

Internalization of EP₄ and Production of Amyloid- β Peptides

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A Role for HSP70 in Protecting against Indomethacin-induced Gastric Lesions*

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A major clinical problem encountered with the use of nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, is gastrointestinal complications. Both NSAID-dependent cyclooxygenase inhibition and gastric mucosal apoptosis are involved in NSAID-produced gastric lesions, and this apoptosis is mediated by the endoplasmic reticulum stress response and resulting activation of Bax. Heat shock proteins (HSPs) have been suggested to protect gastric mucosa from NSAID-induced lesions; here we have tested this idea genetically. The severity of gastric lesions produced by indomethacin was worse in mice lacking heat shock factor 1 (HSF1), a transcription factor for *hsp* genes, than in control mice. Indomethacin administration up-regulated the expression of gastric mucosal HSP70. Indomethacin-induced gastric lesions were ameliorated in transgenic mice expressing HSP70. After indomethacin administration, fewer apoptotic cells were observed in the gastric mucosa of transgenic mice expressing HSP70 than in wild-type mice, whereas the gastric levels of prostaglandin E₂ for the two were indistinguishable. This suggests that expression of HSP70 ameliorates indomethacin-induced gastric lesions by affecting mucosal apoptosis. Suppression of HSP70 expression *in vitro* stimulated indomethacin-induced apoptosis and activation of Bax but not the endoplasmic reticulum stress response. Geranylgeranylacetone induced HSP70 at gastric mucosa in an HSF1-dependent manner and suppressed the formation of indomethacin-induced gastric lesions in wild-type mice but not in HSF1-null mice. The results of this study provide direct genetic evidence that expression of HSP70 confers gastric protection against indomethacin-induced lesions by inhibiting the activation of Bax. The HSP inducing activity of geranylgeranylacetone seems to contribute to its gastroprotective activity against indomethacin.

Nonsteroidal anti-inflammatory drugs (NSAIDs),² such as indomethacin, are a useful family of therapeutics (1). An inhib-

itory effect of NSAIDs on cyclooxygenase (COX) activity is responsible for their anti-inflammatory actions because COX is an enzyme essential for the synthesis of prostaglandins (PGs), such as PGE₂, which have a strong capacity to induce inflammation. On the other hand, NSAID use is associated with gastrointestinal complications (2–4).

Although PGE₂ has 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 (5). We have recently demonstrated that NSAIDs induce apoptosis in cultured gastric cells and at gastric mucosa in a manner independent of COX inhibition (6–10). As for the molecular mechanism governing this apoptosis, we have proposed the following pathway. Permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca²⁺ influx and increases intracellular Ca²⁺ levels, which in turn induces the endoplasmic reticulum (ER) stress response (6, 11, 12). In the ER stress response, an apoptosis-inducing transcription factor, CCAAT enhancer-binding protein homologous transcription factor (CHOP), is induced, as we have previously shown, CHOP is essential for NSAID-induced apoptosis (7). CHOP induces expression of p53 up-regulated modulator of apoptosis (PUMA) and the resulting translocation and activation of Bax. We have already shown that both PUMA and Bax play an important role in NSAID-induced mitochondrial dysfunction, activation of caspases and apoptosis (13). Furthermore, we have suggested that both COX inhibition (measured as a decrease in the gastric PGE₂ level) and gastric mucosal apoptosis are required for the formation of NSAID-induced gastric lesions *in vivo* (10, 14). Therefore, maintenance of gastric PGE₂ levels or protection against gastric mucosal apoptosis is important for protection of gastric mucosa from NSAID-induced lesions.

Heat shock proteins (HSPs) are induced by various stressors, including NSAIDs, and induction of HSPs, especially HSP70, provides cellular resistance to NSAIDs (15, 16). Interestingly, geranylgeranylacetone (GGA), a leading anti-ulcer drug on the Japanese market, has been reported to be a nontoxic HSP inducer (17). We have previously reported that treatment of

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² The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; CHOP, CCAAT enhancer-binding protein homologous transcription factor; COX, cyclooxygenase; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; ER, endoplasmic reticulum; FACS, fluorescence activated cell sorting; GGA, geranylgeranylacetone; HSF, heat shock factor; HSP, heat shock protein; IL, interleukin; PG, prostaglandin; PUMA, p53 up-regulated modulator of apoptosis; RT, reverse transcription; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling; TdT, terminal deoxynucleotidyl transferase; siRNA, small interfering RNA; PI, propidium iodide.

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HSP70 and NSAID-induced Gastric Lesions

cultured gastric mucosal cells with GGA protects cells from NSAID-induced cell death and is accompanied by induction of HSP70 (18). These previous results suggest that HSP70 protects gastric mucosa from NSAID-induced gastric lesions; however, no direct evidence exists. The results also suggest that the protective effect of GGA against NSAID-produced gastric lesions is due to its HSP inducing activity. However, because GGA mediates various other gastroprotective mechanisms, such as an increase in gastric mucosal blood flow, stimulation of surface mucus production, and direct protection of gastric mucosal cell membranes (19–21), it remains unclear whether up-regulation of HSPs represents major mode of gastroprotective activity of GGA against NSAIDs.

Up-regulation of HSP expression by stressors is achieved at the level of transcription through a consensus *cis*-element (heat shock element) and a transcription factor (heat shock factor 1 (HSF1)), which specifically binds to a heat shock element located upstream of the *hsp* genes (22). Disruption to the activity of HSF1 leads to the loss of stressor-induced HSP up-regulation (22, 23). We recently used HSF1-null mice to obtain genetic evidence for a protective role for HSPs against production of gastric lesions (24). However, NSAID-produced gastric lesions, which involve not only irritant-induced mucosal damage but also a decrease in the PGE₂ level, were not tested. Furthermore, although transgenic mice expressing HSP27 display a phenotype of resistance to irritant-induced gastric lesions (25), the effect of artificial up-regulation of HSP70 on the production of gastric lesions has not been tested. In this study, we used HSF1-null mice and transgenic mice expressing HSP70 to examine the role of HSP70 in the pathogenesis of NSAID-produced gastric lesions. The results suggest that HSP70 is protective against the production of indomethacin-induced gastric lesions. Furthermore, the results suggest that HSP70 achieves this protective effect through inhibiting mucosal apoptosis rather than affecting the gastric level of PGE₂.

EXPERIMENTAL PROCEDURES

Chemicals and Animals—RPMI 1640 was obtained from Nissui Pharmaceutical Co. (Osaka, Japan). Paraformaldehyde, fetal bovine serum, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide were obtained from Sigma. Indomethacin was obtained from Wako Co. (Osaka, Japan). A PGE₂ enzyme-linked immunosorbent assay kit was purchased from Cayman Chemical (Ann Arbor, MI). Antibodies against HSP70 were purchased from StressGen (San Diego, CA), and actin and the N-terminal region of Bax (Bax N20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Terminal deoxynucleotidyl transferase (TdT) was obtained from TOYOBO (Osaka, Japan). Biotin 14-ATP, Alexa Fluor 488 goat anti-rabbit (or anti-mice) immunoglobulin G, and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen. Mounting medium for immunohistochemical analysis (VECTASHIELD) was from Vector Laboratories (Burlingame, CA). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) was from Dojindo (Kumamoto, Japan). The RNeasy kit and HiPerFect were obtained from Qiagen, the first strand cDNA synthesis kit was from Takara (Kyoto, Japan), and SYBR GREEN PCR Master Mix was from ABI (Foster City, CA).

HSF1-null mice and their wild-type counterparts (ICR) (10–12 weeks of age and 30–35 g) or transgenic mice expressing HSP70 (gifts from Drs. Angelidis and Pagoulatos (University of Ioannina, Greece)) and their wild-type counterparts (C57/BL6) (6–8 weeks of age and 20–25 g) were prepared as described previously (27). Homozygotic transgenic mice 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—The gastric ulcerogenic response was examined as described previously (14), with some modifications. Mice fasted for 18 h were orally administered with indomethacin. Eight hours later, the animals were sacrificed, after which their stomachs were removed, and the areas of gastric mucosal lesions were measured by an observer unaware of the treatment they had received. Calculation of the scores involved measuring the area of all the lesions in square millimeters and summing the values to give an overall gastric lesion index. The gastric PGE₂ level was determined by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Cell Culture, Measurement of Caspase Activity and K⁺ Efflux, and siRNA Targeting—Human gastric adenocarcinoma (AGS) cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO₂ at 37 °C. The cells were exposed to indomethacin by changing the medium. The cells were cultured for 24 h and then used in experiments. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method as previously described (28). The measurement of caspase-3-like activity was as previously described (9, 11). K⁺ efflux from cells was monitored as previously described (21). The transfection with pcDNA3.1 containing the *hsp70* gene (29) was carried out using Lipofectamine (TM2000) according to the manufacturer's instructions. The cells were used for experiments after a 20-h recovery period.

We used siRNA, with the sequences 5'-ggagcgcaggugugudTdT-3' and 5'-acacaccgucuccaguccdTdT-3', as annealed oligonucleotides for repressing HSP70 expression. AGS cells were transfected with siRNA using HiPerFect transfection reagent according to the manufacturer's instructions. Nonsilencing siRNA (5'-uucuccgaacgugacgudTdT-3' and 5'-acgugacgucgagagaadTdT-3') were used as a negative control.

Fluorescence-activated Cell Sorting (FACS) Analysis—Apoptosis was monitored using PI analysis by FACS as described previously (11). The cells were collected by centrifugation, and the pellets were fixed with 70% ethanol and recentrifuged. The pellets were resuspended in phosphate-citrate buffer (0.2 M Na₂HPO₄ and 4 mM citric acid) and incubated for 20 min at room temperature. After centrifugation, the pellets were resuspended in DNA staining solution (50 mg/ml PI and 10 μg/ml RNase A) and incubated for 20 min at room temperature. Samples were scanned with a FACSCalibur (Becton Dickinson) cell sorter. For measuring only PI-DNA mediated fluorescence spe-

TABLE 1
Primers used

Gene	Forward primer	Reverse primer
<i>hsp25</i>	5'-cctcttccctatccctcgag-3'	5'-ttggctccagactgttcaga-3'
<i>hsp60</i>	5'-cggtgccaataacacaaacg-3'	5'-cttcaggggtgtcacaggt-3'
<i>hsp70</i>	5'-tggtgctgacgaagatgaag-3'	5'-aggctgaagatgagcacggtt-3'
<i>hsp90a</i>	5'-aaaggcagaggctgacaaga-3'	5'-aggggaggcatttcttcagt-3'
<i>hsp90b</i>	5'-gcggaacaagacaagaaaaag-3'	5'-gaagtggctcctccagtcag-3'
<i>atf4</i>	5'-tcaaacctcatgggttctcc-3'	5'-gtgtcatccaacgtggtcag-3'
<i>chop</i>	5'-tgcctttctcttcggacact-3'	5'-tgtgacctctgctggttctg-3'
<i>puma</i>	5'-gacgacctcaacgcacagta-3'	5'-ggagtcccatgatgagattgt-3'
<i>actin</i>	5'-tgcctttctcttcggacact-3'	5'-tgtgacctctgctggttctg-3'
<i>gapdh</i>	5'-tgcctttctcttcggacact-3'	5'-tgtgacctctgctggttctg-3'

cifically, we scanned cells without PI staining and only PI solution (without cells) to determine the signal threshold. For excluding debris and cell fragments, thresholds were set for eliminating the low channels (from 1 to 15), in other words, cells with low PI fluorescence (the G_1 peak was set as channel 400 of total 1024 channels) or for eliminating small cells by adjusting FSC and SSC light scatter parameters (according to the manufacturer's protocols). Apoptotic cells appeared as a hypodiploid peak (sub- G_1 peak) because of nuclear fragmentation and loss of DNA, and we calculated number of cells included this peak and determined the ratio of apoptotic cells to total cells (10,000). For statistical analysis, we measured three different samples in the same experiment.

Real Time RT-PCR Analysis—Total RNA was extracted from gastric tissues or AGS cells using the RNeasy kit according to the manufacturer's protocol. Samples (2.5 μ g of RNA) were reverse-transcribed using the first strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was used in real time RT-PCR (Bio-Rad Chromo 4 system) experiments using iQ SYBR Green Supermix and analyzed with Opticon Monitor software according to the manufacturer's instructions. The real time PCR cycle conditions were 95 °C for 3 min, followed by 45 cycles at 95 °C for 10 s and at 60 °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, glyceraldehyde-3-phosphate dehydrogenase or actin cDNA was used as an internal standard. The primers were designed using the Primer3 website (Table 1).

Immunoblotting Analysis—Total protein was extracted as described previously (30). The protein concentration of each sample was determined by the Bradford method (31). Samples were applied to 8% (HSP70) or 10% (actin, Bax, and cytochrome *c*) polyacrylamide SDS gels and subjected to electrophoresis, after which the proteins were immunoblotted with appropriate antibodies.

Histological, Immunohistochemical, and TdT-mediated Biotinylated UTP Nick End Labeling (TUNEL) Analyses—Gastric tissue samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin before being cut into 4- μ m sections.

For histological examination (hematoxylin and eosin staining), the sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. The samples were mounted with Malinol and inspected with the aid of an Olympus BX51 microscope.

For immunohistochemical analysis, the sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h

with antibody against HSP70 (1:250 dilution) in the presence of 2.5% bovine serum albumin and finally incubated for 1 h with Alexa Fluor 488 goat anti-mouse immunoglobulin G in the presence of DAPI (5 μ g/ml). The samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus BX51).

For TUNEL assay, the sections were incubated first with proteinase K (20 μ g/ml) for 15 min at 37 °C, then with TdT and biotin 14-ATP for 1 h at 37 °C, and finally with Alexa Fluor 488 conjugated with streptavidin for 1 h. The samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus BX51).

Staining of Cells—The cells were cultured on four-well Lab-Tek II glass slides (Nunc). After fixation with 4% formaldehyde for 20 min and permeabilization with 0.5% Triton X-100 for 5 min, nonspecific binding sites were blocked with 3% bovine serum albumin for 30 min. Immunostaining to detect the active form of Bax was performed with a polyclonal antibody (Bax N20) and Alexa Fluor 488 goat anti-rabbit immunoglobulin G. The cells were simultaneously stained with DAPI (5 μ g/ml). The cells were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus BX51).

Statistical Analysis—All of the values are expressed as the means \pm S.E. The Tukey test or the Student's *t* test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of $p < 0.05$.

RESULTS

Enhanced Gastric Ulcerogenic Response Induced by Indomethacin in HSF1-null Mice—The development of gastric lesions following oral administration of indomethacin was compared between wild-type and HSF1-null mice. Administration of indomethacin produced gastric lesions in a dose-dependent manner, and this lesion production was significantly worse in HSF1-null mice relative to wild-type controls (Fig. 1A). This result shows that HSF1 plays an important role in protecting gastric mucosa from indomethacin-induced lesions.

As mentioned above, both a decrease in PGE₂ and mucosal apoptosis play important roles in NSAID-produced gastric lesions, and we have examined these processes in HSF1-null mice. As shown in Fig. 1B, there was no significant difference in the gastric level of PGE₂ between the HSF1-null mice and wild-type mice in either the presence or absence of indomethacin treatment. The level of gastric mucosal apoptosis was determined by TUNEL assay. An increase in the number of TUNEL-positive (apoptotic) cells was observed after the indomethacin

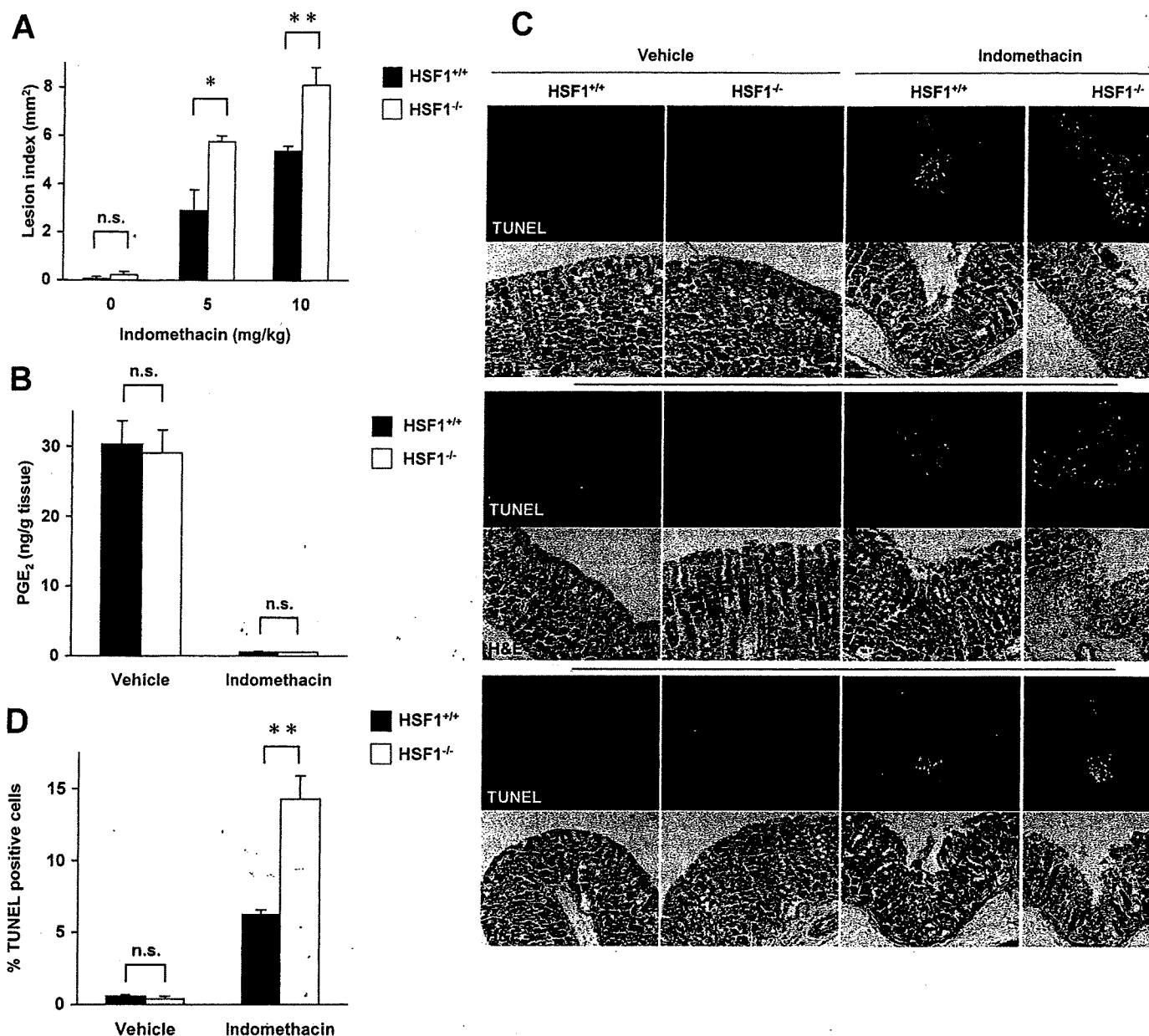


FIGURE 1. Indomethacin-produced gastric lesions in HSF1-null and wild-type mice. HSF1-null mice (-/-) and wild-type mice (ICR) (+/+) were orally administered the indicated doses (A) or 10 mg/kg (B-D) of indomethacin, and their stomachs were removed after 8 h. A, the stomach was scored for hemorrhagic damage. B, the gastric PGE₂ level was determined by enzyme-linked immunosorbent assay. C, sections of gastric tissues were prepared and subjected to hematoxylin and eosin (H&E) staining and TUNEL assay (with DAPI staining) (magnification, X200). D, a ratio of TUNEL-positive cells to total cells in the three sections was determined. A, B, and D, values are means ± S.E. (n = 3-6). **, p < 0.01; *, p < 0.05; n.s., not significant. The experiments shown in Fig. 1 were repeated at least three times, and basically similar results were obtained.

administration, and this increase was more apparent in HSF1-null mice than wild-type mice (Fig. 1, C and D). These results suggest that HSF1 protects the gastric mucosa from lesion formation by inhibiting indomethacin-induced apoptosis rather than by affecting the gastric PGE₂ level.

We monitored the expression of *hsp* mRNAs in gastric tissues by real time RT-PCR. Indomethacin administration up-regulated the expression of *hsp70* mRNA, with this expression significantly lower in indomethacin-treated HSF1-null mice than in the wild-type mice (Fig. 2A). Lack of the *hsf1* gene did not affect the background level of expression of *hsp70* mRNA (Fig. 2A). In contrast, the expression of *hsp25*, *hsp60*, *hsp90α*, and *hsp90β* mRNAs was not affected by either indomethacin

administration or lack of the *hsf1* gene (Fig. 2A). Based on these results, we subsequently focused on HSP70.

Immunoblotting and immunohistochemical analyses demonstrated that indomethacin administration increased the level of HSP70 in gastric mucosa in wild-type mice but not in HSF1-null mice (Fig. 2, B-D). Based on the results in Fig. 2, we proposed that the inability of HSF1-null mice to induce expression of HSP70 is responsible for their phenotypic sensitivity to indomethacin-induced gastric lesions and mucosal apoptosis.

Reduced Gastric Ulcerogenic Response Induced by Indomethacin in Transgenic Mice Expressing HSP70—To test the idea described above, the level of indomethacin-induced gastric lesions was compared between transgenic mice expressing

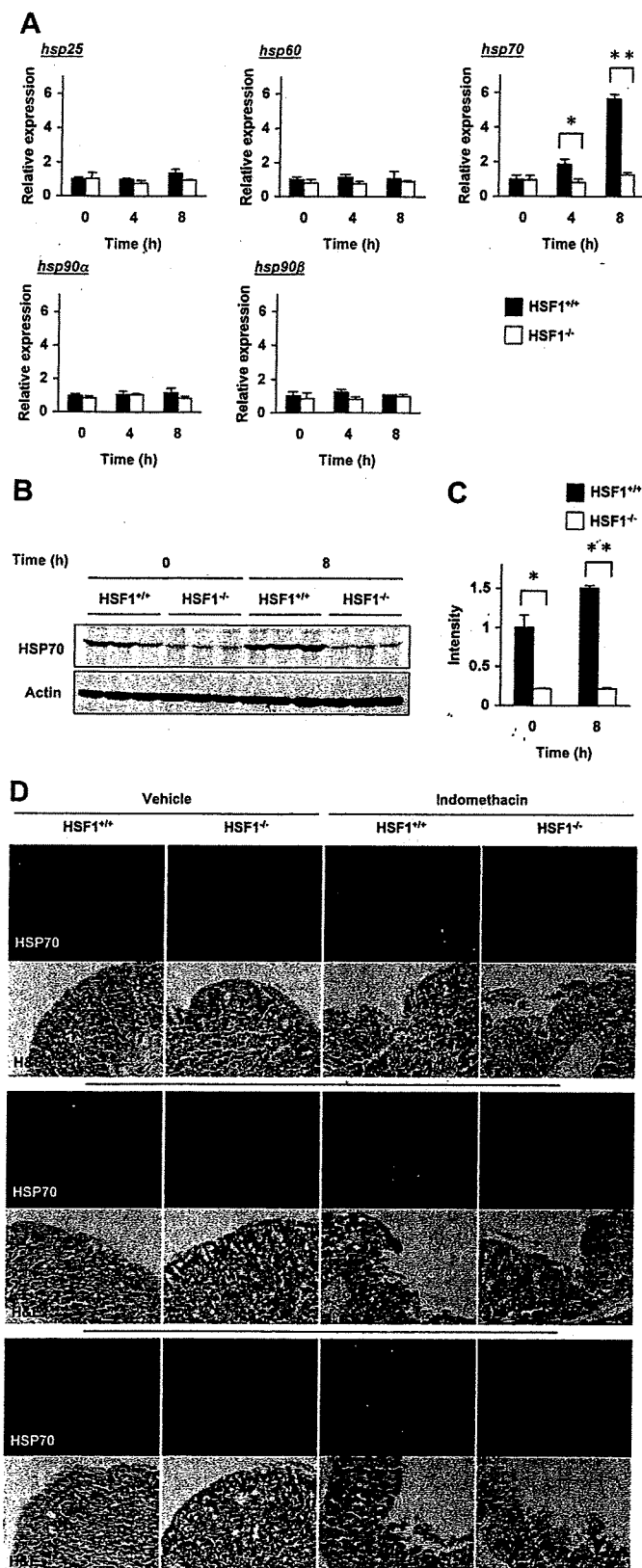


FIGURE 2. Indomethacin-induced expression of HSPs in gastric mucosa. HSF1-null mice ($-/-$) and wild-type mice (ICR) ($+/+$) were orally administered 10 mg/kg indomethacin, and the gastric mucosa was removed after the indicated periods (A) or 8 h (B–D). A, total RNA was extracted and subjected to real time RT-PCR using a specific primer for each gene. The values normalized to the *gapdh* gene are expressed relative to the control sample. The values are given as the means \pm S.E. ($n = 3-4$). **, $p < 0.01$; *, $p < 0.05$. B, whole cell extracts were prepared and the levels of HSP70 and actin were estimated by

immunoblotting. C, the band intensity of HSP70 in B was determined and expressed relative to the control sample. The values are given as the means \pm S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. D, sections of gastric tissues were subjected to hematoxylin and eosin (H&E) staining and immunohistochemical analysis with an antibody against HSP70 and DAPI staining (magnification, $\times 200$). The experiments shown in Fig. 2 were repeated at least three times, and basically similar results were obtained.

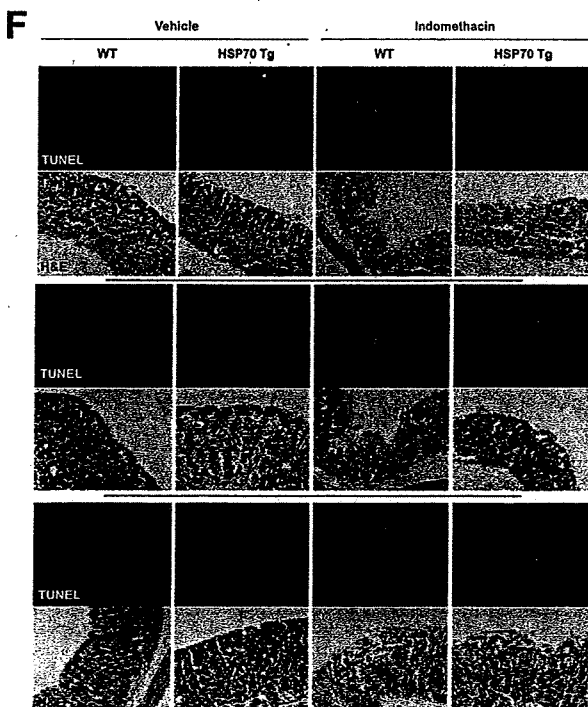
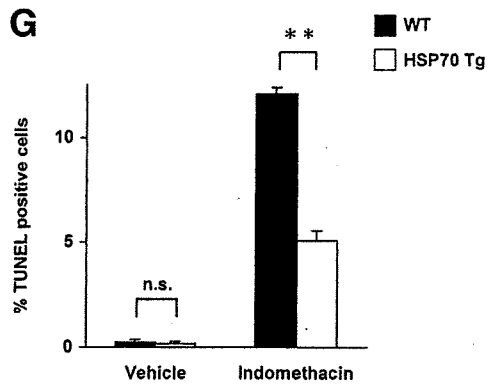
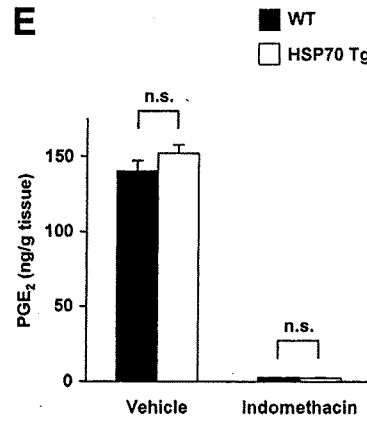
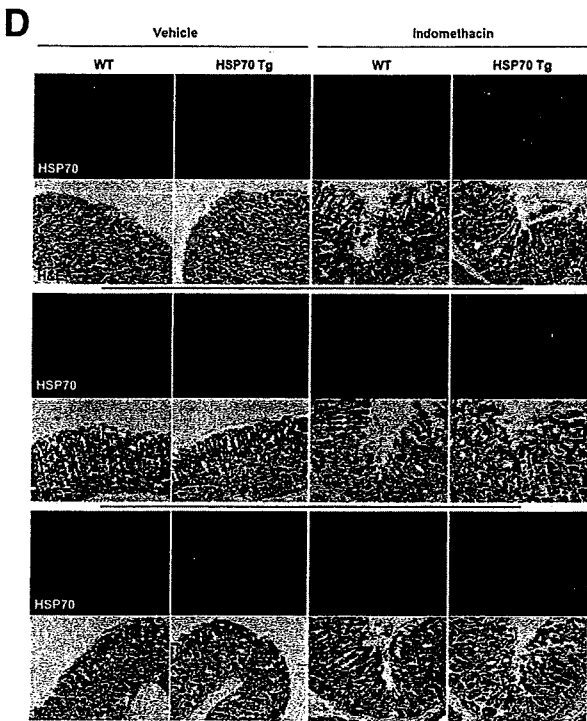
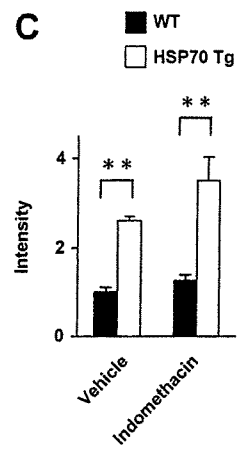
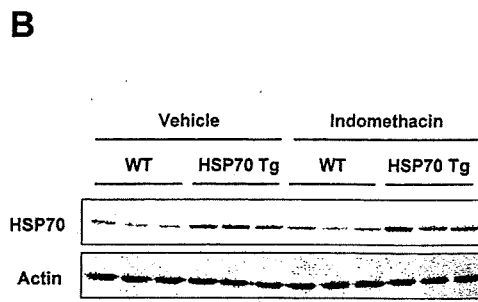
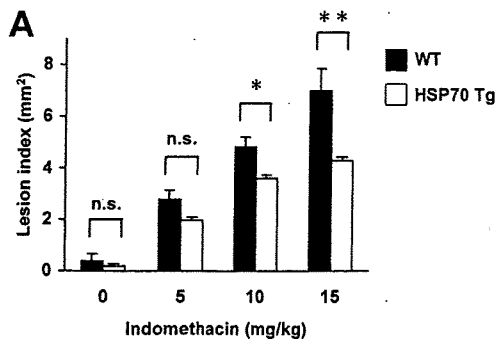
immunoblotting. C, the band intensity of HSP70 in B was determined and expressed relative to the control sample. The values are given as the means \pm S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. D, sections of gastric tissues were subjected to hematoxylin and eosin (H&E) staining and immunohistochemical analysis with an antibody against HSP70 and DAPI staining (magnification, $\times 200$). The experiments shown in Fig. 2 were repeated at least three times, and basically similar results were obtained.

HSP70 and wild-type mice. Relative to control mice, formation of indomethacin-induced gastric lesions was significantly suppressed in transgenic mice expressing HSP70 (Fig. 3A). By immunoblotting and immunohistochemical analyses, we confirmed that HSP70 expression was much higher in the gastric tissues of the transgenic mice than in those of the wild-type mice, regardless of whether or not they were treated with indomethacin (Fig. 3, B–D). These results suggest that HSP70 expression somehow suppresses the formation of indomethacin-induced gastric lesions. As shown in Fig. 3E, there was no significant difference in the gastric level of PGE₂ between transgenic mice expressing HSP70 and wild-type mice. On the other hand, the level of indomethacin-induced gastric mucosal apoptosis was lower in transgenic mice expressing HSP70 than in wild-type mice (Fig. 3, F and G). **Mechanism for the Role of HSP70 in Protecting against Indomethacin-induced Apoptosis**—To understand the molecular mechanism for HSP70-conferred protection against indomethacin-induced cell death *in vitro*, we first examined the effect of siRNA specific for HSP70 on indomethacin-induced cell death in AGS cells. Transfection of cells with siRNA for HSP70 inhibited the expression of HSP70 both in the presence and absence of indomethacin (Fig. 4A). As shown in Fig. 4B, treatment of cells with indomethacin induced cell death in a dose-dependent manner. Transfection of cells with siRNA for HSP70 stimulated this cell death but did not affect cell viability in the absence of indomethacin (Fig. 4B). We also found that overexpression of HSP70 slightly inhibited indomethacin-induced cell death (Fig. 4, C and D). Based on results reported in one of our previous papers (8), we speculated that the cell death evidenced in Fig. 4 (B and D) is mediated by apoptosis. To confirm this idea, we monitored indomethacin-induced apoptosis, using FACS analysis and measurement of caspase-3-like activity. Both analyses showed that indomethacin-induced apoptosis is stimulated by transfection of cells with siRNA for HSP70 (Fig. 4, E and F). The results in Fig. 4 suggest that knocking down of HSP70 stimulated indomethacin-induced apoptosis in AGS cells.

As described above, indomethacin-induced apoptosis is mediated by sequential induction of various cellular phenomena. We next examined which step of this apoptotic pathway is affected by expression of HSP70.

We have previously monitored NSAID-dependent permeabilization of cytoplasmic membranes by monitoring K⁺ efflux from cells (6, 21). The K⁺ concentration in the culture medium increased in the presence of indomethacin, showing that K⁺ efflux from AGS cells was stimulated (Fig. 5A). Down-regulation of expression of HSP70 by siRNA did not affect the K⁺ efflux (Fig. 5B). These results suggest that expression of

HSP70 and NSAID-induced Gastric Lesions



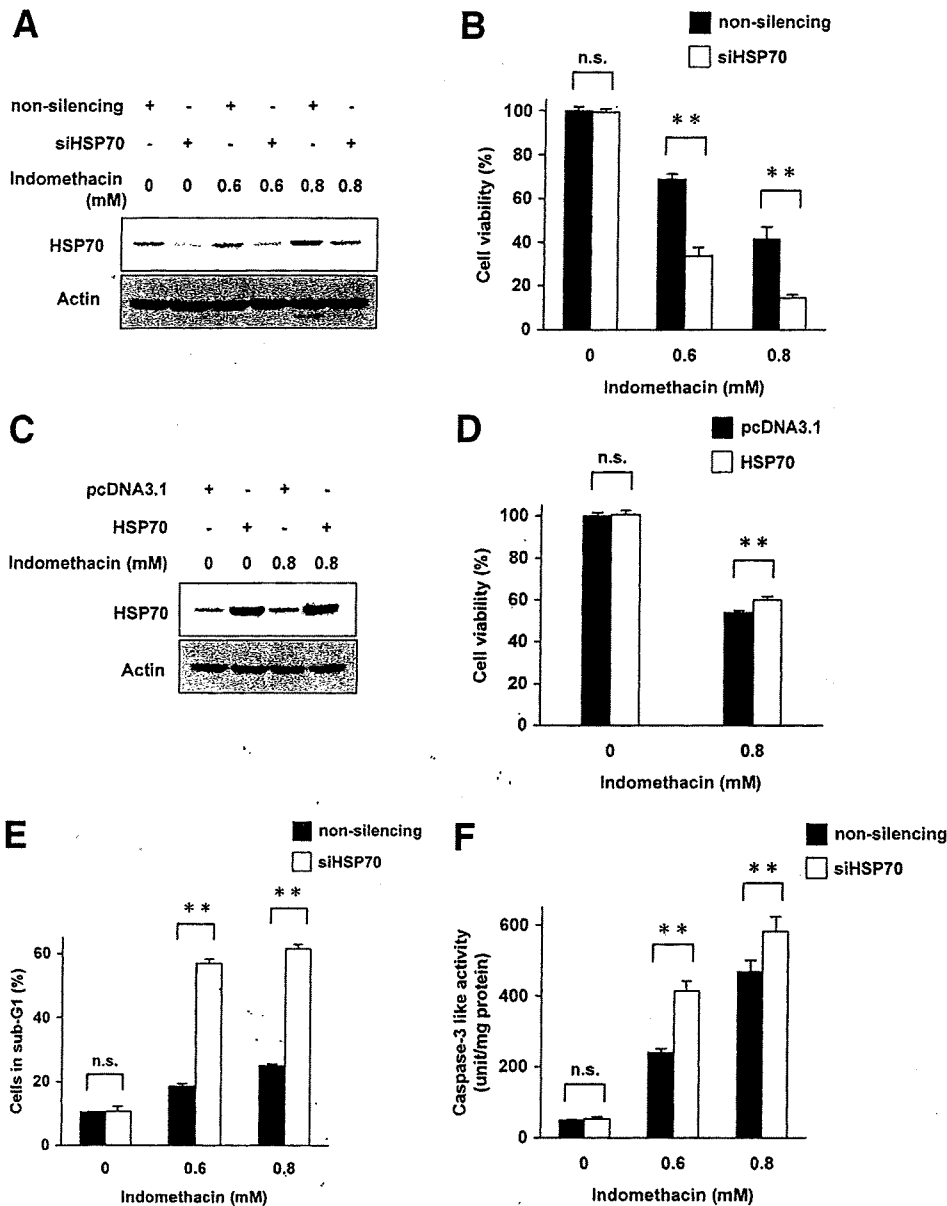


FIGURE 4. Effect of down- (or up)-regulation of expression of HSP70 on indomethacin-induced apoptosis *in vitro*. AGS cells were transfected with siRNA for HSP70 (siHSP70) or nonsilencing siRNA (nonsilencing). After 48 h, the cells were incubated with the indicated concentrations of indomethacin for 24 h (A, B, E, and F). HSP70-overexpressing cells (HSP70) and the mock transfectant control cells (pcDNA3.1) were incubated with the indicated concentrations of indomethacin for 24 h (C and D). A and C, the levels of HSP70 and actin were estimated by immunoblotting. B and D, cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method. E, apoptotic cell number was determined by FACS. F, caspase-3-like activity was measured. B and D–F, values are mean \pm S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; n.s., not significant. The experiments shown in Fig. 4 were repeated at least two times, and basically similar results were obtained.

HSP70 did not affect indomethacin-dependent membrane permeabilization.

Next we examined the effect of expression of HSP70 on the ER stress response, using real time RT-PCR. As shown in Fig. 5C, treatment of cells with indomethacin up-regulated the mRNA expression of not only *chop* and *puma* but also *atf4*,

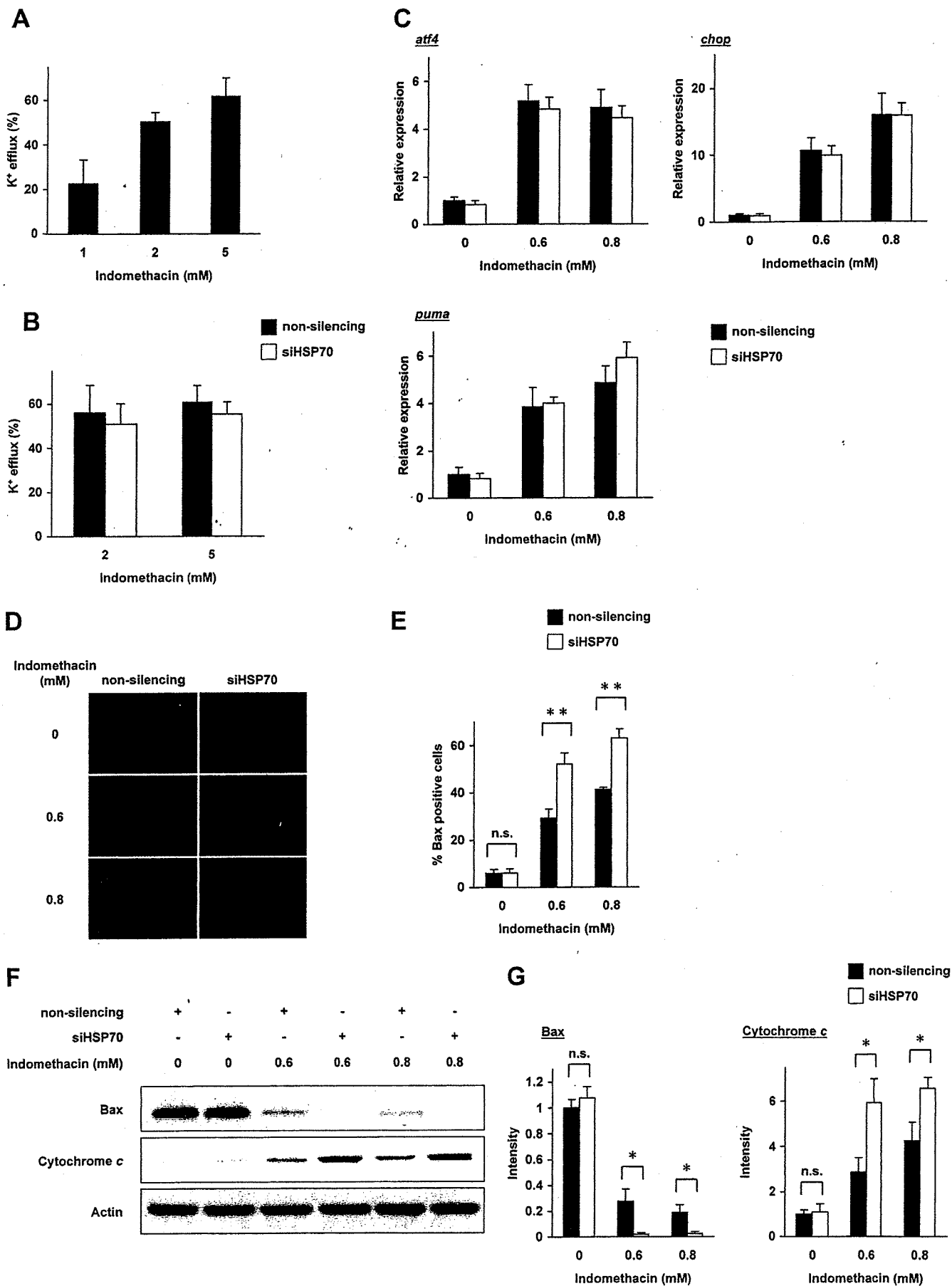
which is an ER stress response-related transcription factor that regulates these genes (13), showing that treatment of AGS cells with indomethacin induces the ER stress response. Down-regulation of expression of HSP70 by siRNA did not affect the indomethacin-dependent up-regulation of expression of these genes (Fig. 5C), suggesting that expression of HSP70 did not affect the ER stress response.

We previously reported that in the pathway for NSAID-induced apoptosis, activation of Bax through conformational change and its resulting translocation from the cytosol to mitochondria occur after induction of ER stress response (13). The effect of expression of HSP70 on activation of Bax was tested by immunostaining analysis using an antibody that specifically recognizes the active form of Bax. This antibody can recognize only the active form in the immunostaining assay but can recognize all forms of Bax in the immunoblotting assay because of the denaturing of proteins in the latter assay (32). As shown in Fig. 5D, the active form of Bax was observed in indomethacin-treated cells but not in control cells, showing that indomethacin induces the conformational change in Bax. Furthermore, the levels of the active form of Bax observed with indomethacin treatment increased in cells transfected with siRNA for HSP70 (Fig. 5D). As shown in Fig. 5E, the number of Bax (active form)-positive cells increased in the presence of indomethacin, and the transfection with siRNA for HSP70 enhanced this increase, suggesting that expression of HSP70 suppresses the indomethacin-dependent activation of Bax.

As shown in Fig. 5 (F and G), the amount of Bax or cytochrome *c* in the cytosol fractions decreased or increased, respectively, in the presence of indomethacin, and these alterations were further enhanced by transfection with siRNA for HSP70. This sug-

FIGURE 3. Indomethacin-produced gastric lesions in transgenic mice expressing HSP70 and in wild-type mice. Transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (C57/BL6) (WT) were administered the indicated doses (A) or 10 mg/kg (B–G) of indomethacin and production of gastric lesions (A), expression of HSP70 (B–D), gastric level of PGE₂ (E), and gastric mucosal apoptosis (F and G) were examined as described in the legends of Figs. 1 and 2 (D and F, magnification, $\times 200$). The values are the means \pm S.E. ($n = 3–6$). **, $p < 0.01$; *, $p < 0.05$; n.s., not significant. The experiments shown in Fig. 3 were repeated at least three times and basically similar results were obtained. H&E, hematoxylin and eosin.

HSP70 and NSAID-induced Gastric Lesions



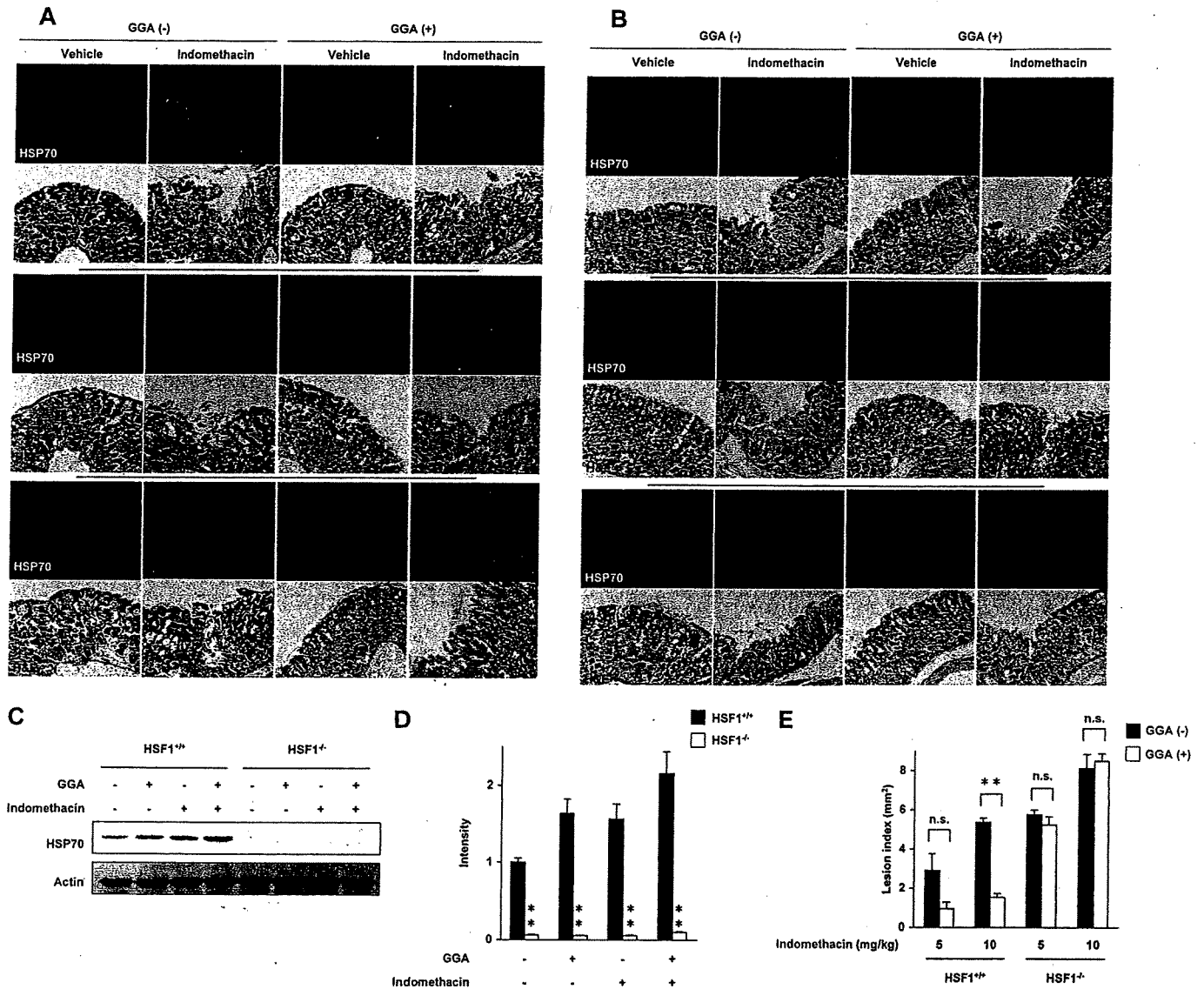


FIGURE 6. Effect of indomethacin and/or GGA on expression of HSP70 and production of gastric lesions. HSF1-null mice (X/X) and wild-type mice (ICR) (+/+) were orally administered 50 mg/kg GGA (10 ml/kg as an emulsion with 5% gum arabic), 1 h after which they were orally administered 10 mg/kg (A–D) or the indicated doses (E) of indomethacin, and the stomach was removed after 8 h. A–D, immunohistochemical and immunoblotting analyses were performed as described in the legend of Fig. 2 (A and B, magnification, $\times 200$). D, experiments shown in C were repeated for six mice/condition, and the results (band intensity of HSP70) were analyzed statistically. The values are the means \pm S.E. ($n = 6$). **, $p < 0.01$. E, gastric mucosal lesions were measured as described in the legend of Fig. 1. The values are the means \pm S.E. ($n = 3-4$). **, $p < 0.01$; n.s., not significant. The experiments shown in Fig. 6 were repeated at least two times, and basically similar results were obtained. H&E, hematoxylin and eosin.

gested that expression of HSP70 suppresses indomethacin-dependent translocation of Bax and mitochondrial outer membrane permeabilization (mitochondrial dysfunction).

Anti-ulcer and HSP-inducing Activities of GGA in HSF1-null Mice—To evaluate the contribution of the HSP inducing activity of GGA to its protective effect against NSAID-produced gastric lesions, we investigated the activities of GGA in HSF1-

null mice. First, we examined the effect of GGA and/or indomethacin on gastric expression of HSP70. An induction of HSP70 expression by indomethacin or GGA alone was observed in the gastric mucosa of wild-type mice (Fig. 6A). Preadministration of GGA enhanced the indomethacin-induced expression of HSP70 (Fig. 6A). On the other hand, HSP70 expression was not observed in HSF1-null mice under

FIGURE 5. Mechanism for the protective effect of HSP70 against indomethacin-induced apoptosis. AGS cells (A) or those transfected with siRNA for HSP70 (siHSP70) or nonsilencing siRNA (nonsilencing) (B–G) were incubated with the indicated concentrations of indomethacin for 10 min (A and B), 12 h (C–E) or 24 h (F and G). A and B, the level of K⁺ efflux was measured using a K⁺ ion-selective electrode. Melittin (10 μ M) was used to establish the 100% level of K⁺ efflux. C, the relative expression of each gene was monitored by real time RT-PCR using a specific primer for each gene. The values normalized to the *actin* gene are expressed relative to the control sample. D, immunostaining with the antibody against the N-terminal region of Bax (Bax N20) and DAPI staining were performed as described under “Experimental Procedures” (magnification, $\times 400$). E, $\sim 400-600$ cells were randomly counted for staining with Bax N20. F, after subcellular fractionation, cytosolic fractions were analyzed by immunoblotting with an antibody against Bax (Bax N20), cytochrome *c*, or actin. G, the band intensity of BAX and cytochrome *c* in three independent experiments (one of them is shown in F) was determined and shown. A–C, E, and G, values are the means \pm S.E. ($n = 3-4$). **, $p < 0.01$; *, $p < 0.05$; n.s., not significant. The experiments shown in Fig. 5 were repeated at least two times, and basically similar results were obtained.

any conditions (Fig. 6B). Similar results were observed in immunoblotting assay (Fig. 6, C and D). Fig. 6E shows the effect of preadministration of GGA on indomethacin-produced gastric lesions in wild-type and HSF1-null mice. Preadministration of GGA significantly suppressed the formation of indomethacin-induced gastric lesions in wild-type mice but not in HSF1-null mice (Fig. 6E). This result shows that HSF1 is required for the anti-ulcer activity of GGA against indomethacin. GGA was administered prior to the administration of indomethacin in Fig. 6E. It seems that preinduction of HSP70 is required to protect against the indomethacin-induced gastric lesions, because indomethacin alone can induce HSP70 to an extent similar to that of GGA alone (Fig. 6D). Overall, the results in Fig. 6 suggest that the loss of the protective effect of GGA against indomethacin in HSF1-null mice is due to the lack of expression of HSP70. In other words, the HSP inducing activity of GGA contributes to its protective effect against the formation of NSAID-produced gastric lesions.

DISCUSSION

A number of previous observations have suggested that HSPs, especially HSP70, play an important role in protecting gastric mucosa against the development of lesions (17, 18, 33–39). However, prior to the current study, little direct evidence, genetic or otherwise, existed to support this idea. We recently addressed this issue by using mice that lack the ability to induce HSPs; HSF1-null mice are more susceptible to ethanol- or hydrochloric acid-produced gastric lesions (24). However, the effect of up-regulation of expression of HSP70 on irritant-induced production of gastric lesions had not been tested. Furthermore, NSAIDs, which are clinically more important as a cause of gastric lesions, were not tested in our previous study. In the present study, we have shown that HSF1-null mice are more susceptible to the formation of indomethacin-induced gastric lesions than controls. Furthermore, we have shown that transgenic mice expressing HSP70 are more resistant to the formation of indomethacin-induced gastric lesions. These results offer direct genetic evidence that expression of HSP70 protects the gastric mucosa against the formation of NSAID-induced gastric lesions. HSP70 normally has a reasonable level of expression in cells, and this may contribute to protection of gastric mucosa.

HSP70 seems to protect gastric mucosa from NSAID-induced lesions through inhibiting apoptosis rather than affecting gastric PGE₂ levels; indomethacin-induced gastric mucosal apoptosis, but not the induced decrease in the gastric level of PGE₂, was ameliorated in transgenic mice expressing HSP70. We also showed that up-regulation or down-regulation of expression of HSP70 in cultured gastric cells make cells resistant or sensitive, respectively, to indomethacin-induced apoptosis. Of the various steps in the NSAID-induced apoptosis pathway (permeabilization of cytoplasmic membranes, stimulation of Ca²⁺ influx across cytoplasmic membranes, increase in the intracellular Ca²⁺ level, induction of the ER stress response (up-regulation of expression of CHOP and PUMA), translocation and activation of Bax, mitochondrial dysfunction, and activation of caspases), the translocation and activation of Bax seems to be the target of HSP70 for conferral of its inhibitory

effect on NSAID-induced apoptosis. This may be concluded because indomethacin-dependent translocation and activation of Bax but not up-regulation of expression of CHOP and PUMA was enhanced by down-regulation of HSP70 expression. Although the mechanism by which HSP70 suppresses the activation of BAX is unclear at present, the direct interaction between HSP70 and Bax may be responsible, as previously reported (40, 41). 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 (42–45). Thus, these effects of HSP70 may also be involved in HSP70-dependent suppression of indomethacin-induced apoptosis. The effect of siRNA for HSP70 on apoptosis was more clear in Fig. 4E (apoptosis monitored by FACS) than Fig. 4F (apoptosis monitored by caspase-3-like activity), and we consider that this is because HSP70 suppresses the apoptotic pathway downstream of caspase-3 and caspase-3-independent pathway, such as apoptosis-inducing factor-induced chromatin condensation.

Of the various HSPs tested, oral administration of indomethacin up-regulated expression of HSP70 only. Although it is already known that NSAIDs induce expression of HSP70 in cultured cells (10, 15, 16), this is the first demonstration that administration of NSAIDs induces expression of HSP70 at the gastric mucosa in animals. Diclofenac (another NSAID) was recently reported to induce expression of HSP70 at the gastric mucosa in humans (46). Combining these results, it seems that induction of HSP70 by NSAIDs at the gastric mucosa plays an important role in protection against the formation of NSAID-produced gastric lesions in humans. Similar specific up-regulation of expression of HSP70 (not a general induction of HSPs) has also been observed for ethanol-induced gastric lesions (24), although various HSPs are induced by these stressors (NSAIDs and ethanol) in cultured cells (15). Thus, expression of HSP70 seems to be specifically regulated at the gastric mucosa in response to stressors, although the mechanism is unknown at present.

In addition to the cytoprotective effect of HSP70, an anti-inflammatory effect of HSP70 was recently revealed. Up-regulation of HSP70 expression by heat shock was found to inhibit the inflammatory stimuli-dependent activation of nuclear factor κ B (NF- κ B), which is responsible for induction of production of various pro-inflammatory cytokines (such as tumor necrosis factor- α , IL-1 β , and IL-6) (47, 48). We recently reported that lipopolysaccharide-induced production of tumor necrosis factor- α , IL-1 β , and IL-6 was inhibited in peritoneal macrophages prepared from transgenic mice expressing HSP70, compared with those from wild-type controls (27). Given that it is well known that pro-inflammatory cytokines stimulate the production of NSAID-induced gastric lesions (49), it is possible that expression of HSP70 suppresses the development of NSAID-induced gastric lesions through its anti-inflammatory effect.

GGA has attracted considerable attention as an HSP inducer, largely because of its clinical value as an anti-ulcer drug and because it can induce HSPs without affecting cell viability (17). We have previously reported that GGA makes cultured gastric cells resistant to indomethacin and simultaneously up-regu-

lates expression of HSP70 (18). In the current study we have shown that artificial expression of HSP70 makes cells resistant to indomethacin. Furthermore, we have revealed that preadministration of GGA suppresses the formation of indomethacin-induced gastric lesions in wild-type mice but not in HSF1-null mice in which up-regulation of gastric mucosal expression of HSP70 by GGA alone and stimulation of the indomethacin-induced expression of HSP70 by GGA were not observed. These results strongly suggest that the HSP inducing activity of GGA contributes to its gastroprotective activity against NSAIDs. These findings should be clinically relevant because it was recently reported that orally administered GGA (at clinical doses) up-regulated HSP70 expression at the gastric mucosa in humans while simultaneously suppressing diclofenac-induced gastric damage (46). Thus, we propose that nontoxic HSP70 inducers are therapeutically beneficial for NSAID-induced gastric lesions.

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HSP70 Confers Protection against Indomethacin-Induced Lesions of the Small Intestine^S

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ABSTRACT

In line with improvements in diagnostic procedures to detect intestinal lesions, it has become clear that nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin induce lesions not only in the stomach but also in the small intestine. However, clinical protocols for the treatment of NSAID-induced lesions of the small intestine have not been established. It is known that heat shock proteins (HSPs), particularly HSP70, confer protection against various stressors, and more recently, the anti-inflammatory activity of HSP70 was revealed. In this study, we examined the effect of expression of HSP70 on indomethacin-induced lesions of the small intestine. The extent of indomethacin-induced lesions to the small intestine was reduced in transgenic mice expressing HSP70 compared with controls. Oral administration of indomethacin increased the expression of HSP70 in the small intestine. Administration of

indomethacin also induced mucosal cell apoptosis and expression of proinflammatory cytokines in the small intestines of control mice, with both of these responses suppressed in the transgenic mice. Geranylgeranylacetone (GGA), a clinically used antiulcer drug, increased expression of HSP70 in the small intestine and suppressed indomethacin-induced lesions of the small intestines in wild-type mice. These results suggest that indomethacin-induced increase in HSP70 expression reduces the extent of lesions to the small intestine by suppressing mucosal cell apoptosis and inflammatory responses. The HSP-inducing activity of GGA seems to contribute to the protective effect of drug against the lesions. Based on these results, we propose that nontoxic HSP70-inducers, such as GGA, would be therapeutically beneficial for treating NSAID-induced lesions of the small intestine.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are an important class of drugs, and 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), such as PGE₂, that have a strong capacity to induce inflammation. NSAID use, however, is associated with gastrointestinal complications. More attention has generally been paid to gastric lesions than lesions of the small intestine, because the latter are

usually asymptomatic and they have been difficult to diagnose. However, recent improvements in the capabilities of diagnostic techniques, such as capsule endoscopy and double-balloon endoscopy, have revealed that lesions of the small intestine occur very frequently and that the small intestine is even more susceptible than gastric tissue to the detrimental effects of NSAIDs (Lanas and Ferrandez, 2006; Maiden et al., 2007). For example, it was reported that 50 to 70% of chronic users of NSAIDs have lesions of the small intestine (Morris et al., 1991; Graham et al., 2005). For gastric lesions, COX-2-selective NSAIDs have been developed as safer alternatives; however, animal and clinical studies have revealed that the safety of long-term use of such COX-2-selective NSAIDs with respect to the small intestine is indistinguishable from that of nonselective NSAIDs (Sigthorsson et al., 2002; Maiden et al., 2007).

The balance between aggressive and defensive factors

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ABBREVIATIONS: CHOP, C/EBP homologous transcription factor; COX, cyclooxygenase; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; FasL, Fas ligand; GGA, geranylgeranylacetone; HSP, heat shock protein; LPS, lipopolysaccharide; MPO, myeloperoxidase; NSAID, nonsteroidal anti-inflammatory drug; PUMA, p53 up-regulated modulator of apoptosis; PG, prostaglandin; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated biotinylated UTP nick end labeling; RT, reverse transcriptase; PCR polymerase chain reaction.

determines the development of gastric lesions. For NSAID-induced gastric lesions, mucosal cell death induced by gastric acid and NSAIDs themselves (aggressive factors) and decreases in the gastric level of PGE₂ (a defensive factor) play an important role. Therefore, drugs that decrease aggressive factors (acid-control drugs, such as histamine-2 receptor antagonists and proton pump inhibitors) or increase defensive factors are therapeutically effective. Compared with gastric lesions, the etiology of NSAID-induced lesions of the small intestine is not clear at present, thus complicating the establishment of clinical protocols for their treatment. However, recent studies suggest that NSAID-induced lesions of the small intestine share some but not all of the aggressive and defensive factors evident with gastric lesions. The direct cytotoxicity (topical effect) of NSAIDs seems to be involved in NSAID-induced lesions of the small intestine (Somasundaram et al., 2000; Basivireddy et al., 2002), and this effect is stimulated by enterohepatic circulation (Reuter et al., 1997). Inflammatory responses, such as the infiltration of neutrophils, stimulate NSAID-induced lesions of the small intestine (Wallace, 1994). Bacterial invasion, bacterial products, bile, and nitric oxide produced by inducible nitric-oxide synthase also seem to damage the small intestinal mucosa to produce lesions (Whittle et al., 1995; Konaka et al., 1999; Jacob et al., 2007). However, acid secretion is not as important in the development of NSAID-induced lesions of the small intestine. Thus, acid-control drugs are not as effective for treating NSAID-induced lesions of the small intestine as they are for treating gastric lesions (Aabakken et al., 1990; Goldstein et al., 2007). On the other hand, a decrease in PGs is one of major causes of NSAID-induced lesions of the small intestine (Kunikata et al., 2002; Tanaka et al., 2002). In fact, several animal and clinical studies have shown that oral administration of PGs is therapeutically effective for treating such lesions (Morris et al., 1994; Watanabe et al., 2008).

Different stressors induce cells to express heat shock proteins (HSPs). Expression of HSPs, especially HSP70, in cultured cells protects them against a range of stressors, including NSAIDs (Mathew and Morimoto, 1998). Interestingly, geranylgeranylacetone (GGA), a leading antiulcer drug on the Japanese market, has been reported to be a nontoxic HSP inducer (Hirakawa et al., 1996; Tomisato et al., 2000). In addition to the cytoprotective effects of HSP70, anti-inflammatory effects have also been suggested (Tang et al., 2007). Thus, it is reasonable to speculate that HSP70 protects against NSAID-induced lesions of the small intestine, thereby acting as a defensive factor in the small intestine as it does in the case of stomach tissue. Although the results of a number of *in vitro* studies support this idea (Urayama et al., 1998), no direct evidence currently exists. In this study, we show that transgenic mice expressing HSP70 are more resistant than wild-type mice to the indomethacin-induced lesions of the small intestine. Furthermore, we show that oral administration of GGA has a protective effect on lesions of this type. These results suggest that HSP70 protects against NSAID-induced lesions of the small intestine and that nontoxic HSP70-inducers, such as GGA, could be therapeutically beneficial in the treatment of such lesions.

Materials and Methods

Chemicals and Animals. Paraformaldehyde, peroxidase standard, and *o*-dianisidine were obtained from Sigma-Aldrich (St. Louis, MO). The PGE₂ enzyme-linked immunosorbent assay kit used here was from Cayman Chemical (Ann Arbor, MI). Indomethacin and quercetin were from Wako Pure Chemicals (Tokyo, Japan). Mayer's hematoxylin, 1% eosin alcohol solution, and malinol were from Muto Pure Chemicals (Tokyo, Japan). Terminal deoxynucleotidyl transferase (TdT) was obtained from Toyobo Company Ltd. (Osaka, Japan). Biotin 14-ATP, Alexa Fluor 488 conjugated with streptavidin, and Alexa Fluor 488 goat anti-mouse immunoglobulin G were purchased from Invitrogen (Carlsbad, CA). GGA was supplied by Eisai Company Ltd. (Tokyo, Japan). Mounting medium for immunohistochemical analysis (VECTASHIELD) was from Vector Laboratories (Burlingame, CA). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) was from Dojindo Laboratories (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 the iQ SYBR Green Supermix was from Bio-Rad Laboratories (Hercules, CA). Transgenic mice expressing HSP70 (a gift from Drs. Angelidis and Pagoulatos, University of Ioannina, Ioannina, Greece), their wild-type counterparts (C57/BL6) (6–8 weeks of age and 20–25 g) and other wild-type mice (ICR, 10–12 weeks of age and 30–35 g) were prepared as described previously (Tanaka et al., 2007). Homozygotic transgenic mice were used in experiments. The experiments and procedures described here were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health and were approved by the Animal Care Committee of Kumamoto University.

Small Intestine Damage Assay. The intestinal ulcerogenic response was examined as described previously (Tanaka et al., 2005a), with some modifications. Indomethacin was orally administered to unfasted mice, the animals were sacrificed 24 h later, and both the jejunum and ileum were removed and treated with formalin for fixation. Samples were opened along the antimesenteric attachment. To calculate lesion scores an observer unaware of the treatment animals had received measured the area of all lesions in square millimeters and summed the values to give an overall lesion index. GGA was orally administered to mice as emulsion with 5% gum arabic at the volume of 10 ml/kg.

Myeloperoxidase (MPO) activity was measured as described previously (Tanaka et al., 2007). Both the jejunum and ileum were removed, rinsed with cold saline, and cut into small pieces. Samples were homogenized in 50 mM phosphate buffer, freeze-thawed, and centrifuged. The protein concentrations of the supernatants were determined by use of the Bradford method. MPO activity was determined in 10 mM phosphate buffer with 0.5 mM *o*-dianisidine, 0.00005% (w/v) hydrogen peroxide, and 20 µg of protein. MPO activity was obtained from the slope of the reaction curve, and its specific activity was expressed as the number of hydrogen peroxide molecules converted per minute per milligram of protein.

Immunoblotting Analysis. Total protein was extracted from the tissues as described previously (Tomisato et al., 2000). The protein concentration of the sample was determined by the Bradford method. Samples were applied to 10% polyacrylamide SDS gels and subjected to electrophoresis, after which the proteins were immunoblotted with appropriate antibodies.

Real-time RT-PCR Analysis. Total RNA was extracted from small intestine tissue with use of an RNeasy kit according to the manufacturer's protocol. Samples (2.5 µg of RNA) were reverse-transcribed with use of a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was used in real-time RT-PCR [Chromo 4 instrument (Bio-Rad)] experiments with use of iQ SYBR GREEN Supermix and analyzed with Opticon Monitor Software according to the manufacturer's instructions. The real-time PCR cycle conditions were 2 min at 50°C followed by 10

min at 90°C and finally 45 cycles of 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, glyceraldehyde-3-phosphate dehydrogenase cDNA was used as an internal standard.

Primers were designed using the Primer3 website (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.www.cgi>). The primers used (name: forward primer, reverse primer) for detection of mouse cDNA included: *hsp70*: 5'-tggtgctgacgaagatgaag-3', 5'-aggtcgaagatgacacgtt-3'; *tnf-α*: 5'-cgtcagccgatttgctatct-3', 5'-cggactccgcaagtctaag-3'; *il-1β*: 5'-gatcccaagcaatacccaaa-3', 5'-ggggaactctgcagactcaa-3'; *il-6*: 5'-ctggagtcacagaaggagtgg-3', 5'-ggtttcccgatgatctcaa-3'; *mip-2*: 5'-accctccaagggttgacttc-3', 5'-ggcacatcaggtacgatccag-3'; *mcp-1*: 5'-ctcactgctgctactcattc-3', 5'-gcttgagggtggttggaaaa-3'; *gapdh*, 5'-aactttggcattgtggaagg-3', 5'-acattgggggttaggaaca-3. Mouse peritoneal macrophages were prepared as described previously (Tanaka et al., 2007).

Immunohistochemical and TUNEL Analyses. Tissue samples from the small intestine were fixed in 4% buffered paraformaldehyde and embedded in paraffin before being cut into 4-μm-thick sections.

For histological examination (hematoxylin and eosin staining), sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. Samples were mounted with Malinol and inspected with the aid of an Olympus BX51 microscope (Olympus America, Inc., Central Valley, PA). For histological evaluation of the tissue damage (histological score), sections were evaluated microscopically by an observer unaware of the treatment the animals had received and were quantified as described previously (Boushey et al., 1999): 0, normal bowel; 1, epithelial loss confined to the villus tip; 2, epithelial detachment from the underlying lamina propria; 3, epithelial detachment involving less than half of the villus; and 4, epithelial detachment involving more than half of the villus and/or ulceration.

For immunohistochemical analysis, sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with antibody against HSP70 (1:250 dilution) in the presence of 2.5% bovine serum albumin, and finally incubated for 1 h with Alexa Fluor 488 goat anti-mouse immunoglobulin G in the presence of DAPI (5 μg/ml). Samples were mounted with VECTASHIELD and inspected by use of fluorescence microscopy (Olympus BX51).

For TdT-mediated biotinylated UTP nick end labeling (TUNEL) assay, sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37°C and then with TdT 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 BX51).

Statistical Analysis. All values are expressed as the mean ± S.E.M. Two-way analysis of variance followed by the Tukey test was used. Differences were considered to be significant for values of $P < 0.05$.

Results

Indomethacin-Induced Lesions of the Small Intestine and Expression of HSP70. The severity of indomethacin-induced lesions in the small intestine was monitored by measurement of a lesion index and MPO activity. We compared the development of lesions in the small intestine after administration of indomethacin between transgenic mice expressing HSP70 and wild-type mice. Indomethacin induced lesions in the small intestine in a dose-dependent manner in wild-type mice, and this production was significantly reduced in transgenic mice expressing HSP70 (Fig. 1A). MPO activity, an indicator of inflammatory infiltration of leukocytes, was increased in wild-type mice in response to the administration of indomethacin. On the other hand, the activity was

lower in indomethacin-administered transgenic mice expressing HSP70 than in wild-type controls (Fig. 1B). The expression of HSP70 did not affect the background level of MPO activity (Fig. 1B). Histological analysis revealed that crypt loss and infiltration of leukocytes in the small intestine could be observed in sections from indomethacin-administered wild-type mice. This intestinal damage was not so apparent, however, in transgenic mice expressing HSP70 (Fig. 1E). These results show that transgenic mice expressing HSP70 are more resistant than wild-type mice to indomethacin-induced lesions of the small intestine.

Using immunoblotting, we monitored expression of HSP70 in the small intestine of indomethacin-administered and untreated transgenic mice expressing HSP70 and wild-type mice. The expression of HSP70 was significantly higher both in indomethacin-treated or untreated transgenic mice expressing HSP70 than in the wild-type controls (Fig. 1, C and D). The results also show that indomethacin administration increases the expression of HSP70 in wild-type mice (Fig. 1, C and D). Immunohistochemical analysis demonstrated that indomethacin administration increased HSP70 levels in the small intestine in wild-type mice and that HSP70 staining was much greater in both indomethacin-treated and untreated transgenic mice expressing HSP70 than in wild-type controls (Fig. 1E). These results show that administration of indomethacin increases the level of HSP70 in the small intestine. To identify cells expressing HSP70, we performed costaining assay. As shown in Supplemental Fig. S1, strong costaining of HSP70 with CD11b (a marker of macrophage), CD4 (a marker of T lymphocyte), and CD31 (a marker of vascular endothelial cell) was observed at the intestinal tissues, especially those from transgenic mice expressing HSP70 or wild-type mice treated with indomethacin. A relatively weak costaining of HSP70 with E-cadherin (a marker of epithelial cells) was also observed, but costaining of HSP70 with MPO (a marker of neutrophil) was not observed (Supplemental Fig. S1). These results suggest that the transgenic mice express HSP70 in various types of cells at the small intestine. Based on the results of Fig. 1, we considered that expression of HSP70 somehow offered protection to the small intestine against indomethacin-induced lesions.

Mechanism for Protective Role of HSP70 against Indomethacin-Induced Lesions. As described in the Introduction, a decrease in the level of PGE₂ (COX inhibition), the presence of mucosal cell apoptosis, and induction of cytokines and chemokines all play important roles in the NSAID-induced production of lesions of the small intestine. Therefore, we compared these factors between transgenic mice expressing HSP70 and wild-type mice. As shown in Fig. 2A, there was no significant difference between transgenic mice expressing HSP70 and wild-type mice either with or without indomethacin treatment in the level of PGE₂ in the small intestine. The extent of mucosal cell apoptosis in the small intestine was also determined in this case by TUNEL assay. An increase in the number of TUNEL-positive (apoptotic) cells in the small intestine of wild-type mice was observed after indomethacin administration, and this increase was clearly suppressed in transgenic mice expressing HSP70 (Fig. 2B). Expression of HSP70 did not affect the background level of apoptosis (Fig. 2B). These results suggest that expression of HSP70 protects the small intestine mucosa from

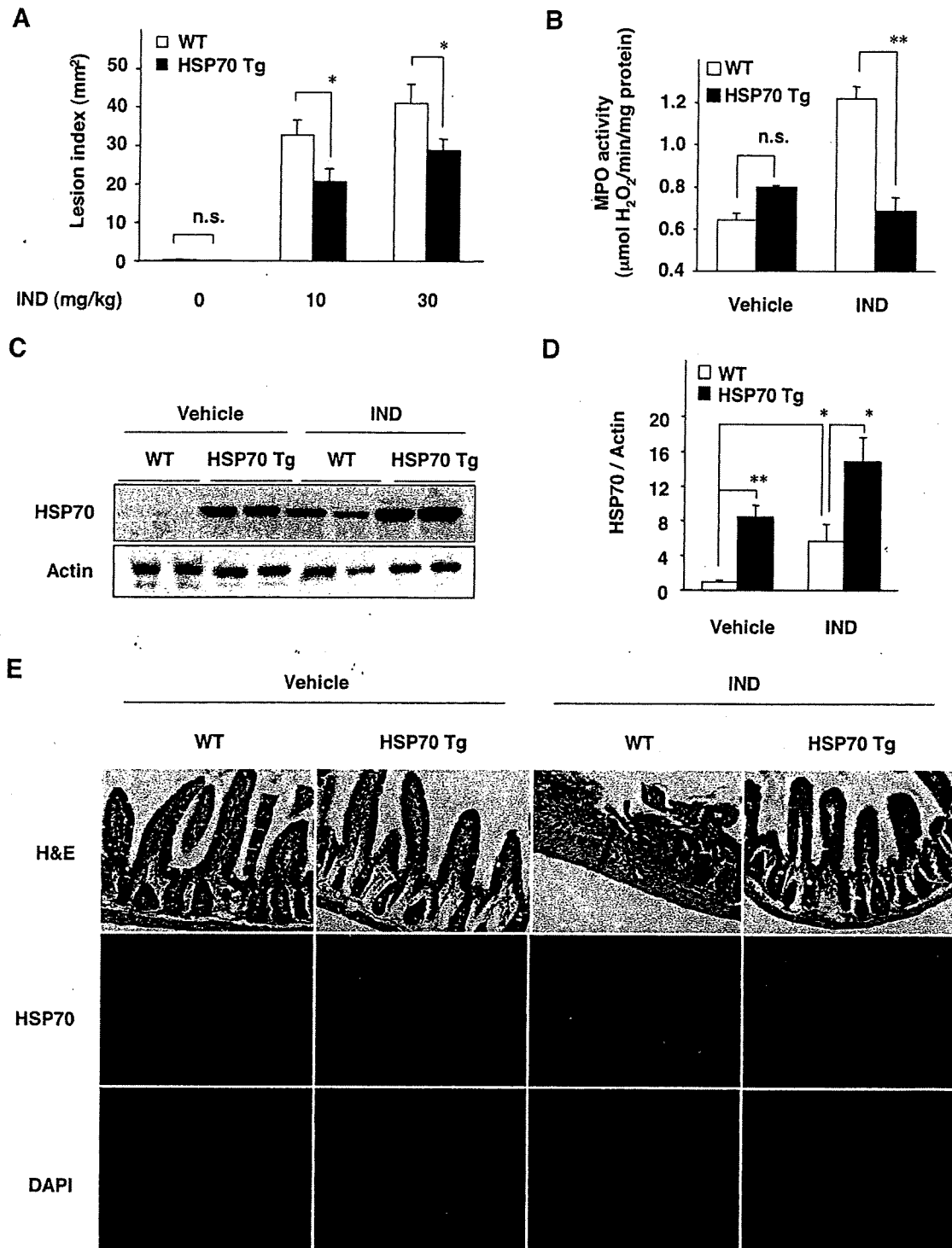


Fig. 1. Indomethacin-induced lesions of the small intestine and expression of HSP70. The indicated doses (A) or 30 mg/kg indomethacin (IND) (B–E) were orally administered to wild-type mice (WT, C57/BL6) and transgenic mice expressing HSP70 (HSP70 Tg), and the small intestine was removed after 24 h. A, the small intestine was scored for hemorrhagic damage ($n = 3-10$). B, small intestine MPO activity ($n = 3-6$) was determined. C, protein extract was prepared and analyzed by immunoblotting with an antibody against HSP70 or actin. D, the band intensity of HSP70 was determined by densitometric scanning, normalized with respect to actin ($n = 6-12$). E, sections of small intestinal tissue were prepared and subjected to H&E staining and immunohistochemical analysis, with an antibody against HSP70. A, B, and D, values are mean \pm S.E.M. ** $P < 0.01$; * $P < 0.05$; n.s., not significant; H&E, hematoxylin and eosin.

lesions by inhibiting indomethacin-induced apoptosis rather than by affecting the level of PGE₂ in the small intestine.

We subsequently compared levels of mRNA expression of various proinflammatory cytokines and chemokines by real-time RT-PCR for transgenic mice expressing HSP70 and wild-type mice. As shown in Fig. 3, mRNA expression levels

of all of the cytokines (*il-1 β* , *il-6*, and *tnf- α*) and chemokines (*mcp-1* and *mip-2*) tested were up-regulated in wild-type mice by the administration of indomethacin. However, the expression of *il-1 β* , *il-6*, and *mip-2* mRNA was significantly lower in indomethacin-treated transgenic mice expressing HSP70 than in wild-type controls (Fig. 3). The expression of

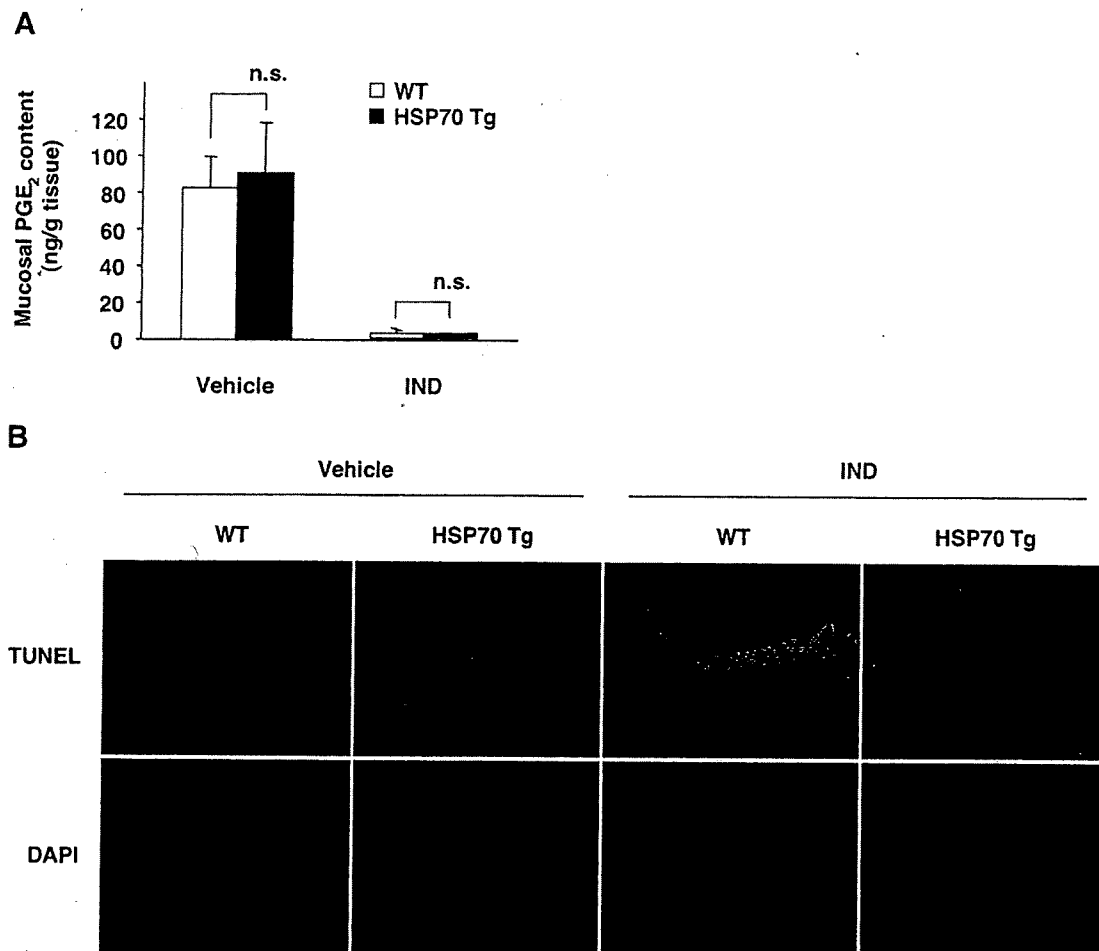


Fig. 2. Indomethacin-induced decrease in PGE₂ level and mucosal cell apoptosis in small intestine. Indomethacin (IND) (30 mg/kg) was orally administered to wild-type (WT, C57/BL6) and transgenic mice expressing HSP70 (HSP70 Tg), and the small intestine was removed after 24 h. **A**, the small intestine PGE₂ level was determined by enzyme-linked immunosorbent assay. Values are mean \pm S.E.M. ($n = 6$). n.s., not significant. **B**, sections of small intestine tissue were prepared and subjected to TUNEL assay and DAPI staining.

HSP70 did not affect the background expression of these genes (Fig. 3). These results suggest that the reduced expression of these proinflammatory cytokines and chemokines in transgenic mice expressing HSP70 is involved in their phenotypic resistance to indomethacin-induced lesions of the small intestine.

The results in Fig. 3 suggest that HSP70 negatively regulates the expression of the proinflammatory cytokines and chemokines under inflammatory conditions. To test this idea in vitro, we compared LPS-stimulated mRNA expression of the proinflammatory cytokines and chemokines in peritoneal macrophages prepared from transgenic mice expressing HSP70 and wild-type mice. As shown in Supplemental Fig. S2, LPS stimulated the mRNA expression of all of these proinflammatory cytokines and chemokines. The expression of *il-1 β* and *il-6* but not other genes was significantly lower in LPS-treated macrophages prepared from transgenic mice expressing HSP70 than from wild-type mice (Supplemental Fig. S2). These results suggest that expression of HSP70 suppresses the expression of these proinflammatory cytokines under inflammatory conditions.

Effect of GGA on Indomethacin-Induced Lesions and Expression of HSP70. We next examined the effect of preadministration of GGA on indomethacin-induced lesions in the small intestine. As shown in Fig. 4A, preadministra-

tion of GGA suppressed the indomethacin-induced lesions in a dose-dependent manner. This GGA administration also suppressed the indomethacin-induced increase in MPO activity (Fig. 4B) but did not affect the background levels of lesions (data not shown) and MPO activity (Fig. 4B). Figure 4C (top) shows the results of histological analysis of small intestinal tissues. Extensive crypt loss, epithelial destruction, and infiltration of leukocytes were observed in sections from indomethacin-administered mice, and the damage was not so apparent in indomethacin-administered mice that were preadministered with GGA (Fig. 4C, top). Histological score analysis revealed that the histological differences were statistically significant (Fig. 4D). We also examined by immunoblotting analysis the effect of GGA and/or indomethacin on the expression of HSP70 in the small intestine. Administration of indomethacin increased the expression of HSP70 (Fig. 4, E and F), whereas GGA significantly stimulated the expression of HSP70 in both the presence and absence of indomethacin treatment (Fig. 4, E and F). Increase in the level HSP70 after the administration of GGA and/or indomethacin was also confirmed by immunohistochemical analysis (Fig. 4C). As shown in Supplemental Fig. S3, strong costaining of HSP70 with CD11b, CD4, and CD31 and weak costaining of HSP70 with E-cadherin were observed at the intestinal tissues, especially those from mice treated with

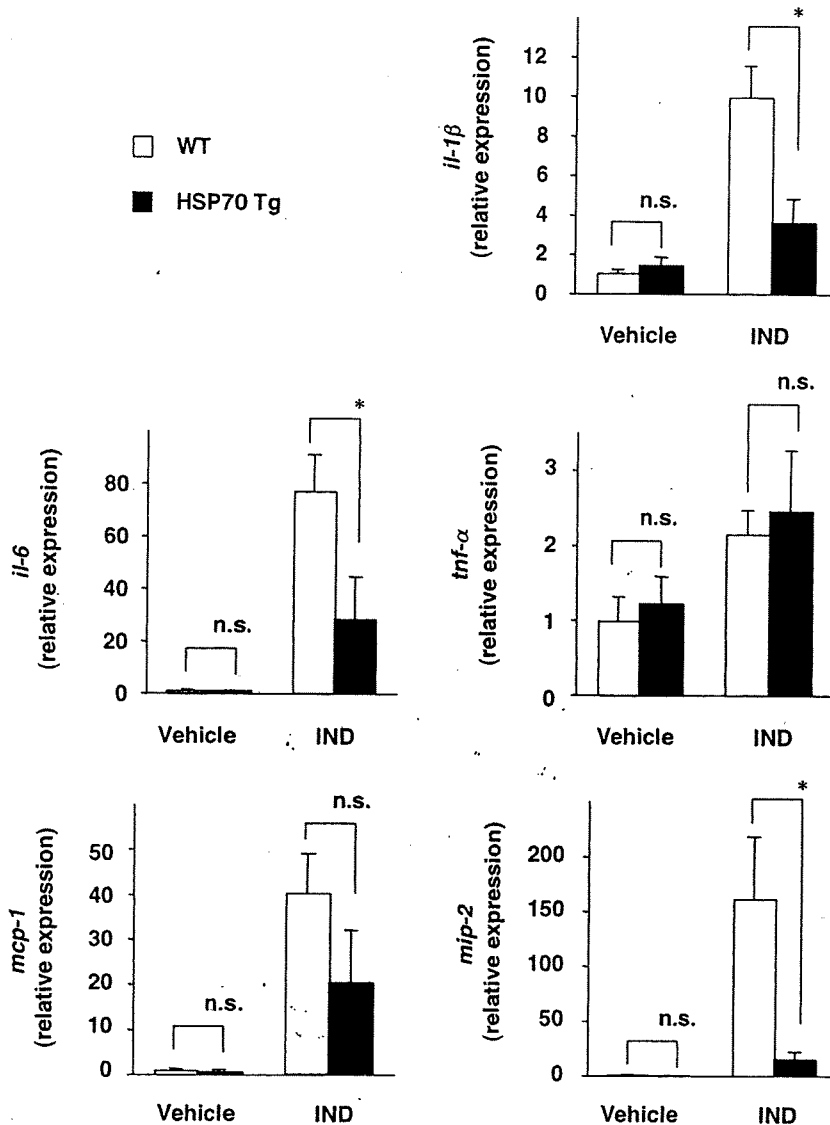


Fig. 3. Indomethacin-induced mRNA expression of various genes in the small intestine. Indomethacin (IND) (30 mg/kg) was orally administered to wild-type (WT, C57/BL6) and transgenic mice expressing HSP70 (HSP70 Tg), and the small intestine was removed after 24 h. Total RNA was extracted and subjected to real-time RT-PCR with use of a specific primer for each gene. Values normalized to the *gapdh* gene are expressed relative to the control sample and given as the mean \pm S.E.M. ($n = 3-9$). *, $P < 0.05$; n.s., not significant; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; H&E, hematoxylin and eosin.

GGA and/or indomethacin. To test the involvement of HSP70 in the protective role of GGA against indomethacin-induced lesions of the small intestine, we examined the effect of preadministration of quercetin (an inhibitor of expression of HSP70) on the protective effect of GGA. As shown in Fig. 4G, preadministration of quercetin diminished the protective effect of GGA against indomethacin-induced lesions of the small intestine, suggesting that GGA suppresses the extent of indomethacin-induced lesions in the small intestine by inducing HSP70.

We also examined the effect of postadministration of GGA on indomethacin-induced lesions of the small intestine. As shown in Fig. 4H, GGA did not significantly suppress the indomethacin-induced lesions when it was administered after the administration of indomethacin, suggesting that preinduction of HSP70 is required for protection against indomethacin-induced lesions of the small intestine.

We also examined the effect of preadministration of GGA on the indomethacin-dependent decrease in the level of PGE₂ and increased level of mucosal cell apoptosis in the small intestine. As shown in Fig. 5A, administration of GGA increased the level of PGE₂ in the small intestine in the absence of indomethacin treatment; however, GGA did not af-

fect this level in the presence of indomethacin. On the other hand, the indomethacin-induced increase in the number of TUNEL-positive cells was suppressed by the preadministration of GGA (Fig. 5B). These results suggest that the GGA-induced expression of HSP70 suppressed the extent of indomethacin-induced lesions by inhibiting indomethacin-induced mucosal cell apoptosis.

Discussion

Many previous studies have used transgenic mice to show that induction of HSP70 expression protects against the development of various diseases, such as inflammatory bowel disease, hypoxic/ischemic brain injury, and spinal and bulbar muscular atrophy (Adachi et al., 2003; Matsumori et al., 2005; Tanaka et al., 2007). In this study, we have gathered evidence to show that HSP70 protects the small intestine against development of NSAID-induced lesions by demonstrating that transgenic mice expressing HSP70 exhibit a phenotype resistant to this disease. In addition to the possibility that HSP70 directly protects the small intestine against NSAIDs, it is also possible that HSP70 affects the

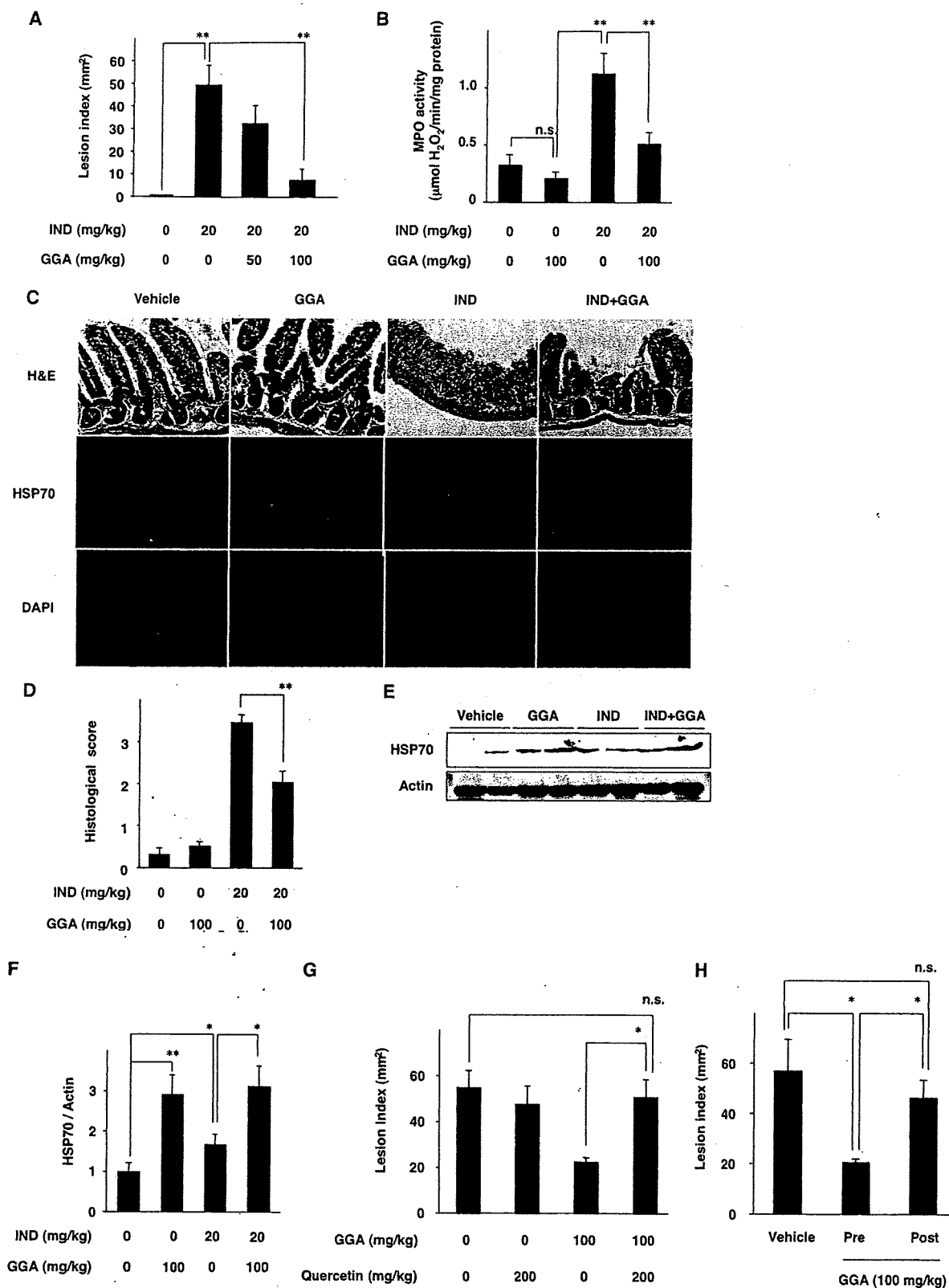


Fig. 4. Effect of GGA on expression of HSP70 and production of lesions in the small intestine. The indicated doses (A) or 100 mg/kg GGA (B–G) (10 ml/kg as an emulsion with 5% gum arabic) were orally administered to wild-type mice (ICR). Quercetin was orally administered 2 h before the GGA administration. Two hours later, 20 mg/kg indomethacin (IND) was orally administered to the mice (A–G). GGA was orally administered 2 h before (Pre) or after (Post) the administration of 20 mg/kg indomethacin (H). The small intestine was removed 24 h (A, B, G, and H) or 4 h (C–F) after the administration of indomethacin. Analyses were performed as described in the legend of Fig. 1 (A–C and E–H) or under *Materials and Methods* (D). A, B, D, and F–H, values are mean \pm S.E.M. [$n = 3-9$ (A), 3–6 (B), 10–16 (D), 5–9 (F), 4–6 (G), 4–9 (H)]. **, $P < 0.01$; *, $P < 0.05$; n.s., not significant.

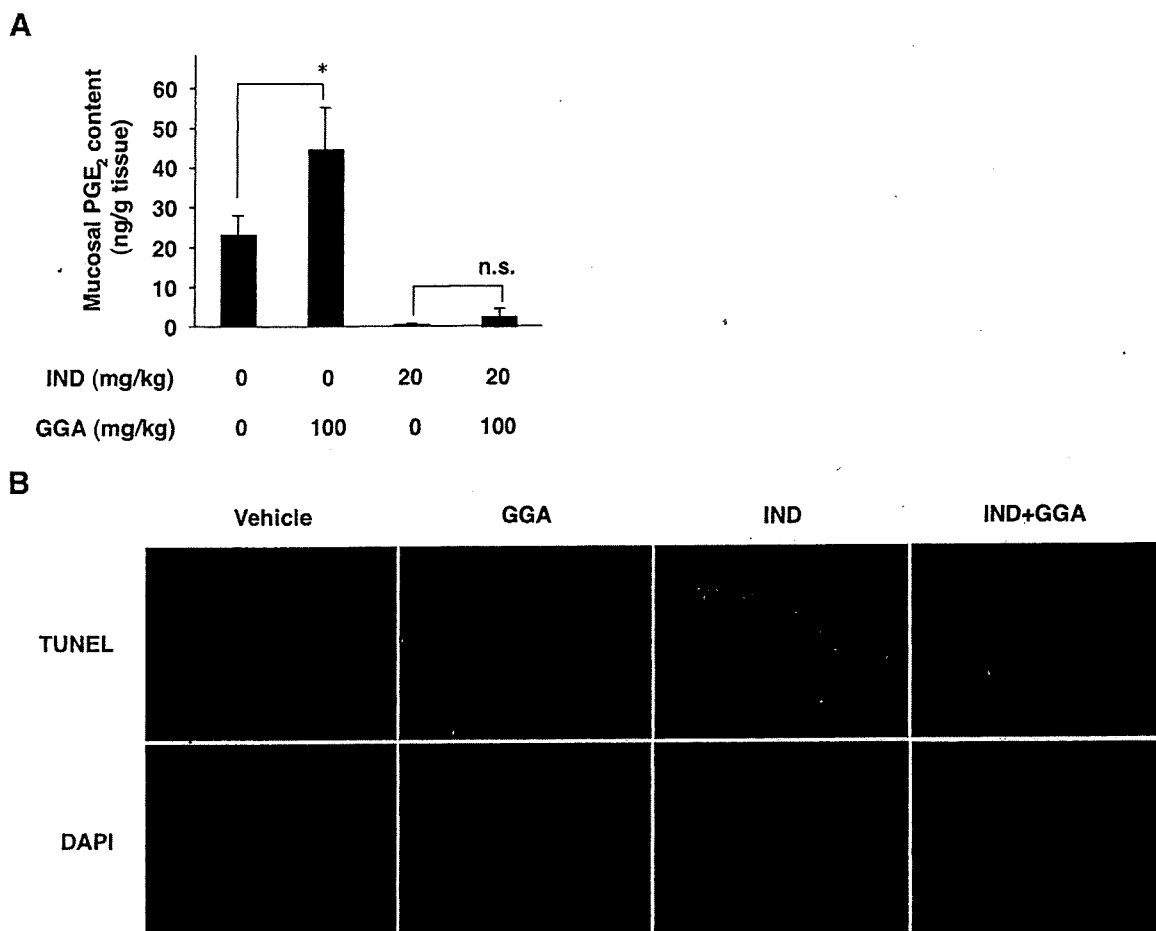


Fig. 5. Effect of GGA on indomethacin-dependent decrease in PGE₂ level and mucosal cell apoptosis. GGA (100 mg/kg) (10 ml/kg as an emulsion with 5% gum arabic) was orally administered to wild-type mice (ICR). Two hours later, 20 mg/kg indomethacin was orally administered to the mice, and the small intestine was removed after 4 h (A) or 24 h (B). Analyses were performed as described in the legend of Fig. 2. Values are mean \pm S.E.M. *, $P < 0.05$; n.s., not significant.

expression of other mediators that would influence NSAID-induced lesions of the small intestine.

HSP70 seems to protect the small intestine from NSAID-induced lesions by inhibiting mucosal cell apoptosis rather than by affecting PGE₂ levels; indomethacin-dependent mucosal cell apoptosis, but not the level of PGE₂ in the small intestine, was reduced in transgenic mice expressing HSP70. Therefore, results in this study also support the notion that NSAID-induced mucosal apoptosis is involved in the production of NSAID-induced lesions of the small intestine. We previously reported that NSAIDs, ibuprofen and nabumetone, have less activity for inducing apoptosis than indomethacin *in vitro* (Tomisato et al., 2004; Arai et al., 2005), suggesting that lesion-inducing activities of these NSAIDs *in vivo* are relatively weak. In fact, we found that ibuprofen and nabumetone produced fewer lesions of the small intestine than indomethacin (Supplemental Fig. S4).

As for the molecular mechanism governing NSAID-induced apoptosis, we proposed the following pathway. Permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca²⁺ influx and increases intracellular Ca²⁺ levels, which in turn induces the endoplasmic reticulum stress response (Tanaka et al., 2005b). In the endoplasmic reticulum stress response, an apoptosis-inducing transcription factor, C/EBP homologous transcription factor (CHOP), is induced and we have shown previously that CHOP is essential for NSAID-

induced apoptosis (Tsutsumi et al., 2004). CHOP induces the expression of p53 up-regulated modulator of apoptosis (PUMA) and the resulting translocation and activation of Bax. We have already shown that both PUMA and Bax play an important role in NSAID-induced mitochondrial dysfunction and in the activation of caspases and apoptosis (Ishihara et al., 2007). Among these various steps in the pathway for NSAID-induced apoptosis, we recently found that translocation and activation of Bax seems to be a target of HSP70 for its inhibitory effect on NSAID-induced apoptosis, because the NSAID-dependent translocation and activation of Bax, but not up-regulation of expression of CHOP and PUMA, was enhanced by the down-regulation of HSP70 expression in cultured cells (Suemasu et al., 2009). Supporting this idea, the inhibitory effect of HSP70 expression on heat shock- or nitric oxide-dependent translocation and activation of Bax and a physical interaction between HSP70 and Bax were reported previously (Gotoh et al., 2004; Stankiewicz et al., 2005). Therefore, the inhibitory effect of HSP70 expression on NSAID-induced apoptosis in the small intestine may be the result of HSP70 inhibition of the NSAID-dependent translocation and activation of Bax. Another mechanism may be also involved in NSAID-induced apoptosis and its suppression by expression of HSP70. For example, Fas/Fas Ligand (FasL) interaction is one of the important mechanisms by which indomethacin induces apoptosis (Maity et al., 2008).