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

Teita Asano, Ken-Ichiro Tanaka, Naoki Yamakawa, Hiroaki Adachi, Gen Sobue, Hidemi Goto, Koji Takeuchi, and Tohru Mizushima

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan (T.A., K-I.T., N.Y., T.M); Nagoya University Graduate School of Medicine, Nagoya, Japan (H.A., G.S., H.G.); and Kyoto Pharmaceutical University, Kyoto, Japan (K.T.)

Received February 10, 2009; accepted May 19, 2009

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. Geranylgeranyacetone (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 anti-inflammatory 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...
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 PGE_2 (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 infection, 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 cytotoxic 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 δ-diamidine were obtained from Sigma-Aldrich (St. Louis, MO). The PGE_2 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 (Venica, 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 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., 2005a), with some modifications. Indomethacin was orally administered to fasted 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 score, observers were unaware of the treatment animals had received. 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.

Immunohistological 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. MPO activity was determined in 10 mM phosphate buffer with 0.5 mM 0-diamididine, 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.

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 were performed 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.

Primer3 was designed using the Primer3 website (http://frodo.wi.mit.edu/ primer3/primer3.3.25/www.cgi). The primers used (name: forward primer, reverse primer) for detection of mouse cDNA included: hsp70: 5'-tggcttgaagatagtaag-3', 5'-aatctgcaagattcattc-3'; tnf-α: 5'-ctgatgagttcgctgtt-3', 5'-tgggattaagctgtaa-3'; il-1β: 5'-gactcgaacgatacaacaaa-3', 5'-gggagacttctgacatac-3'; il-6: 5'-gtggagtcacagaaggtgg-3', 5'-gtgctgcagtagatctea-3'; mtp-2: 5'-actcgcgccaaggtgta-3', 5'-ccaacttaagatgcagca-3'; mcpi: 5'-ctcactctgctgctactcattc-3', 5'-ggtttgccgagtagatctaa-3'; gapdh, 5'-aacctttgcaatttggaag-3', 5'-aaccattttgggtaggaaa-3'; mmtf, 5'-aggtcgaagatgagcacgtt-3', 5'-ggtttgccgagtagatctaa-3'; mtaf, 5'-gatcccaagcaatacccaaa-3'. Samples were maintained 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 0.25% bovine serum albumin, and finally incubated for 1 h with Alexa Fluor 488 goat antibody. Immunohistochemical 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 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).

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 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 and mice that HSP70 staining was much higher 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 maker of T lymphocyte), and CD31 (a maker 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 maker 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 provided protection to the small intestine against indomethacin-induced lesions.
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-1β, 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-1β, 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. 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 ± S.E.M. **P < 0.01; *P < 0.05; n.s., not significant; H&E, hematoxylin and eosin.
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 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-1β 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, preadministration 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...
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 PGE$_2$ and increased level of mucosal cell apoptosis in the small intestine. As shown in Fig. 5A, administration of GGA increased the level of PGE$_2$ in the small intestine in the absence of indomethacin treatment; however, GGA did not affect 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 ± 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.
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).

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 ± S.E.M. * P < 0.05; n.s., not significant.
We examined the expression of Fas and FasL at the small intestine by immunohistochemical analysis and found that indomethacin administration increased the expression of FasL, and this increase was suppressed in transgenic mice expressing HSP70 (Supplemental Fig. S5), suggesting that Fas/FasL interaction is involved in NSAID-induced apoptosis and its suppression by expression of HSP70 at the small intestine. It is also possible that high levels of proinflammatory cytokine secretion, which are reduced significantly by Hsp70, are responsible for suppression of NSAID-induced apoptosis by expression of HSP70 at the small intestine.

In addition to the cytoprotective effect of HSP70, an anti-inflammatory effect of HSP70 has also been reported. For example, up-regulation of HSP70 expression by heat shock inhibits the inflammatory stimuli-dependent activation of nuclear factor-kB, which is responsible for inducing the production of various proinflammatory cytokines (Krappmann et al., 2004). We recently reported that the LFS-induced production of proinflammatory cytokines, including interleukin 1β and interleukin 6, was inhibited in peritoneal macrophages prepared from transgenic mice expressing HSP70 compared with their wild-type controls (Tanaka et al., 2007). As described above, inflammation plays an important role in the production of NSAID-induced lesions of the small intestine, and we found that the indomethacin-dependent elevation of intestinal MPO activity, an indicator of inflammation, was suppressed in transgenic mice expressing HSP70. Furthermore, we found that the indomethacin-induced expression of mRNA for some cytokines and chemokines (iL-1β, iL-6, and miP-2) in the small intestine was suppressed in transgenic mice expressing HSP70. We consider that expression of HSP70 suppresses the expression of these genes in the small intestine through its inhibitory effect on nuclear factor-kB and that this effect is involved in the protective role of HSP70 against NSAID-induced lesions of the small intestine.

GGA has attracted considerable attention as an HSP inducer, largely because of its clinical value as an antiulcer drug and because it can induce HSPs without affecting cell viability (Hirakawa et al., 1996). We reported previously that GGA made cultured gastric cells resistant to indomethacin simultaneously with the up-regulation of expression of HSP70 (Tomisato et al., 2000). It was also reported that GGA made cultured intestinal cells resistant to oxidative stress simultaneously with the up-regulation of expression of HSP70 (Okahara et al., 2006). Furthermore, we found that preadministration of GGA not only increases the intestinal expression of HSP70 but also suppresses the production of indomethacin-induced lesions of the small intestine. The protection by GGA of the small intestine against NSAID-induced lesions was also reported recently (Kamei et al., 2008). These results strongly suggest that oral administration of GGA could also be therapeutically beneficial against NSAID-induced lesions of the small intestine in humans because of its HSP-inducing activity. However, because GGA mediates various other protective mechanisms, such as an increase in mucosal blood flow, stimulation of surface mucus production, and direct protection of cell membranes, these actions of GGA may also be involved in GGA-dependent protection against NSAID-induced lesions of the small intestine.

Gastroprotective drugs, such as GGA, have been used in the treatment of gastric lesions for a long period. However, it is believed that newly developed acid-control drugs (such as histamine-2 receptor antagonists and proton pump inhibitors) are superior to these gastroprotective drugs in curing and preventing gastric lesions. On the other hand, these acid-control drugs seem to be ineffective against NSAID-induced lesions of the small intestine, and the development of new molecules as candidate drugs to treat this disease must pass through the clinical trials process and may encounter the anticipated side effects. Thus, based on the results of this study, we propose that clinical studies be performed to prove the effectiveness of GGA for treating NSAID-induced lesions of the small intestine, given that the safety of GGA has already been shown clinically.

Acknowledgments

We thank Drs. C. E. Angelidis and G. N. Pagoulatos (University of Ioannina, Ioannina, Greece) for generously providing the mice.

References


descopic diagnosis of small bowel ulceration in patients receiving non-steroidal


effects of heat shock protein 70 induced by geranylgeranylacetone on oxidative


Sigthorsson G, Simpson RJ, Walley M, Anthony A, Foster R, Hotz-Beerhofsitz C,
integrity, and pathogenesis of nonsteroidal anti-inflammatory drug enteropathy in

inhibits heat-induced apoptosis upstream of mitochondria by preventing Bax

Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ, Takenaka H,
Tsutsumi S, Hoshino T, Tomisato W, Hwang HJ, Takenaka H, Tsutsumi S, Hoshino T, Hwang HJ, Takenaka H,
Tsuchiya T, Mori M, and Mizushima T (2004) Role of direct cytotoxic effects of NSAIDs in the induction of gastric

response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis.

Dexamethasone protection of rat intestinal epithelial cells against oxidant injury

anti-inflammatory drug (NSAID) induced gastrointestinal damage-potential for
development of gastrointestinal tract safe NSAIDs. Can J Physiol Pharmacol
72:1495–1498.

synthase and microvascular injury in the rat jejunum provoked by indomethacin.

Address correspondence to: Dr. Tohru Mizushima, Graduate School of
Medical and Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honma-
chi, Kumamoto 860-0973, Japan. E-mail: mizu@gpo.kumamoto-u.ac.jp