 2 h 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

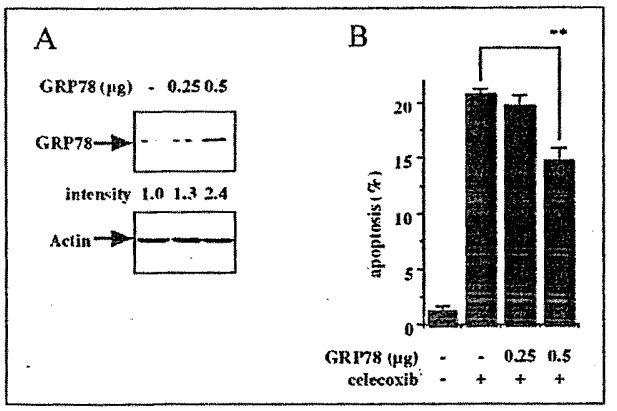

Fig. 3. Effect of over-expression of GRP78 on celecoxib-induced apoptosis (Tsutsumi et al., 2006). AGS cells were transfected with the indicated amount of plasmid for the over-expression of GRP78 and pcDNA3.1 vector (total DNA amounts were fixed at 4 μg). After 48 h, cells were incubated with or without 100 μM celecoxib for 6 h (B). The level of GRP78 protein was estimated by immuno-blotting. Apoptotic cell numbers were determined by FACS (B). Values shown are mean

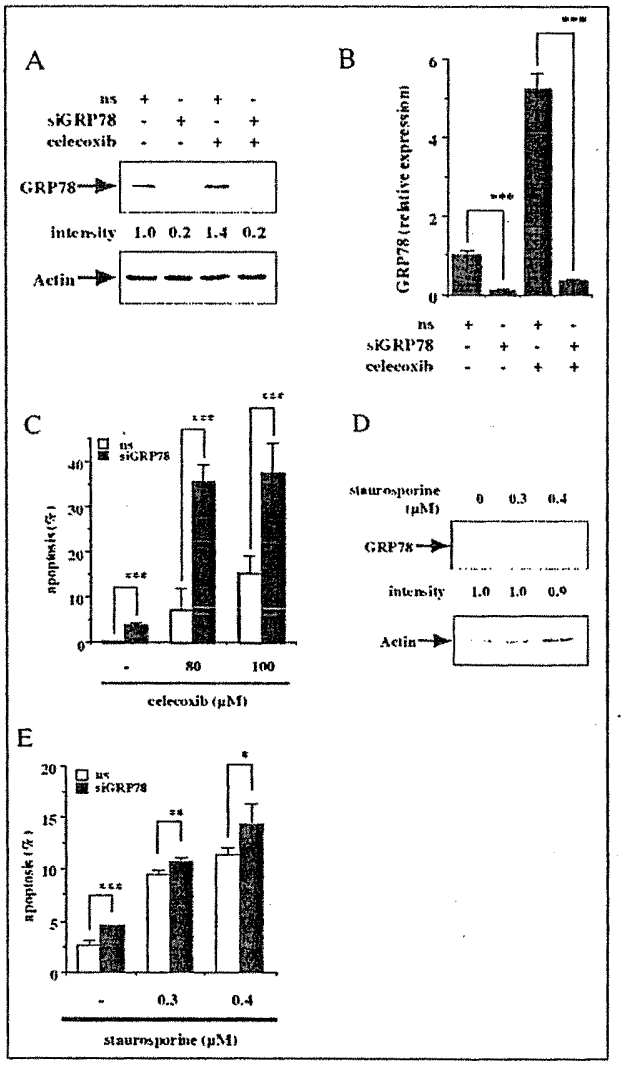

Fig. 4. Effect of GRP78 siRNA on celecoxib-induced apoptosis (Tsutsumi et al., 2006). AGS cells were transfected with 5 μg of siRNA for GRP78 (siGRP78) or non-silencing (ns) siRNA. After 48 h, cells were incubated with or without 80 μM celecoxib (A, B), indicated concentrations of celecoxib (C) or indicated concentrations of staurosporine (D, E) for 6 h. 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

on apoptosis induced by celecoxib, a 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-

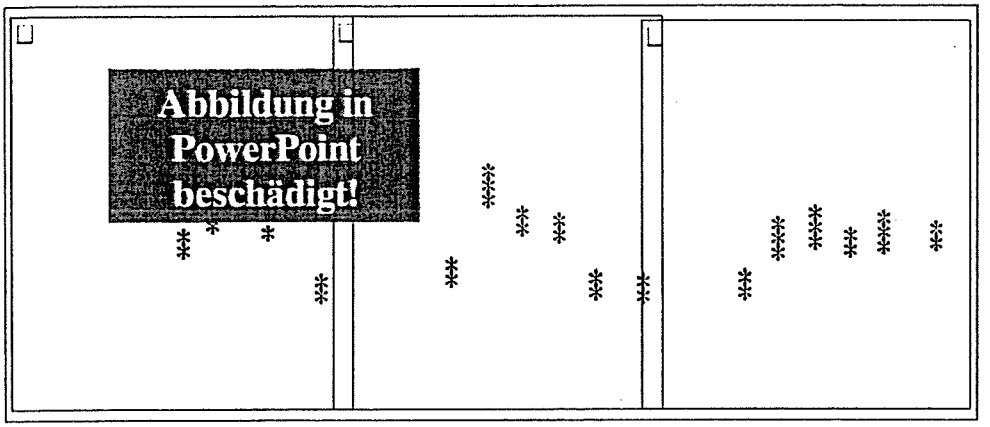

Fig. 5. Effect of SnMP on NSAID-induced apoptosis *in vitro* (Aburaya et al., 2006). Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentrations of indomethacin, diclofenac or ibuprofen in the presence (closed circle) or absence (open squere) of 50 μM SnMP for 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). $^{***}P < 0.001$, $^{**}P < 0.01$,

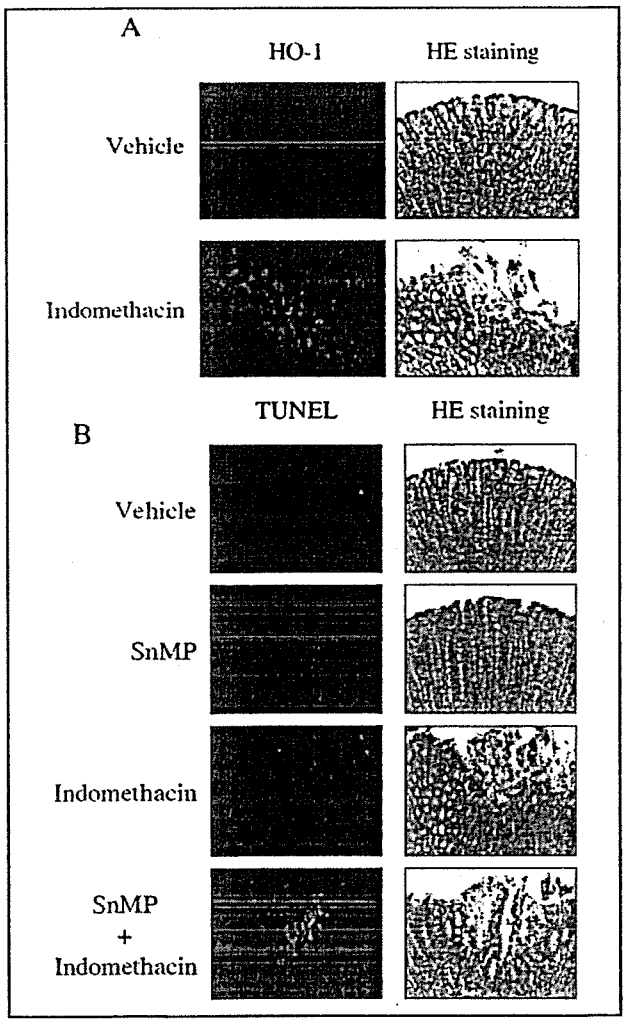

Fig. 6. Up-regulation of HO-1 and induction of apoptosis by indomethacin at gastric mucosa *in vivo* (Aburaya et al., 2006). Rats were intraperitoneally pre-administered with 1 μmol/kg SnMP or vehicle 1 h before the administration of indomethacin (B). Rats were orally administered with 10 mg/kg indomethacin (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)

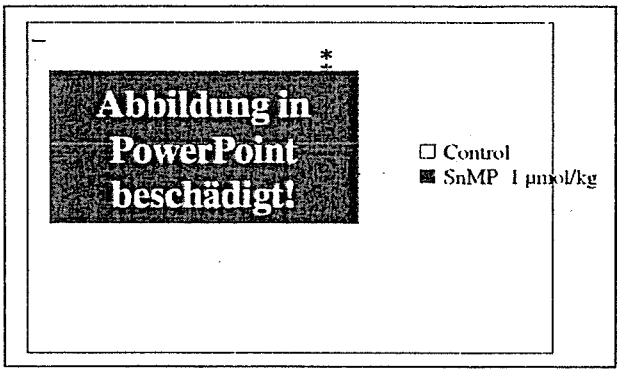

Fig. 7. Effect of SnMP on NSAIDs-induced gastric lesions (Aburaya et al., 2006). Rats were intraperitoneally administered with 1 μmol/kg SnMP or vehicle. After 1 h, animals were administered orally with the indicated doses of indomethacin. After 3 h, the stomach was removed. The stomach was scored for hemorrhagic damage. AValues are given as

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 chemotherapy drug without 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 possible

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.

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Transcriptional activation of ATF6 by endoplasmic reticulum stressors

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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

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transcription of ER stress response-related genes, such as *GRP78* [10,15–20]. Three types of *cis*-acting sequences, ER stress response element (ERSE), ERSE-II and ATF6-binding element, have been reported as consensus sequences for the binding of p50-ATF6 [21–23]. Recently, post-translational modifications of ATF6 other than protein degradation, such as intermolecular disulfide bridge formation and *N*-linked glycosylation, were reported to be involved in ER stressor-induced activation and translocation of ATF6 [24,25]. Thus, it is believed that ATF6 activity is mainly regulated at the post-translational level.

We recently reported that NSAIDs up-regulate the expression of *ATF6* mRNA [7]. Furthermore, some ER stressors (hypoxia and tunicamycin) were also reported to up-regulate *ATF6* mRNA expression [26,27]. In the current study, we have shown that all of the ER stressors tested (celecoxib, tunicamycin and thapsigargin) up-regulate *ATF6* mRNA expression. Furthermore, results in this study suggest that this up-regulation is due to the ER stressor-induced generation of p50-ATF6 and contributes to enhance the ER stress response.

Materials and methods

Chemicals and plasmids. Antibodies against ATF6, GRP78, lamin and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The RNeasy kit, small interfering RNAs (siRNAs) and HiPerFect transfection reagent were from Qiagen (Valencia, CA). Lipofectamine (TM2000) and pcDNA3.1 plasmid were obtained from Invitrogen (Carlsbad, CA). HilyMax was from Dojindo Laboratories (Kumamoto, Japan).

Cell culture, transfection, and real-time RT-PCR analysis. AGS, HCT-15, HeLa and Kato III are human carcinoma cell lines derived from stomach (AGS and Kato III), colon (HCT-15) or uterine cervix (HeLa) tissue. Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO_2 at 37 C.

Transfection of AGS cells with plasmids was carried out using Lipofectamine (TM2000) or HilyMax according to the manufacturer's protocol. On the other hand, cells were transfected with siRNA using HiPerFect transfection reagent according to the manufacturer's instructions.

Real-time RT-PCR was done as described previously [7].

Immuno-blotting analysis. The protein concentration of samples was determined by the Bradford method. Samples were applied to polyacrylamide SDS gels, subjected to electrophoresis, and the resultant proteins then immuno-blotted with their respective antibodies.

Statistical analysis. All values are expressed as the mean ± standard deviation (SD). 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

Up-regulation of ATF6 mRNA expression by various ER stressors

We recently reported that treatment of AGS cells with 80 μM celecoxib for 6 h caused a five-fold increase in the amount of *ATF6* mRNA [7]. In the current study, we have used real-time RT-PCR to examine the effect of various ER stressors, including celecoxib, on *ATF6* mRNA expression. As shown in Fig. 1A, thapsigargin (2 μM) up-regulated *ATF6* mRNA expression in AGS cells in an incubation period-dependent manner. This is the first demonstration that thapsigargin up-regulates the level of *ATF6* mRNA. This time-course profile was similar to that of thapsigargin-induced up-regulation of *GRP78* mRNA expression, although the extent of up-regulation was more marked for *GRP78* than *ATF6* (Fig. 1B). We also examined the effect of other ER stressors (5 μg/ml tunicamycin and 100 μM celecoxib) on *ATF6* mRNA expression and found that both of these ER stressors up-regulate expression of *ATF6* mRNA (Fig. 1C and D). We also confirmed that these concentrations of chemicals up-regulate expression

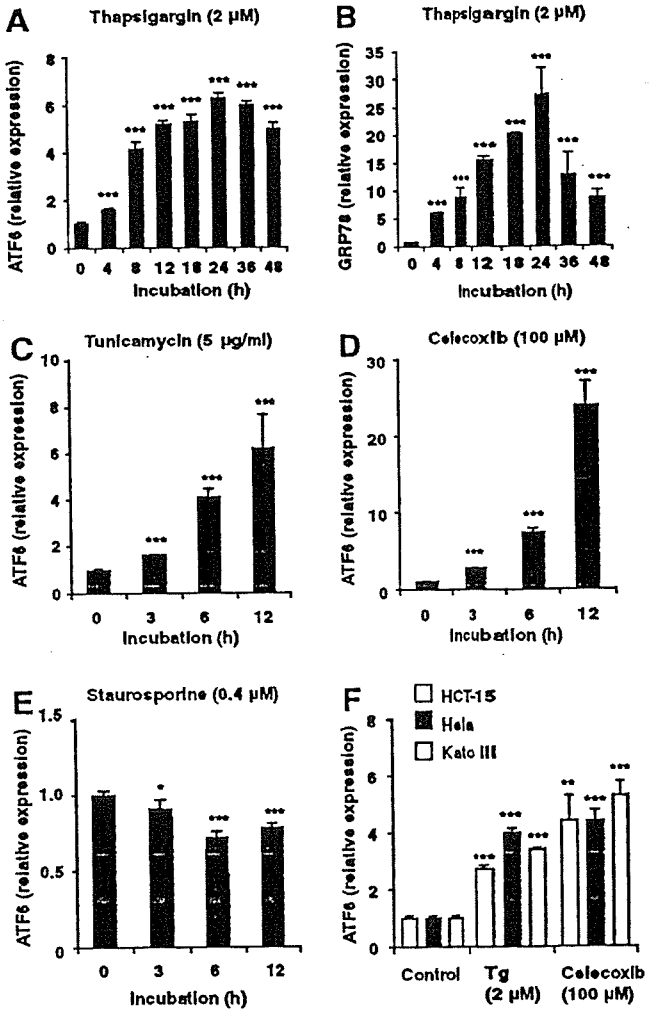

Fig. 1. Up-regulation of *ATF6* mRNA expression by ER stressors. AGS cells (A–E) or HCT-15, HeLa and Kato III cells (F) were incubated with the indicated concentrations of thapsigargin (Tg) (A,B,F), tunicamycin (C), celecoxib (D,F) or staurosporine (E) for the specified time periods (A–E) or 12 h (F). Total RNA was extracted and subjected to real-time RT-PCR using primers specific for *ATF6* or *GRP78*. Values were normalized to actin gene expression and expressed relative to the control sample (time 0 or without drugs). Values are given as means ± SD ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

of *GRP78* mRNA (data not shown). In contrast, staurosporine, which is known to lack ER stress response-inducing activity [7], did not up-regulate (but rather slightly down-regulated) expression of *ATF6* mRNA (Fig. 1E).

As shown in Fig. 1F, both thapsigargin and celecoxib up-regulated the level of *ATF6* mRNA in all of the cell lines tested (HCT-15, HeLa and Kato III). Overall, the results in Fig. 1 show that ER stressors generally up-regulate the levels of *ATF6* mRNA in various human cells, suggesting that this up-regulation is mediated by the ER stress response.

Mechanism for up-regulation of ATF6 mRNA levels by ER stressors

In general, up-regulation of the levels of distinct mRNAs is due to either transcriptional activation of the gene or stabilization of the mRNA. We examined the e ect of ER stressors on the stability of *ATF6* mRNA. After treatment with thapsigargin or celecoxib, cells were further incubated in the presence of actinomycin D, an inhibitor of RNA synthesis, and the amount of *ATF6* mRNA was determined. As shown in Fig. 2A, the amount of *ATF6* mRNA was indistinguishable between thapsigargin-treated and non-treated cells. This demonstrates that thapsigargin does not a ect the stability of *ATF6* mRNA. On the other hand, the results in Fig. 2B show that celecoxib slightly stabilizes *ATF6* mRNA; however, this stabilization was not as distinct as that recently reported for *CHOP* mRNA (Fig. 2C) [28]. In conclusion, the results in Fig. 2A–C suggest that the up-regulation of *ATF6* mRNA levels by ER stressors is generally due to transcriptional activation rather than mRNA stabilization; however, it is possible that celecoxib-dependent stabilization of *ATF6* mRNA contributes to the up-regulation of *ATF6* mRNA levels in celecoxib-treated cells.

Next, we examined the contribution of the ER stress response to the up-regulation of *ATF6* mRNA levels by ER stressors. All of the three ER transmembrane proteins, IRE1, PERK and ATF6, are involved in ER stressor-induced gene expression directly (ATF6) or indirectly (IRE1 and PERK). IRE1 splices *XBP-1* mRNA, resulting in translation of the active form of this protein and PERK phosphorylates eukaryotic initiation factor-2α (eIF-2α) leading to activation of ATF4 expression [29,30]. Both XBP-1 and ATF4 are involved in ER stressor-induced gene expression [13]. Thus, three transcription factors, p50-ATF6, XBP-1 and ATF4, were candidates for mediating ER stressor-induced up-regulation of *ATF6* mRNA levels. At first, we examined the contribution of XBP-1 and ATF4 to the ER stressor-induced up-regulation of *ATF6* mRNA levels by use of siRNA techniques. As shown in Fig. 2D and E, transfection with a given siRNA clearly suppressed the thapsigargin- or celecoxib-induced mRNA expression of its target gene. On the other hand, transfection with siRNA for XBP-1 or ATF4 did not drastically a ect the up-regulation of *ATF6* mRNA, although slight inhibition

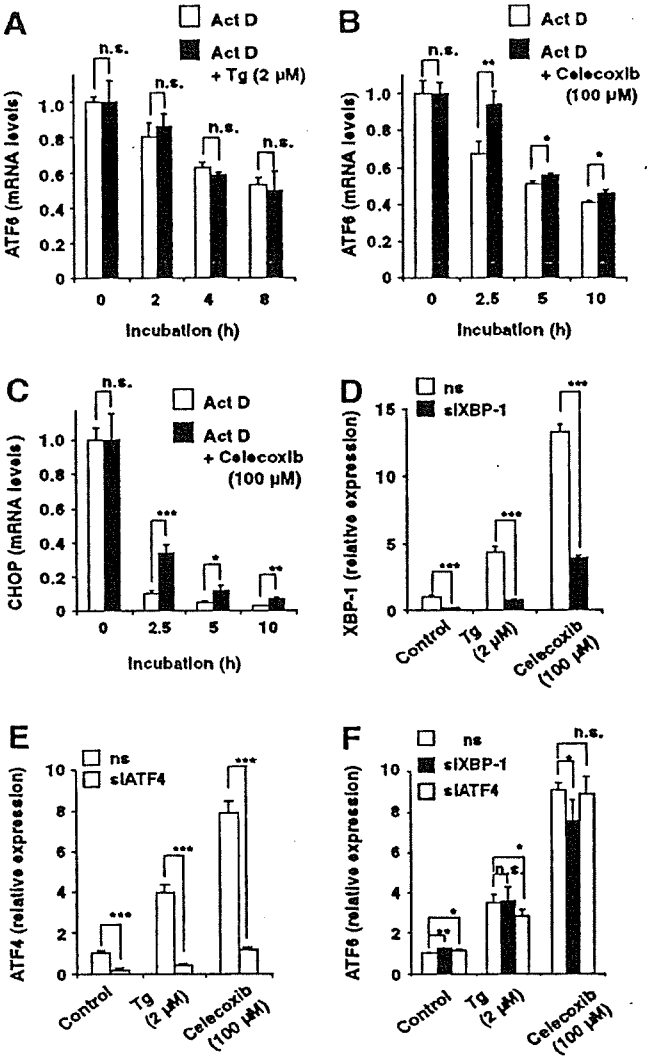

Fig. 2. Stability of *ATF6* mRNA in the presence of ER stressors and e ect of siRNA for XBP-1 or ATF4 on the ER stressor-induced up-regulation of *ATF6* mRNA expression. AGS cells were pre-incubated with or without thapsigargin (Tg) (A) or celecoxib (B,C), at the indicated concentrations, for 6 h and further incubated with 1 μg/ml actinomycin D (Act D) for the specified time periods under the same conditions as the pre-incubation step (A–C). AGS cells were transfected with siRNA for XBP-1 (siXBP-1) or ATF4 (siATF4) or with non-silencing (ns) siRNA. After 24 h, cells were incubated with or without the indicated concentration of thapsigargin (Tg) or celecoxib for 6 h (D–F). The levels of *ATF6* (A,B,F), *CHOP* (C), *XBP-1* (D) and *ATF4* (E) mRNA were estimated by real-time RT-PCR experiments as described in the legend of Fig. 1. Values are given as means ± SD ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. n.s., Not significant.

was observed in thapsigargin-treated cells transfected with siRNA for ATF4 and in celecoxib-treated cells transfected with siRNA for XBP-1 (Fig. 2F). Based on the results in Fig. 2F and the results with AEBSF shown in Fig. 3 (see below), it may be concluded that neither XBP-1 nor ATF4 is a main transcription factor responsible for ER stressor-induced up-regulation of *ATF6* mRNA expression.