HSP70 CONFER PROTECTION AGAINST INDOMETHACIN-INDUCED
LESIONS OF THE SMALL INTESTINE

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Abbreviations: BSA, bovine serum albumin; CHOP, C/EBP homologous transcription factor; COX, cyclooxygenase; DAPI, 4’, 6-diamidino-2-phenylindole dihydrochloride; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FasL, Fas
ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGA, geranylgeranylacetone; H₂-blocker, histamine 2-receptor antagonist; H & E, hematoxylin and eosin; HSP, heat shock protein; IL, interleukin; iNOS, inducible NO synthase; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; MPO, myeloperoxidase; NF-κB, nuclear factor kappa B; NO, nitric oxide; NSAID, non-steroidal anti-inflammatory drug; PUMA, p53 up-regulated modulator of apoptosis; PG, prostaglandin; PPI, proton pump inhibitor; S.E.M., standard error of the mean; TdT, terminal deoxynucleotidyl transferase; TNF, tumor necrosis factor; TUNEL, TdT-mediated biotinylated UTP nick end labelling.
ABSTRACT

In line with improvements in diagnostic procedures to detect intestinal lesions, it has become clear that non-steroidal 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 to 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 pro-inflammatory cytokines in the small intestines of control mice, with both these responses suppressed in the transgenic mice. Geranylgeranyacetone (GGA), a clinically used anti-ulcer 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 appears to contribute to the drug’s protective effect against the lesions. Based on these results, we propose that non-toxic HSP70-inducers, such as GGA, would be therapeutically beneficial for treating NSAID-induced lesions of the small intestine.
Introduction

Non-steroidal 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 PGE$_2$, which have a strong capacity to induce inflammation. NSAID use, however, is associated with gastrointestinal complications. More attention has generally been paid to gastric lesions rather than lesions of the small intestine, because the latter are usually asymptomatic and their diagnosis was difficult to make. 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 50-70% 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 non-selective 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 (H\(_2\)-blockers) and proton pump inhibitors (PPIs)) or increase defensive factors are therapeutically effective. Compared to 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 (NO) produced by inducible NO synthase (iNOS) also seem to damage the small intestinal mucosa to produce lesions (Whittle et al., 1995; Konaka et al., 1999; Jacob et al., 2007). On the other hand, 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 compared to their effect on 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, a number of 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,
geranylgeranylation (GGA), a leading anti-ulcer drug on the Japanese market, has been reported to be a non-toxic 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. While 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 non-toxic HSP70-inducers, such as GGA, could be therapeutically beneficial in the treatment of such lesions.
Methods

Chemicals and Animals. Paraformaldehyde, peroxidase standard and o-dianisidine were obtained from Sigma (St. Louis, MO). The PGE₂ enzyme-linked immunosorbent assay (ELISA) 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 (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 gifted from Eisai (Tokyo, Japan). 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 was obtained from Qiagen (Valencia, CA), the first-strand cDNA synthesis kit was from GE Healthcare (Buckinghamshire, UK), and the iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). Transgenic mice expressing HSP70 (a gift from Drs. Angelidis and
Pagoulatos (University of Ioannina, Greece), their wild-type counterparts (C57/BL6) (6–8 weeks of age and 20 to 25 g) and other wild-type mice (ICR, 10–12 weeks of age and 30 to 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 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, 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. Unfasted mice were orally administered indomethacin and 24 h later the animals were sacrificed and both the jejunum and ileum were removed and treated with formalin for fixation. Samples were opened along the anti-mesenteric attachment. Calculation of lesion scores involved measuring the area of all lesions in square millimetres by an observer unaware of the treatment animals had received, and summing 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 previously described (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 using 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 expressed as the number of hydrogen peroxide molecules converted per min per mg 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 using an RNeasy kit according to the manufacturer’s protocol. Samples (2.5 μg RNA) were reverse-transcribed using 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 using iQ SYBR GREEN Supermix and analysed 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 (GAPDH) 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’-aggtcgaagatgagcacgtt-3’; *tnf-α*: 5’-cgtcagccgatgtctatct-3’, 5’-cggactccgaaagtctaaag-3’; *il-1β*: 5’-gatcccaagcaatacccaaa-3’, 5’-ggggaactctgcagactcag-3’; *il-6*: 5’-ctggcagcagcagagactg-3’, 5’-ggtttgccgagtagatctc-3’; *mip-2*: 5’-accctggcagctgactt-3’, 5’-ggcactcagcgacggtc-3’; *mcp-1*: 5’-ctccctgcgctactcact-3’, 5’-gcttgaggtggttgaaa-3’; *gapdh*: 5’-aactttggcattgtggaagg-3’, 5’-acacattgggggtaggaaca-3.

Mouse peritoneal macrophages were prepared as described previously (Tanaka et al., 2007).

**Immunohistochemical and TdT-mediated Biotinylated UTP Nick End Labelling (TUNEL) Analyses.** Small intestine tissue samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin before being cut into 4-µm-thick sections.
For histological examination (hematoxylin and eosin (H & E) 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. 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 quantified as described (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 one-half of the villus; 4, epithelial detachment involving more than one-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 (BSA), 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 using fluorescence microscopy (Olympus BX51).
For TUNEL assay, sections were incubated first with proteinase K (20 µg/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. 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 ANOVA 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 between transgenic mice expressing HSP70 and wild-type mice the development of lesions in the small intestine after administration of indomethacin. 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. 1C and D). The results also show that indomethacin-administration increases the expression of HSP70 in wild-type mice (Fig. 1C 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 co-staining assay. As shown in supplemental Fig. S1, strong co-staining 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 co-staining of HSP70 with E-cadherin (a maker of epithelial cell) was also observed, however, co-staining 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 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 PGE$_2$ (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. We therefore compared these factors between transgenic mice expressing HSP70 and wild-type mice. As shown in Fig. 2A, there was no significant difference in the small intestinal level of PGE$_2$ between transgenic mice expressing
HSP70 and wild-type mice either with or without indomethacin treatment. 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 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 pro-inflammatory 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 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 HSP70 did not affect the background expression of these genes (Fig. 3).

These results suggest that the reduced expression of these pro-inflammatory 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 pro-inflammatory cytokines and chemokines under inflammatory conditions. To test this idea in vitro, we compared LPS-stimulated mRNA expression of the pro-inflammatory cytokines and chemokines in peritoneal macrophages prepared from the transgenic mice expressing HSP70 and the wild-type mice. As shown in supplemental Fig. S2, LPS stimulated the mRNA expression of all of these pro-inflammatory 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 pro-inflammatory cytokines under inflammatory conditions.
Effect of GGA on Indomethacin-induced Lesions and Expression of HSP70.

We next examined the effect of pre-administration of GGA on indomethacin-induced lesions in the small intestine. As shown in Fig. 4A, pre-administration 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 (upper panels) 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 pre-administered with GGA (Fig. 4C, upper panels). 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. 4E and F), while GGA significantly stimulated the expression of HSP70 in both the presence and absence of indomethacin treatment (Fig.
4E and F). Increase in the level HSP70 following the administration of GGA and/or indomethacin was also confirmed by immunohistochemical analysis (Fig. 4C). As shown in supplemental Fig. S3, strong co-staining of HSP70 with CD11b, CD4 and CD31 and weak co-staining of HSP70 with E-cadherin was 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 pre-administration of quercetin (an inhibitor of expression of HSP70) on the protective effect of GGA. As shown in Fig. 4G, pre-administration 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 post-administration 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 pre-induction of HSP70 is required for protection against indomethacin-induced lesions of the small intestine.
We also examined the effect of pre-administration 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 pre-administration 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

A number of previous studies have used transgenic mice to show that induction of HSP70 expression is protective 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 resistant phenotype 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 expression of other mediators that would influence NSAID-induced lesions of the small intestine.

HSP70 appears to protect the small intestine from NSAID-induced lesions by inhibiting mucosal cell apoptosis rather than by affecting PGE$_2$ levels; indomethacin-dependent mucosal cell apoptosis but not a decrease in the level of PGE$_2$ 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 \textit{in vitro} (Tomisato et al., 2004; Arai et al., 2005), suggesting that lesion-inducing activities of these NSAIDs \textit{in vivo} are relatively weak. In fact, we found that ibuprofen and nabumetone produced less 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 Ca$^{2+}$ influx and increases intracellular Ca$^{2+}$ levels, which in turn induces the endoplasmic reticulum (ER) stress response (Tanaka et al., 2005b). In the ER stress response, an apoptosis-inducing transcription factor, C/EBP homologous transcription factor (CHOP), is induced and we have previously shown 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, as well as 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 previously reported (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 due to HSP70’s 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 important mechanism by which indomethacin induces apoptosis (Maity et al., 2008). 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 pro-inflammatory cytokine secretion, which are reduced significantly by Hsp70 is 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 kappa B (NF-κB), which is responsible for inducing the production of various pro-inflammatory cytokines (Krappmann et al., 2004). We recently reported that the LPS-induced production of pro-inflammatory cytokines, including IL-1β and IL-6, was inhibited in peritoneal macrophages prepared from transgenic mice expressing HSP70 compared to 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 here 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 NF-κB 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 due to its clinical value as an anti-ulcer drug and because it can induce HSPs without affecting cell viability (Hirakawa et al., 1996). We previously reported 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 (Ohkawara et al., 2006). Furthermore, we found here that pre-administration 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 recently reported elsewhere (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 owing to 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.

Gastro-protective 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 H2-blockers and PPIs) are superior to these gastro-protective drugs in curing/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 should 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.
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Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ, Takenaka H,

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


Dexamethasone protection of rat intestinal epithelial cells against oxidant injury

inflammatory drug (NSAID) induced gastrointestinal damage--potential for
development of gastrointestinal tract safe NSAIDs. *Can J Physiol Pharmacol*

**72**:1493-1498.

Watanabe T, Sugimori S, Kameda N, Machida H, Okazaki H, Tanigawa T, Watanabe K,

bowel injury by low-dose enteric-coated aspirin and treatment with misoprostol:

Footnotes

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Legends for Figures

**Fig. 1.** Indomethacin-induced lesions of the small intestine and expression of HSP70. Wild-type mice (WT, C57/BL6) and transgenic mice expressing HSP70 (HSP70 Tg) were orally administered the indicated doses (A) or 30 mg/kg of indomethacin (IND) (B-E) 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, D, values are mean ± S.E.M. **P<0.01; *P<0.05; n.s., not significant.

**Fig. 2.** Indomethacin-induced decrease in PGE$_2$ level and mucosal cell apoptosis in small intestine. Wild-type (WT, C57/BL6) and transgenic mice expressing HSP70...
(HSP70 Tg) were orally administered 30 mg/kg indomethacin (IND) and the small intestine was removed after 24 h. A, the small intestine PGE$_2$ level was determined by ELISA. Values are mean ± 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.

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

Fig. 4. Effect of GGA on expression of HSP70 and production of lesions in the small intestine. Wild-type mice (ICR) were orally administered indicated doses (A) or 100 mg/kg (B-G) of GGA (10 ml/kg as an emulsion with 5% gum arabic). Quercetin
was orally administered 2 h before the GGA administration. Two hours later the mice were orally administered 20 mg/kg of indomethacin (IND) (A-G). GGA was orally administered 2 h before (Pre) or after (Post) the administration of 20 mg/kg indomethacin (H). Small intestine was removed 24 h (A, B, G, H) or 4 h (C-F) after the administration of indomethacin. Analyses were performed as described in the legend of Fig. 1 (A-C, E-H) or in materials and methods (D). A, B, D and F-H, values are mean ± S.E.M.  

\[ n = 3-9 \text{ (A), 3-6 \text{ (B), 10-16 \text{ (D), 5-9 \text{ (F), 4-6 \text{ (G), 4-9 \text{ (H).}}] \] **P<0.01; *P<0.05; n.s., not significant.

**Fig. 5. Effect of GGA on indomethacin-dependent decrease in PGE\(_2\) level and mucosal cell apoptosis.** Wild-type mice (ICR) were orally administered 100 mg/kg of GGA (10 ml/kg as an emulsion with 5% gum arabic). Two hours later they were orally administered 20 mg/kg of indomethacin 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.
Figure 2

A

Mucosal PGE$_2$ content (ng/g tissue)

Vehicle

IND

WT

HSP70 Tg

n.s.

B

Vehicle

IND

WT

HSP70 Tg

TUNEL

DAPI
Figure 5

(A) Mucosal PGE$_2$ content (ng/g tissue)

| IND (mg/kg) | 0 | 0 | 20 | 20 |
| GGA (mg/kg) | 0 | 100 | 0 | 100 |

* indicates statistical significance (p < 0.05), n.s. indicates not significant.

(B) TUNEL and DAPI images for different treatments:

- Vehicle
- GGA
- IND
- IND+GGA