

# Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis

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Received 19.9.03; revised 05.2.04; accepted 06.2.04; published online 07.5.04  
Edited by H Ichijo

## Abstract

Apoptosis induced by nonsteroidal anti-inflammatory drugs (NSAIDs) is involved not only in the production of NSAID-induced gastric lesions but also in the antitumor activity of these drugs. The endoplasmic reticulum (ER) stress response is a cellular mechanism that aids in protecting the ER against ER stressors and is involved in ER stressor-induced apoptosis. Here, we examine the relationship between this response and NSAID-induced apoptosis in cultured guinea-pig gastric mucosal cells. Exposure of cells to indomethacin, a commonly used NSAID, induced GRP78 as well as CHOP, a transcription factor involved in apoptosis. Three factors that positively regulate CHOP expression (ATF6, ATF4 and XBP-1) were activated and/or induced by indomethacin. NSAIDs other than indomethacin (diclofenac, ibuprofen and celecoxib) also induced CHOP. Monitoring of the transcriptional activities of ATF6 and CHOP by luciferase assay revealed that both were stimulated in the presence of indomethacin. Furthermore, indomethacin-induced apoptosis was suppressed in cultured guinea-pig gastric mucosal cells by expression of the dominant-negative form of CHOP, or in peritoneal macrophages from CHOP-deficient mice. These results suggest that ER stress response-related proteins, particularly CHOP, are involved in NSAID-induced apoptosis.

*Cell Death and Differentiation* (2004) 11, 1009–1016.

doi:10.1038/sj.cdd.4401436

Published online 7 May 2004

**Keywords:** NSAIDs; apoptosis; endoplasmic reticulum; CHOP; gastric mucosal cells

**Abbreviations:** ASK1, apoptosis signal-regulating kinase 1; ATF, activating transcription factor; CHOP, C/EBP homologous transcription factor; COX, cyclooxygenase; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; ERSE, ER stress response element; FBS, fetal bovine serum; GRP,

glucose-regulated protein; IRE1, protein-kinase and site-specific endoribonuclease; JNK, c-jun NH<sub>2</sub>-terminal kinase; MTT, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NSAIDs, nonsteroidal anti-inflammatory drugs; PEK, pancreatic eIF2 kinase; PERK, protein kinase R-like ER kinase; PG, prostaglandin; TRAF2, tumor necrosis factor receptor-associated factor; UPR, unfolded protein response; XBP-1, X box binding protein

## Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently used classes of medicines in the world and account for nearly 5% of all prescribed medications.<sup>1</sup> NSAIDs have great efficacy in the treatment of pain, inflammation and fever on account of their inhibition of cyclooxygenase (COX) activity. COX is essential for the synthesis of prostaglandins (PGs), which have a strong involvement in the induction of inflammation. Recently, clinically beneficial properties of NSAIDs other than anti-inflammation have been revealed. Epidemiological studies revealed that prolonged use of aspirin or other NSAIDs reduces the risk of cancer and Alzheimer's disease.<sup>2–4</sup> These activities of NSAIDs were confirmed by a number of *in vivo* and *in vitro* experiments. Since these activities of NSAIDs cannot be fully explained by the COX-inhibition,<sup>5,6</sup> COX-independent actions of NSAIDs are necessary to be identified.

NSAID administration is associated with gastrointestinal complications, such as gastric ulcers.<sup>7</sup> In the United States, about 16 500 people die per year as a result of NSAID-associated gastrointestinal complications.<sup>8</sup> We recently suggested that direct cytotoxic effects of NSAIDs (such as induction of apoptosis), that is, COX-independent are involved in NSAID-induced gastric lesions *in vivo*.<sup>9</sup> Furthermore, in addition to inhibition of angiogenesis and cell growth by NSAIDs, induction of apoptosis by NSAIDs is also involved in their antitumor activities.<sup>6</sup> Therefore, elucidation of the mechanism of NSAID-induced apoptosis is important to understand the mechanism of both NSAID-induced gastric lesions and their antitumor activities. We previously reported that activation of caspases (caspase-3, -8 and -9) is accompanied with induction of apoptosis by NSAIDs.<sup>10</sup> However, the upstream pathways of apoptosis induced by NSAIDs remain unknown.

Accumulation of unfolded protein in the endoplasmic reticulum (ER) induces the ER stress response, otherwise known as the unfolded protein response (UPR). In the mammalian ER stress response, three types of ER transmembrane proteins are important: protein-kinase and site-specific endoribonuclease (IRE1), protein kinase R-like ER kinase/pancreatic eIF2 kinase (PERK/PEK) and activating transcription factor 6 (ATF6).<sup>11–13</sup> The mammalian ER stress response can be separated into two phases, adaptation and

apoptosis. Cells initially adapt to the accumulation of unfolded proteins by inducing ER-resident stress proteins (molecular chaperons) such as glucose-regulated protein (GRP) 78 and GRP94.<sup>14–17</sup> These proteins refold the unfolded proteins in an attempt to maintain homeostasis in the ER. However, if this adaptation does not prove sufficient, the apoptotic response is initiated, by both ATF6- and ATF4-dependent activation of C/EBP homologous transcription factor (CHOP).<sup>18</sup> In this study, we revealed that NSAIDs induce ER stress response. We found that indomethacin induces both GRP78 and CHOP. It also causes activation of ATF6, ATF4 and X box binding protein (XBP-1). Experiment using the dominant-negative form of CHOP and cells from CHOP-deficient mice implicated that the activation of CHOP is involved in NSAID-induced apoptosis.

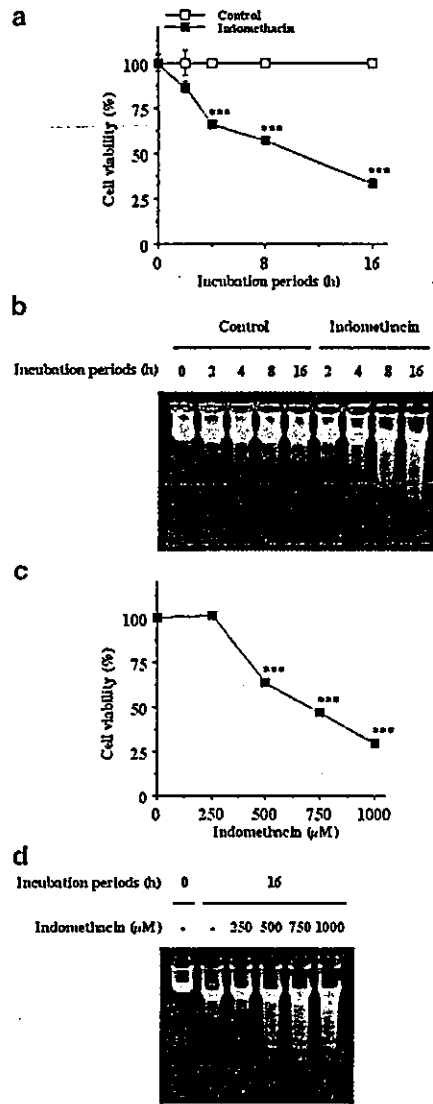
## Results

### Induction of GRP78 and CHOP by indomethacin associated with apoptosis

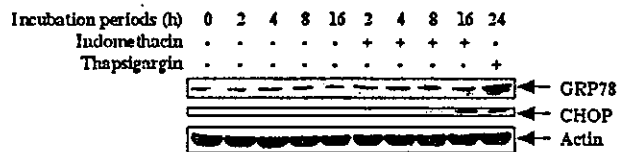
We have previously reported that long-term (16 h) treatment of primary cultures of guinea-pig gastric mucosal cells with NSAIDs (1 mM for indomethacin) induces apoptosis.<sup>10</sup> In order to reveal the pathway underlying this apoptosis, we used DNA microarray techniques to search for genes whose expression is stimulated by indomethacin under these apoptotic conditions, successfully identifying *GRP78* as one such gene (S Mima *et al.*, unpublished results). In the present study, we first confirmed the indomethacin-dependent induction of GRP78 in gastric mucosal cells. Figure 1 shows the time-course and dose-response of apoptosis induced by indomethacin. A decrease in cell viability and apoptotic DNA fragmentation were observed when cells were treated with 0.5–1 mM indomethacin for 4–16 h (Figure 1), this being consistent with our previously reported results.<sup>10</sup> We also confirmed the induction of apoptosis by showing the chromatin condensation and caspase-3 activation under the conditions (data not shown). Furthermore, we showed that almost all cells were not stained with propidium iodide,<sup>10</sup> showing that necrosis was not induced under the conditions (less than 1 mM) (data not shown). In subsequent experiments, we treated cells with 1 mM indomethacin for 16 h in order to examine the mechanism underlying this apoptosis.

The effect of indomethacin on the expression of GRP78 was examined by immunoblotting analysis (Figure 2). GRP78 was present in nontreated control cells and increased in response to treatment with thapsigargin (an inhibitor of sarco ER Ca<sup>2+</sup> ATPase and a representative inducer of the ER stress response), as previously described.<sup>19</sup> Indomethacin slightly but significantly increased the amount of GRP78 (Figure 2). We also examined the effect of indomethacin on the expression of another ER stress response-related protein, CHOP, a transcription factor, which induces apoptosis. CHOP was not present in nontreated cells, but was induced by thapsigargin, this being consistent with previously reported results.<sup>19</sup> Indomethacin clearly induced CHOP, and to a greater extent than thapsigargin (Figure 2).

We also examined the effect of indomethacin on the expression of *GRP78* and *CHOP* mRNA by Northern blotting analysis (Figure 3). Similar to thapsigargin, indomethacin

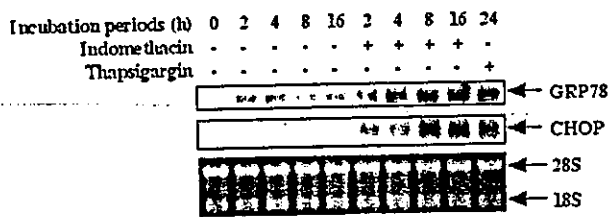


**Figure 1** Apoptosis induced by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM (a, b) or the indicated concentrations (c, d) of indomethacin for the indicated periods (a, b) or 16 h (c, d). Cell viability was determined by the MTT method. Values are expressed as mean  $\pm$  S.E.M. ( $n = 4$ ). \*\*\* $P < 0.001$  (a, c). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (b, d)



**Figure 2** Immunoblotting analysis for induction of GRP78 and CHOP by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin or 2  $\mu$ M thapsigargin (positive control) for the indicated periods. Whole cell extracts (20  $\mu$ g protein for GRP78, 5  $\mu$ g protein for actin and 30  $\mu$ g protein for CHOP) were analyzed by immunoblotting with an antibody against GRP78, actin or CHOP

increased the amount of *GRP78* and *CHOP* mRNA, suggesting that these ER stress response-related proteins are induced at the level of transcription. Given that CHOP is a



**Figure 3** Northern blotting analysis for induction of *GRP78* and *CHOP* mRNA by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin or 2  $\mu$ M thapsigargin (positive control) for the indicated periods. The level of the *GRP78* and *CHOP* mRNA was monitored by Northern blotting analysis. Bands of ribosomal RNA (28S and 18S) stained with ethidium bromide are shown

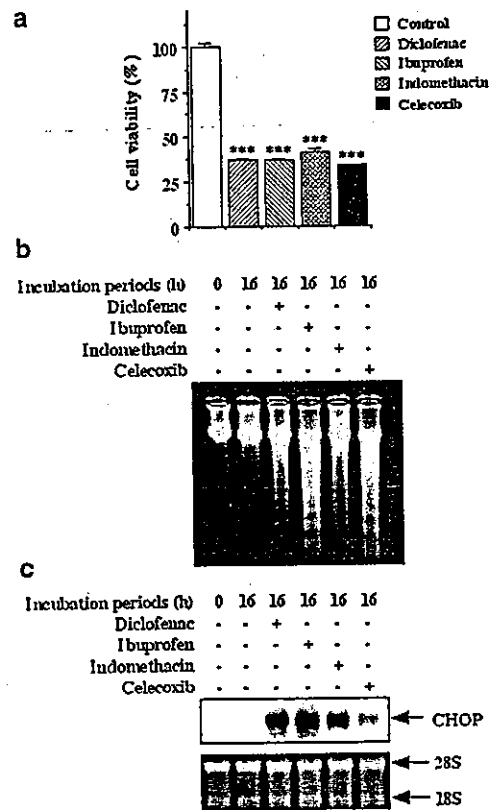
transcription factor involved in the induction of apoptosis, we then focused on *CHOP* induction by indomethacin in order to understand the mechanism of NSAID-induced apoptosis.

Induction of apoptosis is not specific for indomethacin, but is observed in response to NSAIDs in general. Given that we have previously found that aspirin, diclofenac, ibuprofen and celecoxib also induce apoptosis,<sup>9</sup> we examined the effect of some of these agents on *CHOP* mRNA induction. As well as indomethacin (1 mM), treatment of cells with diclofenac (1 mM), ibuprofen (2 mM) or celecoxib (80  $\mu$ M) caused a decrease in cell viability and apoptotic DNA fragmentation, confirming that apoptosis is induced under these conditions (Figure 4a and b). These NSAIDs increased the amount of *CHOP* mRNA (Figure 4c). There are two subtypes of COX, these being COX-1 and COX-2, but COX activity in gastric mucosal cells is mainly derived from COX-1. With the exception of celecoxib, which is COX-2 specific, NSAIDs used in Figure 4 can inhibit both subtypes.<sup>20</sup> Therefore, if the induction of *CHOP* mRNA by indomethacin is caused by COX inhibition in gastric mucosal cells (in other words, inhibition of COX-1), higher concentrations of celecoxib would be required to reveal an effect. Given that the reverse was the case, it appears that *CHOP* induction by NSAIDs is not related to inhibition of COX and PG synthesis in gastric mucosal cells. Furthermore, we found that the addition of PGE<sub>2</sub> to the culture medium did not attenuate apoptosis and *CHOP* induction by indomethacin (data not shown).

We have previously established conditions under which other gastric stressors (ethanol, hydrogen peroxide and hydrochloric acid) induce apoptosis in primary cultures of guinea-pig gastric mucosal cells.<sup>21</sup> We therefore examined whether these gastric stressors induce *CHOP* under apoptotic conditions. Treatment of cells with 4% ethanol for 6 h, 300  $\mu$ M hydrogen peroxide for 6 h or 20 mM hydrochloric acid for 8 h caused a decrease in cell viability and apoptotic DNA fragmentation (Figure 5a and b). However, none of these gastric stressors induced *CHOP* mRNA (Figure 5c). Therefore, induction of *CHOP* (and possibly the ER stress response) is not generally observed for gastric stressor-induced apoptosis, but is specific for NSAID-induced apoptosis.

#### Activation of ATF6 by indomethacin

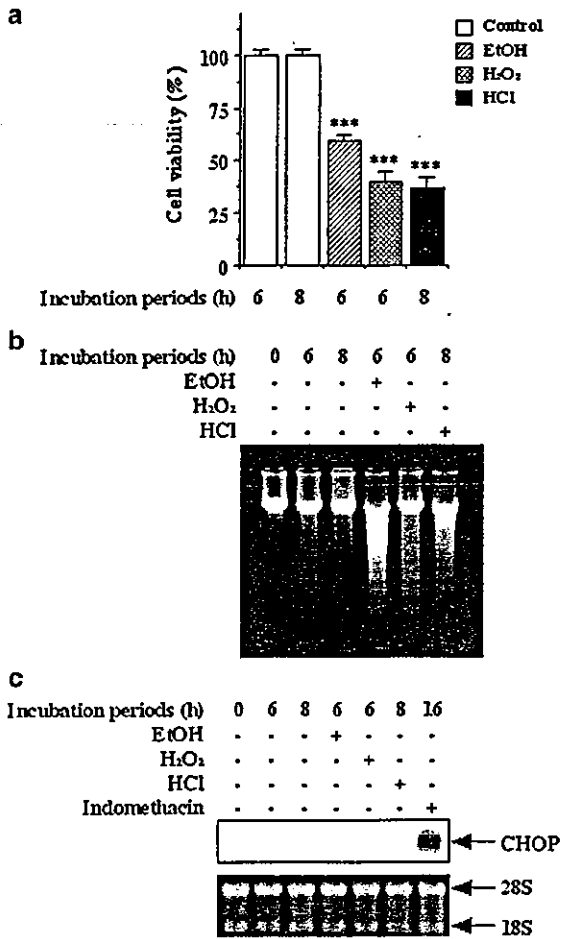
ER stress response element (ERSE) has been identified in the promoter of ER chaperons and *CHOP* genes.<sup>22</sup> ER trans-



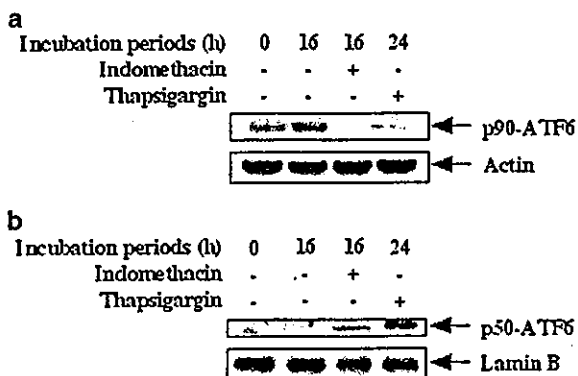
**Figure 4** Induction of *CHOP* mRNA by NSAIDs other than indomethacin. Cultured gastric mucosal cells were incubated with 1 mM diclofenac, 2 mM ibuprofen, 1 mM indomethacin or 80  $\mu$ M celecoxib for 16 h. Cell viability was determined by the MTT method. Values are expressed as mean  $\pm$  S.E.M. ( $n=4$ ). \*\*\* $P < 0.001$  (a). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (b). The level of *CHOP* mRNA was monitored by Northern blotting analysis. Bands of ribosomal RNA (28S and 18S) stained with ethidium bromide are shown (c)

membrane-localized p90-ATF6 (the inactive form of ATF6 for ERSE-dependent transcription) is cleaved into p50-ATF6, which translocates to the nucleus where it specifically binds to ERSE to activate the transcription from ERSE.<sup>13</sup> The induction of *GRP78* and *CHOP* mRNA by indomethacin (Figure 3) suggests that p90-ATF6 is activated (cleaved) into p50-ATF6. Immunoblotting analysis was used to confirm this point. Immunoblotting of whole cell extracts revealed that the p90-ATF6 band disappeared following treatment of cells with indomethacin, as well as with thapsigargin (Figure 6a). Unfortunately, due to the presence of cross-reaction bands around p50-ATF6, we could not detect p50-ATF6 in whole cell extracts (data not shown). We therefore prepared nuclear extracts in which p50-ATF6 should be enriched. As shown in Figure 6b, the p50-ATF6 band was indeed detected in extracts prepared from indomethacin- or thapsigargin-treated cells, but not from control cells. As expected, the p90-ATF6 band was not detected in any nuclear extracts (data not shown). These results suggest that ATF6 is activated in the presence of indomethacin.

For further confirmation of this point, the transcriptional activity of ATF6 was measured using a reporter plasmid where the ERSE of the *CHOP* gene was inserted.<sup>23</sup>



**Figure 5** Induction of *CHOP* mRNA by gastric stressors other than NSAIDs. Cultured gastric mucosal cells were incubated with 4% ethanol (EtOH) for 6 h, 300  $\mu$ M hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 6 h, 20 mM hydrochloric acid (HCl) for 8 h or 1 mM indomethacin for 16 h. Cell viability was determined by the MTT method. Values are mean  $\pm$  S.E.M. ( $n=4$ ). \*\*\* $P<0.001$  (a). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (b). The level of *CHOP* mRNA was monitored by Northern blotting analysis. Bands of ribosomal RNA (28S and 18S) stained with ethidium bromide are shown (c)



**Figure 6** Activation of ATF6 by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin for 16 h or 2  $\mu$ M thapsigargin for 24 h (positive control). Whole cell extracts (100  $\mu$ g protein for ATF6 and 5  $\mu$ g protein for actin) (a) or nuclear extracts (30  $\mu$ g protein for ATF6 and 5  $\mu$ g protein for lamin B) (b) were analyzed by immunoblotting with an antibody against ATF6, actin or lamin B

Compared with control cells (without ERSE in the promoter), treatment with indomethacin stimulated luciferase activity to a similar extent to that observed with thapsigargin in cells that have ERSE in the reporter plasmid (Figure 7). The stimulation of luciferase activity by thapsigargin was much the same as that previously reported.<sup>23</sup> The results illustrated in Figures 6 and 7 suggest that ATF6 is activated in the presence of indomethacin, which in turn induces the transcription of the *CHOP* gene from ERSE.

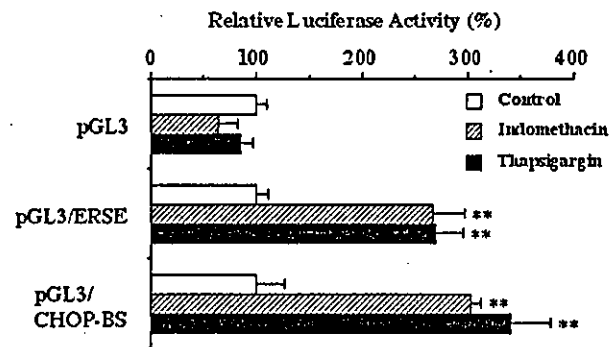
We also measured the transcriptional activity of CHOP using a reporter plasmid where the CHOP binding site of the Rous sarcoma virus long terminal repeat was inserted.<sup>23</sup> Compared with control cells (without the CHOP binding site in the promoter), treatment with indomethacin stimulated luciferase activity to a similar extent to that seen with thapsigargin in cells that have the CHOP binding site in the reporter plasmid (Figure 7). Taken together, the results illustrated in Figures 2, 3 and 7 suggest that the induction of CHOP by indomethacin is involved in altering the transcription of CHOP-regulated genes, including those genes involved in apoptosis.

#### Induction of ATF4 and XBP-1 by indomethacin

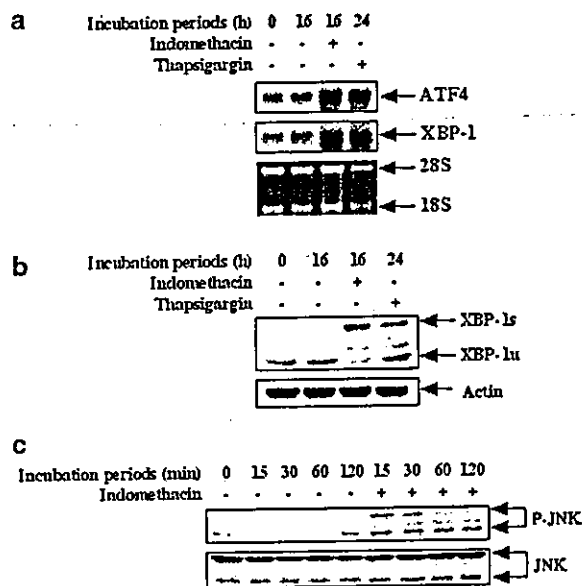
In addition to regulation by ATF6 through ERSE, expression of the *CHOP* gene is positively regulated by other transcription factors, such as ATF4 and XBP-1.<sup>24</sup> The induction of ATF4 is dependent on PERK/PEK, whereas XBP-1 is dependent on both ATF6 and IRE1.<sup>11,12</sup> ATF4 has been shown to bind to the promoter of the *CHOP* gene (the upstream region of ERSE) and activate *CHOP* transcription,<sup>25</sup> while XBP-1 binds to ERSE.<sup>24</sup> As shown in Figure 8a, both *ATF4* and *XBP-1* mRNA were increased in the presence of indomethacin, as well as thapsigargin. The induction of *ATF4* mRNA suggests that PERK/PEK is activated in the presence of indomethacin.

#### Activation of XBP-1 and JNK by indomethacin

IRE1 play an important role in ER stressor-dependent apoptosis. IRE1 activates caspase-12, XBP-1 and apopto-



**Figure 7** Activation by indomethacin of transcription from ERSE and the CHOP binding site. Cultured gastric mucosal cells were cotransfected with pRL-SV40 (internal control plasmid with the *Renilla reniformis* luciferase gene) and pGL3 (*Photinus pyralis* luciferase gene with SV40 promoter), pGL3/ERSE or pGL3/CHOP-BS. After 24 h, cells were incubated with 1 mM indomethacin or 2  $\mu$ M thapsigargin (positive control) for 8 h. Fire fly luciferase activity was measured, normalized for *Renilla reniformis* luciferase activity, and expressed relative to the activity in the absence of these chemicals (control). Values are mean  $\pm$  S.E.M. ( $n=3$ ). \*\* $P<0.01$



**Figure 8** Induction and/or activation of ATF4, XBP-1 and JNK by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin or 2  $\mu$ M thapsigargin (positive control) for indicated periods. The level of ATF4 or XBP-1 mRNA was monitored by Northern blotting analysis. Bands of ribosomal RNA (28S and 18S) stained with ethidium bromide are illustrated (a). Whole cell extracts (30  $\mu$ g protein for XBP-1, 5  $\mu$ g protein for actin and 20  $\mu$ g protein for JNK) were analyzed by immunoblotting with an antibody against XBP-1, JNK or phosphorylated JNK (P-JNK) (b, c). XBP-1s, spliced form of XBP-1; XBP-1u, unspliced form of XBP-1 (b)

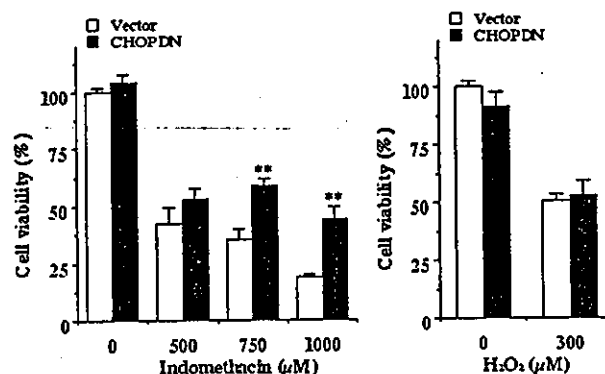
sis-signal-regulating kinase 1 (ASK1), all of which are involved in apoptosis.<sup>26,27</sup> We here examined the effect of NSAIDs on these IRE1-dependent pathway. Since antibody against mouse caspase-12 did not work for immunoblotting experiments of guinea-pig proteins, we could not reveal the effect of NSAIDs on caspase-12 activation.

IRE1 splices XBP-1 mRNA to converting it into a potent activator for transcription from ERSE. Both unspliced (inactive) and spliced (active) form of XBP-1 protein was observed in cells treated with indomethacin or thapsigargin, whereas only unspliced form of XBP-1 protein was observed in control cells (Figure 8b). This result suggests that IRE1 is activated in the presence of indomethacin.

ER stressors activate ASK1 through IRE1 and activated ASK1 induces apoptosis through activation (phosphorylation) of c-Jun NH<sub>2</sub>-terminal kinase (JNK). As shown in Figure 8c, phosphorylation of JNK was strongly induced by treatment of cells with indomethacin. Therefore, the ASK1 pathway seems to be activated by indomethacin.

### Involvement of CHOP in indomethacin-induced apoptosis

In order to test whether the induction of CHOP by indomethacin is involved in indomethacin-induced apoptosis, we examined the effect of expression of the dominant-negative form of CHOP. Gastric mucosal cells were cotransfected with an enhanced green fluorescent protein (EGFP) expression plasmid and an expression plasmid for the dominant-negative form of CHOP prior to treatment with indomethacin (Figure 9).



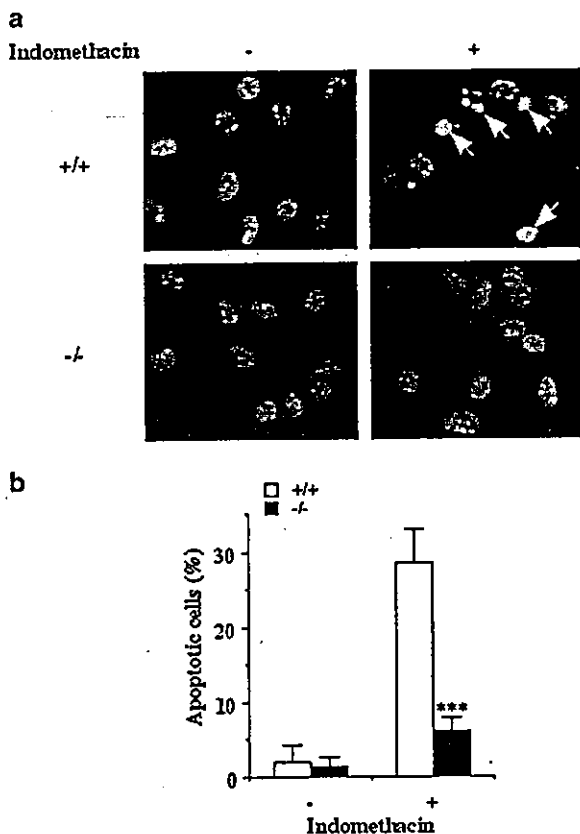
**Figure 9** Prevention of indomethacin-induced apoptosis by the expression of the dominant-negative form of CHOP. Cultured gastric mucosal cells were cotransfected with 3  $\mu$ g of pEGFP-C1 (EGFP expression plasmid) and 10  $\mu$ g of pOPRSVI-L133/L140ACHOP (expression plasmid for dominant-negative form of CHOP, CHOPDN) or vector. After 24 h, the cells were incubated with the indicated concentrations of indomethacin for 12 h or with 300  $\mu$ M hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 6 h. Cells were observed by fluorescence microscope and EGFP-positive cells were counted and expressed relative to numbers of vector-transfected cells without stress. Values are expressed as mean  $\pm$  S.E.M. (n = 3). \*\*P < 0.01

Since the transfection efficiency was not so high in primary culture of gastric mucosal cells, we measured the cell viability by counting EGFP-positive cells instead of MTT method, which was used in other experiments. The indomethacin-dependent decrease in cell viability was partially suppressed by expression of the dominant-negative form of CHOP (Figure 9). In contrast, expression of this form of CHOP did not alter the effect of hydrogen peroxide (Figure 9), which did not induce CHOP under the conditions (Figure 5). This result was consistent, even when higher concentrations of hydrogen peroxide were used (data not shown). Thus, the induction of CHOP appears to be involved in indomethacin-induced apoptosis.

For further confirming the involvement of CHOP in indomethacin-induced apoptosis, we used CHOP-deficient mice. Since primary culture of gastric mucosal cells has not been established in mouse, we used peritoneal macrophages. We previously reported that NO-induced apoptosis significantly decreased in peritoneal macrophages from CHOP-deficient mice.<sup>23</sup> Peritoneal macrophages from wild-type mice or CHOP-deficient mice were treated with indomethacin for 24 h. As shown in Figure 10, indomethacin-induced chromatin condensation was observed in peritoneal macrophages from wild-type mice, but not so apparently in those from CHOP-deficient mice. This result also strongly suggests that the induction of CHOP is involved in indomethacin-induced apoptosis.

### Discussion

In this study, we have demonstrated that NSAIDs activate the ER transmembrane protein ATF6 via its cleavage, and suggested that cleaved ATF6 stimulates the transcription of the CHOP gene from ERSE. That this CHOP induction is involved in NSAID-induced apoptosis was suggested by showing that expression of the dominant-negative form of CHOP partially suppresses the apoptosis induced by



**Figure 10** Peritoneal macrophages from CHOP-deficient mice are resistant to indomethacin-induced apoptosis. Peritoneal macrophages from wild-type (+/+) or CHOP-deficient (-/-) mice were treated with 1 mM indomethacin for 24 h. Cells were stained with Hoechst dye 33258 and observed under a fluorescence microscope. Arrows indicate condensed chromatin (a). Apoptotic cells with condensed chromatin were counted and expressed relative to total cells. Values are expressed as mean  $\pm$  S.E.M. ( $n = 3$ ). \*\*\* $P < 0.001$  (b)

indomethacin and that indomethacin-induced apoptosis was significantly inhibited in peritoneal macrophages from CHOP-deficient mice. We also showed that other factors, namely ATF4 and XBP-1, which positively regulate the *CHOP* transcription, are induced by NSAIDs, suggesting that not only ATF6 but also ATF4 and XBP-1 are involved in the induction of *CHOP* by NSAIDs. We consider that the concentrations of NSAIDs required for apoptosis *in vitro* are possible *in vivo* associating with gastric ulceration in animal models, as discussed in our previous paper.<sup>10</sup>

Suppression of NSAID-induced apoptosis by expression of the dominant-negative form of *CHOP* was only partial (Figure 9). Furthermore, expression of the wild-type *CHOP* did not induce apoptosis in the absence of stressors (data not shown). These data suggest that *CHOP*-independent mechanisms are also involved in NSAID-induced apoptosis in gastric mucosal cells. ER stress activates ASK1 through IRE1 and tumor necrosis factor receptor-associated factor 2 (TRAF2) and activated ASK1 induces apoptosis through JNK.<sup>28</sup> On the other hand, caspase-12, an ER-associated caspase, may also be involved in NSAID-induced apoptosis. ER stress causes procaspase-12 to cluster at the ER membrane where it is activated via IRE1 and TRAF2.<sup>11</sup>

Activated caspase-12 activates caspase-9 and -3.<sup>11</sup> We here showed that JNK is activated in the presence of indomethacin and observation of spliced form of XBP-1 in cells treated with indomethacin suggested that IRE1 is activated under the conditions. Therefore, we consider that these *CHOP*-independent mechanisms are also involved in NSAID-induced apoptosis in gastric mucosal cells.

As described in Introduction, NSAID-induced apoptosis is involved in NSAID-induced gastric lesions *in vivo*. Therefore, results in this paper suggest that *CHOP* is involved in NSAID-induced gastric lesions. We also showed that not only the apoptotic ER stress response but also the adaptive response (induction of GRP78) is induced by NSAIDs. Previously, many papers have reported that the expression of GRP78 in cells suppresses apoptosis.<sup>18,29</sup> Therefore, induction of GRP78 by NSAIDs may be involved in protection of gastric mucosal cells from NSAID-induced apoptosis. We previously reported that geranylgeranylacetone, an inducer of cytosolic molecular chaperons (heat-shock proteins) and an antiulcer drug, protects gastric mucosal cells from apoptosis, resulting in suppression of gastric lesions.<sup>30</sup> We propose that an inducer of ER molecular chaperons (such as GRP78) may become a new type of antiulcer drug. On the other hand, induction of GRP78 by NSAIDs may weaken the antitumor activities of NSAIDs through inhibiting NSAID-induced apoptosis in tumor cells. Since it was reported that GRP78 is overexpressed in some tumor cells,<sup>31</sup> an inhibitor of GRP78 may have clinical benefit, because it may make tumor cells sensitive to NSAIDs.

Epidemiological studies have shown that prolonged use of aspirin or other NSAIDs reduces the risk of Alzheimer's disease.<sup>2</sup> Treatment of amyloid precursor protein transgenic mice with some NSAIDs suppressed the development of amyloid plaques pathology<sup>32</sup> and NSAIDs lowered the level of amyloid  $\beta$ -protein in cultured cells.<sup>5</sup> Although it was recently reported that some NSAIDs inhibit  $\gamma$ -secretase that is involved in amyloid  $\beta$ -protein production,<sup>33</sup> the mechanism for decrease in amyloid  $\beta$ -protein production by NSAIDs is not fully understood. We showed that induction of GRP78 and *CHOP* by indomethacin was not specifically observed in gastric mucosal cells but observed in other cells, such as HEK293 (human embryonic kidney) cells (data not shown). Since GRP78 was shown to decrease amyloid  $\beta$ -protein production,<sup>34</sup> we propose that GRP78 induced by NSAIDs is involved in inhibition of amyloid  $\beta$ -protein production by NSAIDs and their anti-Alzheimer's disease activity.

## Materials and Methods

### Chemicals, media and animals

Fetal bovine serum (FBS) was obtained from Gibco. Indomethacin was purchased from Wako Co., while ibuprofen, diclofenac and 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were from Sigma Co. Celecoxib was obtained from LKT Laboratories Inc. Pronase E and type I collagenase were purchased from Kaken Pharmaceutical Co. and Nitta Gelatin Co., respectively. An antibody against GRP78 was from StressGen Biotechnologies Corp. Antibodies against XBP-1, *CHOP*, actin, JNK and lamin B were from Santa Cruz Biotechnology Inc. An antibody against phosphorylated JNK was from Cell Signaling Corp. An antibody against ATF6 was kindly provided by Dr. K Mori (Kyoto University).

Lipofectamine PLUS was obtained from Invitrogen Corp. The RNeasy kit was from Qiagen Co., the Rediprime II DNA Labeling system, [ $\alpha$ - $^{32}$ P]dCTP (3000 Ci/mmol) from Amersham Pharmacia Biotech Co., and the Dual Luciferase Assay System from Promega Corp. Male guinea-pigs weighing 200–300 g were purchased from Shimizu Co. The experiments and procedures described here were approved by the Animal Care Committee of Okayama University.

## Plasmid

A *Photinus pyralis* luciferase reporter (pGL3) and an internal standard plasmid harboring the *Renilla reniformis* luciferase gene (pRL-SV40) were obtained from Promega Corp. A plasmid in which the ERSE of the human CHOP gene or the CHOP binding site of the Rous sarcoma virus long terminal repeat was inserted (pGL3/ERSE or pGL3/CHOP-BS, respectively) was produced as described previously.<sup>23</sup> The mammalian expression plasmid for the dominant-negative form of CHOP (pOPRS-VI-L133/L140ACHOP) was as described elsewhere.<sup>13,23,35</sup> A plasmid expressing EGFP (pEGFP-C1) was obtained from CLONTECH Laboratories Inc.

## Cell culture and transfection

Gastric mucosal cells were isolated from guinea-pig fundic glands, as described previously.<sup>36</sup> After treatment with type I collagenase (80  $\mu$ g/ml) and Pronase E (1 mg/ml), cells were washed with medium containing FBS to stop the reaction of these enzymes. Then, cells ( $6 \times 10^5$  cells/wells) were cultured for 1 day in RPMI 1640 containing 0.3% v/v FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in type-I collagen-coated plastic culture plates (Iwaki) in 5% CO<sub>2</sub>/95% air at 37°C. After removing nonadherent cells by washing with RPMI 1640, cells that were attached to the plate at about 50% confluence were used. Guinea-pig gastric mucosal cells prepared under these conditions have been previously characterized, with the majority (about 90%) of such cells being identified as pit cells.<sup>36,37</sup> Cells were exposed to gastric stressors by changing the entire bathing medium.

Peritoneal macrophages were prepared from wild-type or CHOP-deficient mice as described previously.<sup>23</sup> Female mice were intraperitoneally given with 3 ml of 10% polyepitone (Difco), and peritoneal cells were harvested after 3 days. Cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS. After 2 days incubation, nonadherent cells were removed by three times washing with PBS and adherent cells were cultured in the presence or absence of indomethacin.

Transfection of cultured guinea-pig gastric mucosal cells with plasmids was carried out using Lipofectamine PLUS according to the manufacturer's protocols.

## Cell viability assay

Cell viability was determined by the MTT method as described previously.<sup>10</sup> After the treatment with NSAIDs or other gastric irritants, cells were incubated with MTT solution at the final concentration of 1 mg/ml in PBS for 2 h. Isopropanol and hydrochloric acid were added to the final concentrations of 50% and 20 mM, respectively. The optical density at 570 nm was determined by spectrophotometer using a reference wavelength of 630 nm.

Apoptotic DNA fragmentation was monitored as previously described.<sup>38</sup> Cells were collected using a rubber policeman and suspended in 70  $\mu$ l of lysis buffer, consisting of 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, and 0.5% sodium-*N*-lauroylsarcosinate. Proteinase K was added to a final

concentration of 1 mg/ml, and the lysate was incubated at 50°C for 2 h. RNaseA was then added to a final concentration of 0.5 mg/ml and incubated at 50°C for 30 min. These samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5  $\mu$ g/ml ethidium bromide.

Apoptotic chromatin condensation was monitored as described previously.<sup>21</sup> Cells were washed with PBS and fixed with 4% v/v formaldehyde for 1 h. Cells were then washed with PBS, stained with 10  $\mu$ g/ml Hoechst dye 33258 and observed under a fluorescence microscope.

## Immunoblotting and Northern blotting analyses

Whole cell extracts were prepared as described previously,<sup>21</sup> as were nuclear extracts.<sup>39</sup> The protein concentration of the samples was determined by the Bradford method. Samples were applied to 8, 10 or 12% polyacrylamide gels containing SDS, subjected to electrophoresis, and proteins were then immunoblotted with each antibody.

Total RNA was extracted from the cells using an RNeasy kit according to the manufacturer's protocols. Samples (5  $\mu$ g RNA) were separated by agarose (1%) gel electrophoresis in the presence of 6.3% formaldehyde and blotted onto nylon membranes. DNA probes for *GRP78*, *CHOP*, *ATF4* and *XBP-1* were amplified by PCR as described<sup>23,40,41</sup> and labeled using the Rediprime II DNA Labeling system according to the manufacturer's instructions. After hybridization and washing, membranes were analyzed with BAS2000A (FUJIX).

## Luciferase assay

Cells were transfected with 2  $\mu$ g of each of the *Photinus pyralis* luciferase reporter plasmids (pGL3 and its derivatives) and 0.4  $\mu$ g of internal standard plasmid bearing the *Renilla reniformis* luciferase reporter (pRL-SV40). *Photinus pyralis* luciferase activity in cell extracts was measured using the Dual Luciferase Assay System and then normalized for *Renilla reniformis* luciferase activity.

## Statistical analysis

All values are expressed as the mean  $\pm$  standard error (S.E. M.). One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison was used for evaluation of differences between the groups. A Student's *t*-test for unpaired results was performed to test differences between two groups. Results were considered to be significant for values of  $P < 0.05$ .

## Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan. We thank Dr. K Mori (Kyoto University) and S Akira (Osaka University) for the antibody against ATF6 and the expression plasmid for the dominant-negative form of CHOP, respectively.

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## Cytotoxic Synergy between Indomethacin and Hydrochloric Acid in Gastric Mucosal Cells

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Received February 12, 2004; accepted April 8, 2004

Orally ingested non-steroidal anti-inflammatory drugs (NSAIDs) and acid in gastric secretions are gastric irritants that co-exist at the surface of the gastric mucosa. Here, we examined the individual and combined effects of indomethacin, a typical NSAID, and hydrochloric acid on cell death in primary cultures of guinea pig gastric mucosal cells. Indomethacin alone (at concentrations less than 200  $\mu\text{M}$ ) did not induce apoptosis; however, hydrochloric acid-induced apoptosis was stimulated in the presence of indomethacin (50–200  $\mu\text{M}$ ). Isobologram analysis confirmed the presence of a cytotoxic synergy between indomethacin and hydrochloric acid. The synergistic response between the two gastric irritants was also observed for necrosis. Given that the  $\text{IC}_{50}$  value of indomethacin for inhibition of prostaglandin synthesis is about 5 nM, the synergistic response between indomethacin and hydrochloric acid appears to be independent of the inhibition of cyclooxygenase activity by indomethacin.

**Key words** cytotoxic synergy; hydrochloric acid; indomethacin; gastric mucosal cell; apoptosis; necrosis

The gastric mucosa is frequently exposed to different types of irritants (such as alcohol, acid, pharmaceutical drugs and bacteria). These irritants can cause gastric mucosal cell death, resulting in the production of gastric lesions *in vivo*. Using primary cultures of guinea pig gastric mucosal cells, we recently reproduced this gastric irritant-induced cell death *in vitro* and revealed that all of the gastric irritants tested (ethanol, hydrogen peroxide, hydrochloric acid and non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin) induced both necrosis and apoptosis of cells depending on the treatment conditions used. Short-term treatment of cells with relatively high concentrations of these irritants and long-term treatment of cells with relatively low concentrations of them induce necrosis and apoptosis, respectively.<sup>2,3)</sup> We have also previously shown that endogenous factors that protect the gastric mucosa from such irritants (heat shock proteins (HSPs) and prostaglandins (PGs)) or anti-ulcer drugs suppress apoptosis and necrosis in this cell model.<sup>4–9)</sup> This suggests that this *in vitro* system is useful for understanding the mechanisms underlying irritant-induced gastric lesions *in vivo*.

Most previous studies (including ours) pertaining to irritant-induced cell death *in vitro* have examined the effects of a single irritant on gastric mucosal cells. However, *in vivo*, multiple irritants co-exist simultaneously at the gastric mucosal surface. For example, when an NSAID is orally administered, the gastric mucosa is exposed not only to the NSAID but also to gastric acid (hydrochloric acid). Such an exposure to multiple irritants can give rise to gastric lesions. On this basis, in order to understand the mechanism of production of gastric lesions *in vivo*, it is important to examine what takes place in gastric mucosal cells *in vitro* when a combination of several irritants is used. In this study, we identified a synergistic response between indomethacin and hydrochloric acid in the induction of both apoptosis and necrosis in guinea pig gastric mucosal cells under primary culture. Furthermore, from the results obtained it is suggested that this synergistic response cannot be explained by an inhibitory effect of indomethacin on cyclooxygenase (COX) activity with a subse-

quent down regulation of PG synthesis.

### MATERIALS AND METHODS

**Chemicals and Media** Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, New York, U.S.A.). RPMI 1640 was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Pronase E and type I collagenase were purchased from Kaken Pharmaceutical Co. (Kyoto, Japan) and Nitta Gelatin Co. (Osaka, Japan), respectively. Hydrochloric acid, indomethacin, PD98059, SP600125 and SB203580 were from Wako Co. (Tokyo, Japan). Hoechst 33342 (Ho 342), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and propidium iodide (PI) were from Sigma Co. (Tokyo, Japan). Male guinea pigs (4 weeks of age) were purchased from Shimizu Co., LTD (Kyoto, Japan). ELISA kit used for PGE<sub>2</sub> analysis was from Cayman Chemical Co (Ann Arbor, Michigan, U.S.A.).

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 Institute of Health and were approved by the Animal Care Committee of Okayama University.

**Cell Culture** Gastric mucosal cells were isolated from guinea pig fundic glands as described previously.<sup>9)</sup> Isolated gastric mucosal cells ( $6 \times 10^5$  cells/dish) were cultured for 12 h in RPMI 1640 containing 0.3% FBS, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin in type-I collagen-coated plastic culture plates (Iwaki) under the conditions of 5% CO<sub>2</sub>/95% air and 37 °C. After removing non-adherent cells by washing with RPMI 1640, cells that were attached to plates at about 50% confluence were used. Guinea pig gastric mucosal cell preparations cultured under these conditions have been previously characterized, with the majority (about 90%) of cells being identified as pit cells.<sup>10,11)</sup>

**Treatment of Cells with Hydrochloric Acid and Indomethacin** Cells were exposed to hydrochloric acid and/or indomethacin by replacement of the entire bathing medium with fresh medium containing these irritants at con-

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concentrations as noted in the text. Under the conditions of our culture medium, 10, 15, 18, 20 and 22 mM hydrochloric acid (final concentrations) caused the pH values of 7.8, 7.3, 6.8, 6.3, 5.8 and 4.8, respectively. Since we pre-warmed the medium under the conditions of 5% CO<sub>2</sub>/95% air and 37 °C before the use of cell culture, these pH values were maintained from start to the end of cell culture (after 24 h).

For monitoring cell viability, cells were incubated for 2 h with MTT solution at a final concentration of 0.5 mg/ml. Cells were exposed to isopropanol and hydrochloric acid used at final concentrations of 50% (v/v) and 20 mM, respectively. The optical density of each sample at 570 nm was determined by spectrophotometer using a reference wavelength of 630 nm.<sup>12)</sup>

PGE<sub>2</sub> levels in culture media in the presence of different concentrations of indomethacin were determined by ELISA as previously described.<sup>13)</sup>

**DNA Fragmentation Assay** Apoptotic DNA fragmentation was monitored as previously described.<sup>11)</sup> Briefly, cells were collected with a rubber policeman and suspended in 20 μl of lysis buffer, consisting of 50 mM Tris-HCl (pH7.8), 10 mM EDTA, and 0.5% sodium-*N*-lauroylsarcosinate. Proteinase K was added to give a final concentration of 1 mg/ml, and the lysate was incubated at 50 °C for 2 h. RNaseA was then added to give a final concentration of 0.5 mg/ml and the solution was incubated again at 50 °C for a further 30 min. Samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide.

**Nuclear Staining Assay** Cells were washed with PBS and incubated with 0.17 mM Ho 342 and 100 μg/ml PI for

20 min, following which they were analyzed using fluorescence microscopy as previously described.<sup>5)</sup>

**Statistical Analysis** All values are expressed as the mean ± standard error (S.E.M.). A one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison was used for the evaluation of differences between groups. A Student's *t*-test for unpaired results was performed for the evaluation of differences between two groups. Differences were considered to be significant for values of *p* < 0.05.

Isobologram analysis was used for analyzing the combined effects of hydrochloric acid and indomethacin on cell death. The synergy between these irritants was estimated by determining a combination index (CI) value. The combined effect is one of summation when CI=1, synergism when CI < 1, and antagonism when CI > 1.<sup>14,15)</sup>

RESULTS AND DISCUSSION

**A Synergistic Response between Indomethacin and Hydrochloric Acid in the Induction of Apoptosis** In a first set of experiments we measured the sensitivity of primary cultures of gastric mucosal cells to exposure to indomethacin. Treatment of cells for 24 h with indomethacin at concentrations lower than 200 μM did not significantly decrease cell viability (Fig. 1A). On this basis we used concentrations of indomethacin less than 200 μM when examining

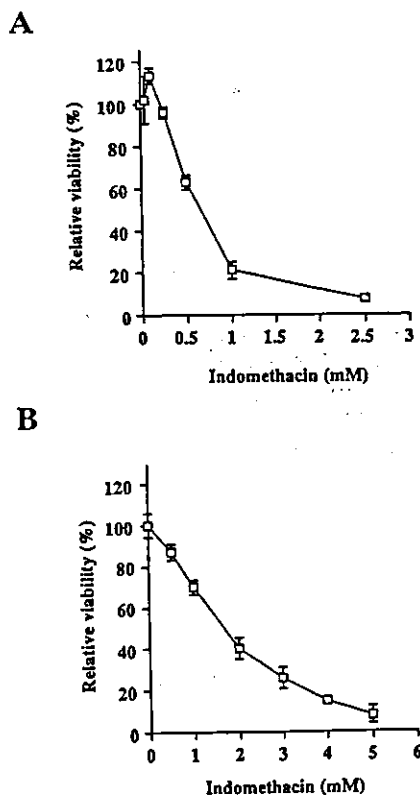


Fig. 1. Cell Death Induced by Exposure to Indomethacin  
Cultured gastric mucosal cells were treated with indicated concentrations of indomethacin for 24 h (A) or for 1 h (B). Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3).

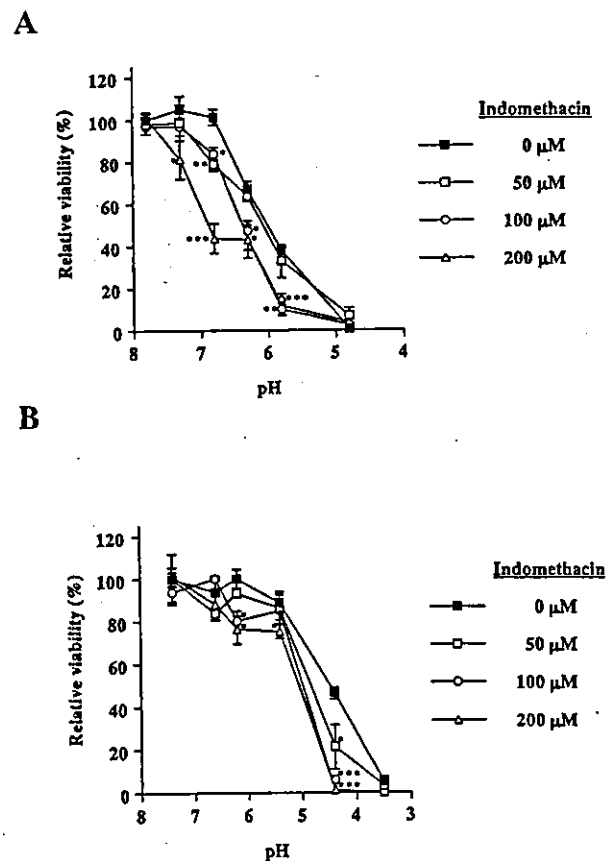


Fig. 2. Cell Death Induced by the Combined Use of Indomethacin and Hydrochloric Acid  
Cultured gastric mucosal cells were treated with different concentrations of hydrochloric acid (0, 10, 15, 18, 20, 22 mM) in the presence of indicated concentrations of indomethacin for 24 h (A) or for 1 h (B). The pH value of the medium was determined and plotted. Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3). \*\*\**p* < 0.001; \*\**p* < 0.01; \**p* < 0.05.

the combined effects of indomethacin and hydrochloric acid on the induction of cell death in cultured gastric mucosal cells.

Cells were exposed for 24 h to a range of concentrations of hydrochloric acid in the presence of different concentrations of indomethacin (Fig. 2A). In the absence of indomethacin, cell viability in the presence of hydrochloric acid decreased in a dose-dependent manner. In the presence of indomethacin (50, 100, 200  $\mu\text{M}$ ), the decrease in cell viability was observed at lower concentrations of hydrochloric acid (higher pH values) than when acid alone was used. Indomethacin did not

affect the degree of hydrochloric acid-induced cell death when it was employed at concentrations lower than 50  $\mu\text{M}$  (data not shown). Based on previous reports from this laboratory, it was postulated that the acid-induced cell death observed here and its up-regulation by indomethacin were most likely to be mediated by apoptosis. In support of this, DNA fragmentation assays showed that apoptotic DNA fragmentation was induced in a dose-dependent manner in the presence of hydrochloric acid and up-regulated in the presence of indomethacin (Fig. 3A). Moreover, in double-staining experiments with PI and Ho 342, we tested for the presence of

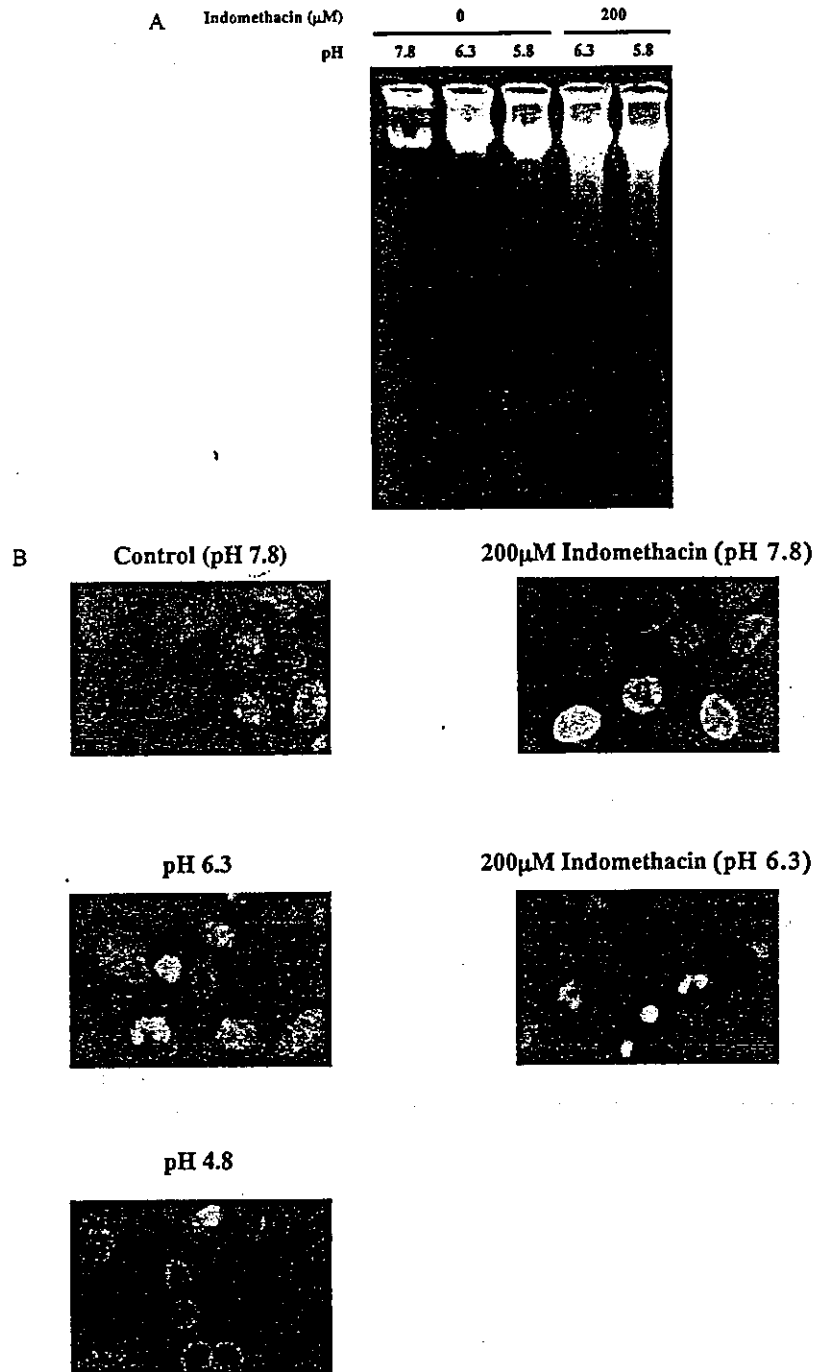


Fig. 3. Apoptotic DNA Fragmentation and Chromatin Condensation by a Combination between Indomethacin and Hydrochloric Acid

Cultured gastric mucosal cells were treated with various concentrations of hydrochloric acid in the presence or absence of 200  $\mu\text{M}$  indomethacin for 24 h. The pH value of the medium was determined and shown. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (A). After staining with both PI and Ho 342, cells were observed under fluorescence microscope. For positive control of necrosis, cells were cultured under the conditions of pH 4.8 (B).

apoptosis on the basis that living cells and apoptotic cells would not stain pink with propidium iodide, but that necrotic cells would stain in this way due to a loss of membrane integrity. Furthermore, Ho 342 can visualize the chromatin condensation of apoptotic cells.<sup>16)</sup> As shown in Fig. 3B, cells treated both hydrochloric acid and indomethacin under the above conditions were not stained with propidium iodide and showed the chromatin condensation. Further confirmation of induction of apoptosis by measuring cytochrome c release from mitochondria may be necessary.

In order to address the issue of a synergistic relationship between indomethacin and hydrochloric acid in the induction of apoptosis, CI values were calculated from the results of experiments presented in Figs. 1A and 2A. The CI value was 0.39, showing that a synergistic response for apoptosis was obtained when indomethacin and hydrochloric acid were used in unison.

**A Synergistic Response between Indomethacin and Hydrochloric Acid for Necrosis** As described in the Introduction, short-term (1 h) treatment of cells with relatively high concentrations of gastric irritants induces necrosis.<sup>1,2)</sup> We next examined the synergy between indomethacin and hydrochloric acid in relation to the induction of necrosis. The sensitivity of gastric mucosal cells to short-term (1 h) indomethacin treatment is shown in Fig. 1B. As shown in Fig. 2B, in the presence of indomethacin, a decrease in cell viability was observed at lower concentrations of hydrochloric acid (higher pH values) than in the absence of indomethacin. It was concluded that the cell death induced in this manner was mainly mediated by necrosis, given that the cell death was not accompanied by apoptotic DNA fragmentation and that pink nuclear staining was observed in double-staining experiments with PI and Ho 342 (data not shown).

The CI values between indomethacin and hydrochloric acid for necrosis were calculated from the results of experiments presented in Figs. 1B and 2B. The CI value was 0.56, showing that indomethacin and hydrochloric acid used in a combined manner also act synergistically to produce necrosis in gastric mucosal cells. The overall response, however, was not as strong as that for apoptosis.

**Mechanism of the Synergistic Response between Indomethacin and Hydrochloric Acid** NSAIDs inhibit COX activity and as such they decrease the level of circulating PGs. In turn, PGs, and PGE<sub>2</sub> in particular, have cytoprotective effects on the gastric mucosa.<sup>7,8,17,18)</sup> We previously reported that the primary culture of guinea pig gastric mucosal cells produced PGE<sub>2</sub> continuously even without stressors.<sup>19)</sup> On this basis we considered whether or not the effect of indomethacin on hydrochloric acid-induced apoptosis such as that seen in Fig. 2A might be caused by an indomethacin-induced inhibition of COX activity. PGE<sub>2</sub> levels in culture media after 1 h treatment with a range of concentrations of indomethacin were measured by ELISA and compared to the effect of indomethacin on the IC<sub>50</sub> (concentration required for 50% reduction of cell viability) values of hydrochloric acid for apoptosis. As shown in Fig. 4, much higher concentrations of indomethacin were required to decrease the IC<sub>50</sub> value than were required to decrease PGE<sub>2</sub> levels. This inhibitory effect of indomethacin on PGE<sub>2</sub> levels was basically same when the period of treatment was prolonged to 24 h (data not shown). It seems that the synergistic

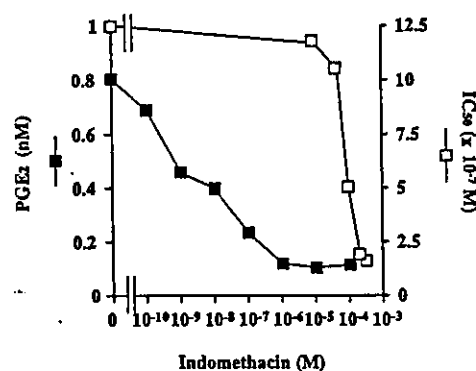


Fig. 4. Relationship between Inhibition of PG Synthesis and the Synergistic Response by Indomethacin

Cultured gastric mucosal cells were treated for 24 h with different concentrations of hydrochloric acid in the presence of a range of indomethacin concentrations. Cell viability was determined by the MTT method. The IC<sub>50</sub> values for hydrochloric acid (concentration of H<sup>+</sup> required for 50% reduction of cell viability) were determined. PGE<sub>2</sub> levels in culture media were determined by ELISA after 1 h treatment of indomethacin.

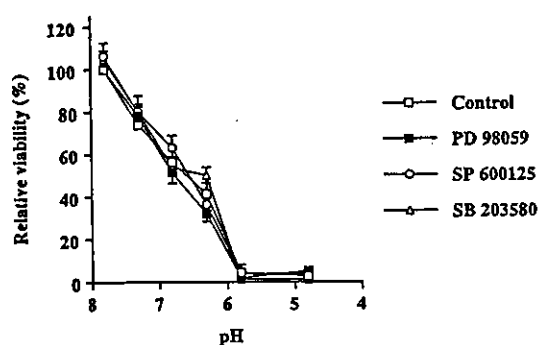


Fig. 5. Effect of Inhibitors of MAPKs on the Cytotoxic Synergy between Indomethacin and Hydrochloric Acid

Cultured gastric mucosal cells were pre-treated for 1 h with 10 μM PD98059 (MEK1/ERK inhibitor), SP600125 (an inhibitor for JNK) and SB203580 (an inhibitor for p38). They were then treated for 24 h with different concentrations of hydrochloric acid in the presence 200 μM indomethacin. The pH value of the culture medium was determined and plotted. Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n = 3).

response between indomethacin and hydrochloric acid for apoptosis cannot be explained by an inhibitory effect of indomethacin on COX activity and subsequent PG synthesis.

Recently, some NSAIDs were reported to activate mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-jun NH<sub>2</sub>-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38).<sup>20,21)</sup> Given that these MAPKs are known to be involved in apoptosis,<sup>22,23)</sup> we used specific inhibitors (PD98059 for MEK1/ERK, SP600125 for JNK and SB203580 for p38) to test the possibility that indomethacin could affect the hydrochloric acid-induced apoptosis via altered MAPK activity. Gastric mucosal cells were pre-treated with each of three inhibitors and then treated with indomethacin and hydrochloric acid for 24 h. As shown in Fig. 5, none of inhibitors reduced the level of hydrochloric acid-induced apoptosis in the presence of indomethacin. Based on previous reports where these inhibitors were used,<sup>22,23)</sup> the concentrations used in the experiments described here should have been sufficient to inhibit their targets. As such, we suggest that these particular MAPKs are not involved in the synergistic response between indomethacin and hydrochloric acid for hydrochloric acid-induced apoptosis.

# Adaptive Cytoprotection Induced by Pretreatment with Ethanol Protects Against Gastric Cell Damage by NSAIDs

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In this study, we examined adaptive cytoprotection against NSAIDs in human gastric carcinoma cells in culture. Pretreatment of cells with low (nontoxic) concentrations of ethanol protected cells from cell death induced by subsequent exposure to NSAIDs. The adaptive cytoprotection against NSAIDs induced by ethanol was not attenuated by pretreatment of cells with inhibitors of protein synthesis or prostaglandin synthesis, thus inferring that neither newly synthesized proteins nor prostaglandins are involved in this process. Furthermore, treatment of cells with the low concentration of ethanol did not affect the synthesis and secretion of mucin. In *in vivo* experiments on rats, oral preadministration of a low dose of ethanol protected the gastric mucosa from gastric lesions induced by subsequent oral administration of NSAIDs. One possible explanation for this *in vivo* phenomenon is that the adaptive cytoprotection induced by ethanol protects the gastric mucosa from the direct cytotoxic effect of NSAIDs.

**KEY WORDS:** adaptive cytoprotection; ethanol; NSAID; gastric lesion; gastric irritant.

Nonsteroidal antiinflammatory drugs (NSAIDs) are one of the most frequently used classes of medicines in the world and account for nearly 5% of all prescribed medications (1). NSAIDs have great efficacy in the treatment of pain, inflammation, and fever on account of their inhibition of cyclooxygenase (COX) activity. COX is the rate-limiting enzyme for the synthesis of prostaglandins (PGs), which have a strong involvement in the induction of inflammation. On the other hand, NSAID administration is associated with gastrointestinal complications, such as gastric ulcers (2). About 15–30% of persons who are chronic users of NSAIDs suffer from gastrointestinal ulcers and bleeding (3–6). In the United States, about 16,500

people die per year as a result of NSAID-associated gastrointestinal complications (7).

The inhibition of COX activity by NSAIDs was previously believed to be fully responsible for the gastrointestinal side effects of these drugs (8) given that PGs have a strong cytoprotective effect on the gastrointestinal mucosa (9). We recently suggested that in addition to COX inhibition by NSAIDs, the direct cytotoxicity of NSAIDs is also responsible for their producing gastric lesions (Tomisato et al., submitted). Therefore, a protocol should be found to protect gastric mucosal cells from the direct cytotoxicity of NSAIDs.

Adaptive cytoprotection refers to phenomenon in which the gastric mucosa develops enhanced resistance to a gastric irritant by virtue of the preadministration of low doses of that same irritant or another (mild irritant) *in vivo* (10). Adaptive cytoprotection is also observed *in vitro*; the pretreatment of cultured gastric mucosal cells with low concentrations of an irritant (mild irritant) induces a cell phenotype that is resistant to subsequent exposure of

Manuscript received July 31, 2003; accepted October 31, 2003.

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the cells to high concentrations of the same or another irritant (11–14). Using the human gastric carcinoma cell line, AGS, which has been shown to be morphologically and functionally consistent with normal gastric mucosal cells (11, 15, 16), we recently reported that pretreatment of cells with low concentrations of ethanol made cells resistant to subsequent exposure to high concentrations of ethanol (17). However, no reports have been published to date concerning *in vitro* adaptive cytoprotection against NSAIDs. In this study, we found that low (nontoxic) concentrations of ethanol specifically results in adaptive cytoprotection against NSAIDs in AGS cells. We also found that preadministration of a low dose of ethanol in rats reduced the production of gastric lesions induced by subsequent administration of NSAIDs.

## MATERIALS AND METHODS

**Chemicals and Media.** Fetal bovine serum (FBS) was from Gibco (Grand Island, NY). Ham F12 was from Nissui (Tokyo). Indomethacin was from Wako Co. (Tokyo). Ibuprofen, diclofenac, cycloheximide, actinomycin-D, 2,2'-azinobis(ethylbenzothiazolin-6-sulfonic acid)-2NH<sub>4</sub> (ABTS), and 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma Co. (Tokyo). [<sup>3</sup>H]Glucosamine (18.7 Ci/mmol) was from Amersham Biosciences Co. (Tokyo). Horseradish peroxidase-conjugated soybean agglutinin lectin (SBA) was from Seikagaku Co. (Tokyo). Male Wistar rats weighing 160–190 g were purchased from Shimizu Co. (Kyoto, Japan).

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 Institute of Health and were approved by the Animal Care Committee of Okayama University.

**Cell Culture and Treatment with NSAIDs.** AGS cells were cultured in Ham F12 medium containing 10% FBS, as described previously (11, 15, 16). AGS cells (2 × 10<sup>5</sup> cells per well in 24-well plates) were cultured for 24 hr and used for experiments. NSAIDs were dissolved in DMSO and control experiments (without NSAIDs) were performed in the presence of same concentrations of DMSO. Cells were exposed to NSAIDs by replacement of the entire bathing medium with fresh medium containing NSAIDs. For monitoring cell viability, cells were incubated with MTT solution at a final concentration of 0.5 mg/ml for 2 hr. Isopropanol and hydrochloric acid were added to cells at final concentrations of 50% and 20 mM, respectively. The optical density of each sample at 570 nm was determined by spectrophotometer using a reference wavelength of 630 nm (18).

**Measurement of Incorporation of [<sup>3</sup>H]Glucosamine into Cells.** Measurement of incorporation of [<sup>3</sup>H]glucosamine into cells was performed as described previously (19). After incubation with [<sup>3</sup>H]glucosamine, cells were washed with phosphate-buffered saline (PBS) and treated with the solution containing Tris/HCl (pH 7.2) and 2% Triton X-100. Samples were incubated for 15 min on ice in the presence of 50% ethanol and washed with 70% ethanol. The precipitates were suspended with 1 N NaOH and neutralized by acetic acid. The radioactivities were calculated using a liquid scintillation counter.

**Determination of Mucin Levels in Culture Media.** Preparation of mucin fractions from culture media was performed as described previously (20, 21). Mucin fractions were purified and concentrated by the freeze-dry method and ethanol precipitation. The amount of mucin in samples was determined by the enzyme-linked lectin-binding assay (20, 21). Samples (100 μg protein) were loaded on polystyrene 96-well ELISA plates (Iwaki) and incubated at 4°C for 12 hr. After washing with PBS, samples were incubated with 200 μl blocking buffer (1% BSA in PBS) for 2 hr at 37°C. After washing with PBS, samples were then incubated with 50 μl SBA solution (10 μg/ml SBA in PBS) for 1 hr at 37°C. After further washing with PBS, samples were finally incubated with 100 μl ABTS solution (1 mM ABTS, 0.1 M citrate [pH 4.0], 0.03% hydrogen peroxide) for 40 min at room temperature. The optical density at 412 nm was then measured.

**Gastric Damage Assay.** Rats (24 hr fasted) were orally administered with NSAIDs with 1% methylcellulose in a volume of 5 ml/kg animal. In some experiments, 30 min before the oral administration of NSAIDs, ethanol was orally administered in a volume of 5 ml/kg animal. Three hours after the oral administration of NSAIDs, rats were anesthetized and stomachs removed and scored for hemorrhagic damage by an observer unaware of the treatment that the rats had received. The score involved measuring the area of all lesions in millimeters and summing the values to give an overall gastric lesion index.

**Statistical Analysis.** All values are expressed as the mean ± standard error (SE). One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison was used for the evaluation of differences between groups. A Student *t*-test for unpaired results was performed for the evaluation of differences between two groups. Differences were considered to be significant for values of *P* < 0.05.

## RESULTS AND DISCUSSION

**Adaptive Cytoprotection Against NSAIDs Induced by Ethanol.** We first measured the sensitivity of AGS cells to diclofenac, one of the NSAIDs. Treatment of cells with diclofenac (5 mM) for 2 hr decreased cell viability to about 50% of the control (Figure 1A). We also measured the sensitivity of AGS cells to ethanol and found that treatment of cells with ethanol less than 5% (v/v) for 1 hr did not affect cell viability (Figure 1B), thus showing that low concentrations of ethanol as such serve only as a "mild irritant" for AGS cells. Similar results were obtained when cell death was monitored by the trypan blue exclusion test (data not shown). We showed that short-term (1- to 2-hr) treatment of gastric mucosal cells with relatively high concentrations of NSAIDs and long-term (16-hr) treatment of them with relatively low concentrations of NSAIDs induced necrosis and apoptosis, respectively (19, 22). Therefore, cell death by exposure to 5 mM diclofenac for 2 hr was likely to be mediated by necrosis. We confirmed this point by showing that the cell death caused by this form of diclofenac treatment was not accompanied by apoptotic DNA fragmentation and chromatin condensation (data not shown).

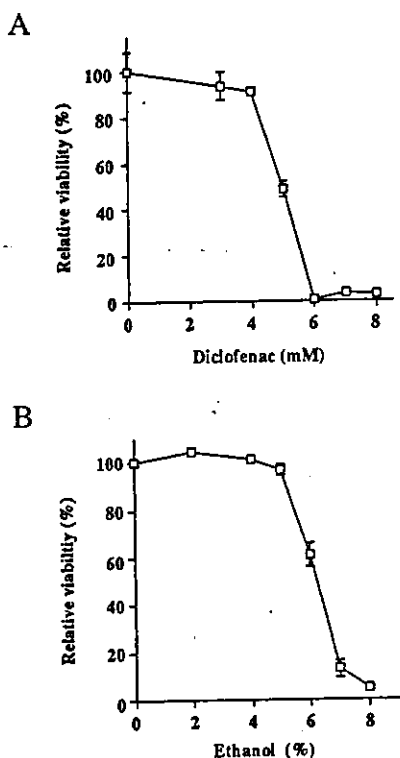


Fig 1. Cell death induced by exposure to diclofenac or ethanol. AGS cells were treated with the indicated concentrations of diclofenac for 2 hr (A) or ethanol for 1 hr (B). Cell viability was determined by the MTT method. Values are mean  $\pm$  SE ( $n = 3$ ).

AGS cells were pretreated with low concentrations (1–5%) of ethanol for 1 hr and subsequently treated with diclofenac (5 mM) for 2 hr (Figure 2A). The pretreatment of cells with 2–4% ethanol significantly attenuated the cell death caused by a subsequent exposure of the cells to diclofenac compared to that when the pretreatment step was not included (Figure 2A). We considered these findings to be indicative of adaptive cytoprotection and refer to this process as such in the subsequent sections of this paper. The adaptive cytoprotection against diclofenac was dependent on the concentrations of ethanol used and was most apparent when 3% ethanol was used (Figure 2A). Adaptive cytoprotection against diclofenac was observed when pretreatment with 3% ethanol was carried out for 0.5, 1, or 2 hr, with the effect most apparent for the 1-hr pretreatment (Figure 2B). Furthermore, the adaptive cytoprotection induced by ethanol was also observed when diclofenac was used at concentrations of 4.5 and 5.5 mM (Figure 2C).

We also examined the effect of ethanol pretreatment on cell death induced by NSAIDs other than diclofenac. We found that treatment of cells with 5 mM indomethacin or 10 mM ibuprofen decreased cell viability to about 50%

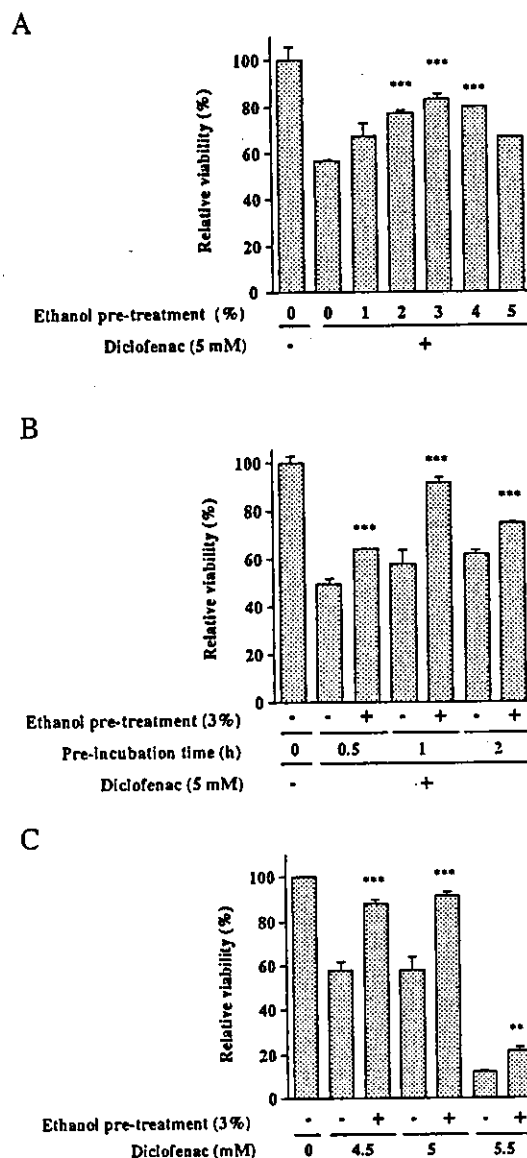


Fig 2. Ethanol-induced adaptive cytoprotection against diclofenac. AGS cells were pretreated with the indicated concentrations (A) or 3% (B, C) ethanol for 1 hr (A, C) or the indicated times (B). Cells were further treated with diclofenac at 5 mM (A, B) or the indicated concentrations (C) for 2 hr. Cell viability was determined by the MTT method. Values are mean  $\pm$  SE ( $n = 3$ ). \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ .

(data not shown). As shown in Figure 3, pretreatment of cells with 3% ethanol for 1 hr made cells resistant to subsequent exposure to indomethacin or ibuprofen. Therefore, the adaptive cytoprotection induced by ethanol is not specific for diclofenac but is observed against various NSAIDs in general.

**Adaptive Cytoprotection Against NSAIDs Induced by Various Gastric Irritants.** Given that, in addition to ethanol and NSAIDs, gastric mucosal cells *in vivo* are

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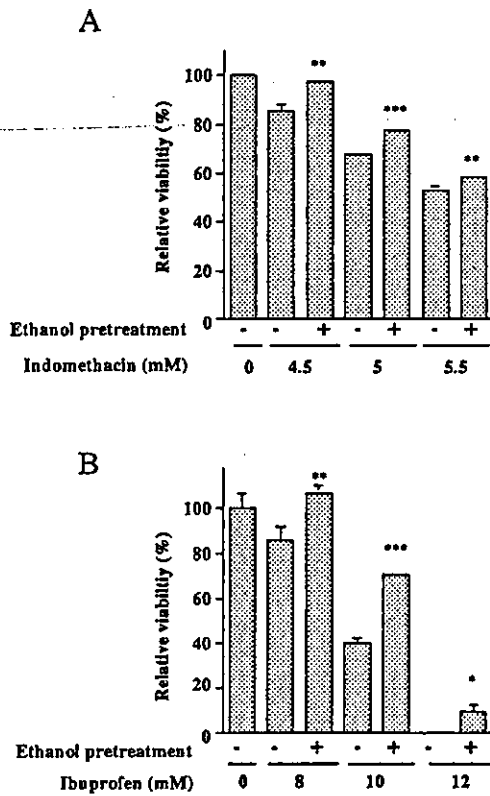


Fig 3. Ethanol-induced adaptive cytoprotection against NSAIDs other than diclofenac. AGS cells were pretreated with 3% ethanol for 1 hr. Cells were further treated with the indicated concentrations of indomethacin (A) or ibuprofen (B) for 2 hr. Cell viability was determined by the MTT method. Values are mean  $\pm$  SE ( $n = 3$ ). \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ .

exposed to various gastric irritants, such as oxidative stressors and acids, we examined the process of adaptive cytoprotection against diclofenac induced by gastric irritants other than ethanol (diclofenac, hydrogen peroxide, and hydrochloric acid). We first measured the sensitivity of AGS cells to hydrogen peroxide and hydrochloric acid and found that treatment of cells with hydrogen peroxide lower than 0.3 mM or hydrochloric acid lower than 10 mM did not affect cell viability (data not shown). As shown in Figure 4, none of the gastric irritants (diclofenac, hydrogen peroxide, and hydrochloric acid) induced adaptive cytoprotection against diclofenac. Therefore, it seems that the effect of ethanol is specific in its action to bring about adaptive cytoprotection against diclofenac.

**Mechanism of Ethanol-Induced Adaptive Cytoprotection Against NSAIDs.** Previous *in vivo* and *in vitro* studies suggested that various molecular mechanisms, such as induction of heat shock proteins (HSPs), stimulation of PG synthesis, and stimulation of mucin synthesis and secretion, are involved in the process of adaptive cytoprotection under various conditions. We examined here

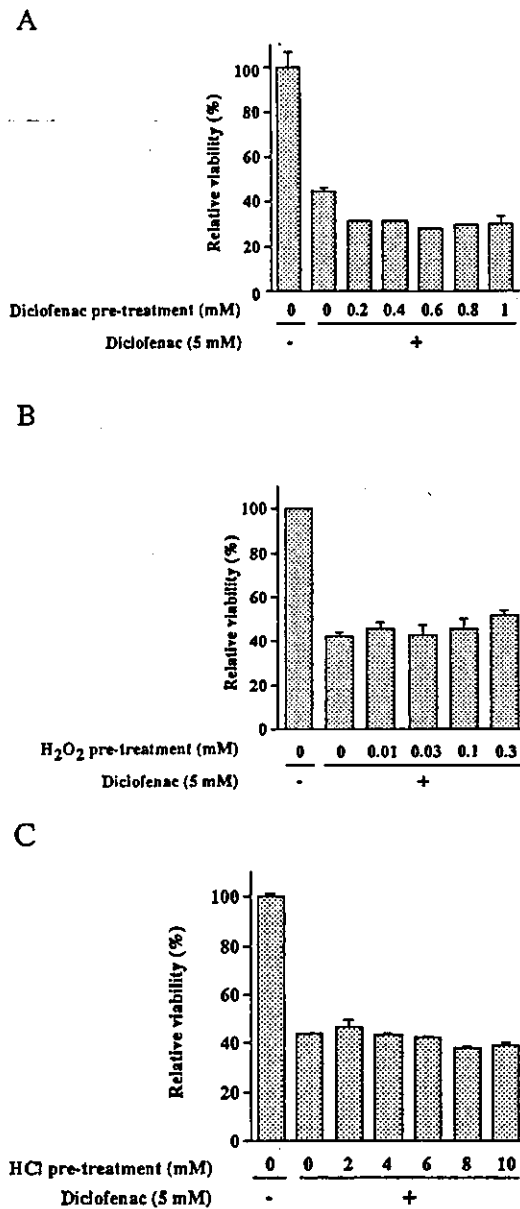
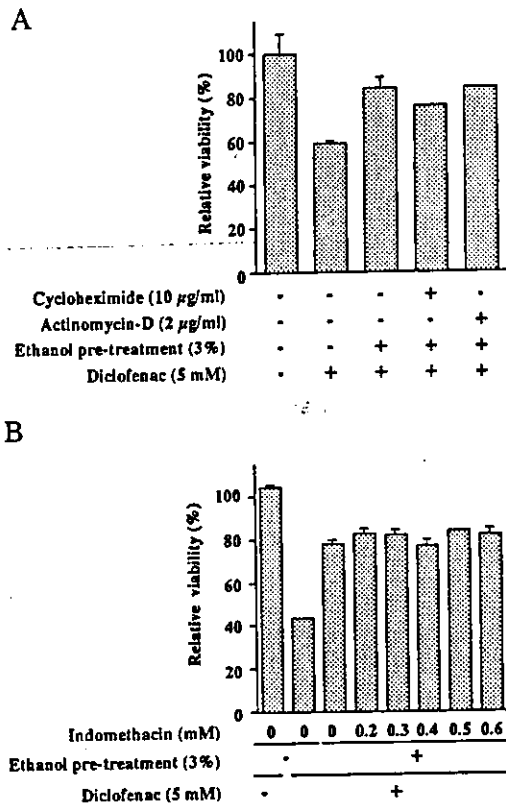


Fig 4. Adaptive cytoprotection against diclofenac induced by various irritants. AGS cells were pretreated with the indicated concentrations of diclofenac (A), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (B), or hydrochloric acid (HCl) for 1 hr. Cells were further treated with diclofenac (5 mM) for 2 hr. Cell viability was determined by the MTT method. Values are mean  $\pm$  SE ( $n = 3$ ).

whether or not these mechanisms might be responsible for the adaptive cytoprotection against NSAIDs induced by ethanol in AGS cells.

Preinduction of HSPs protects gastric mucosal cells from cell death induced by various gastric irritants including NSAIDs (22–27). Furthermore, induction of HSPs was suggested to be involved in heat stress-induced





**Fig 5.** Effect of an inhibitor of protein synthesis or PG synthesis on ethanol-induced adaptive cytoprotection. AGS cells were initially treated with cycloheximide (CHX; 10 µg/ml) or actinomycin-D (Act-D; 2 µg/ml) for 0.5 hr (A) or indicated concentrations of indomethacin for 1 hr (B), then with 3% ethanol for 1 hr, and, finally, with diclofenac (5 mM) for 2 h. Cell viability was determined by the MTT method. Values are mean  $\pm$  SE ( $n = 3$ ).

adaptive cytoprotection against ethanol (25). To test the contribution of HSPs to the adaptive cytoprotection identified in the present study, we examined the effect of an inhibitor of transcription (actinomycin-D) or translation (cycloheximide) on the ethanol-induced adaptive cytoprotection against diclofenac seen here. Neither actinomycin-D (2 µg/ml) nor cycloheximide (10 µg/ml) was found to inhibit the adaptive cytoprotection process (Figure 5A). We confirmed that these inhibitors decreased to less than 5% of control the incorporation of [<sup>35</sup>S]methionine into acid-insoluble fractions (data not shown), thereby showing that both of these inhibitors inhibited transcription or translation almost completely. It therefore seems that induction of distinct proteins such as HSPs is not involved in the adaptive cytoprotection against NSAIDs.

Prostaglandins (PGs), especially PGE<sub>2</sub>, have cytoprotective effects on gastric mucosa as a consequence of various physiological mechanisms (28–30). We have previously shown that PGE<sub>2</sub> protects cultured gastric mucosal cells from ethanol-induced apoptosis (30, 31). Further-

more, we recently showed that pretreatment of AGS cells with 3% ethanol stimulated PG synthesis and that this stimulation was partly responsible for inducing adaptive cytoprotection against ethanol (17). To test whether or not the stimulation of PGE<sub>2</sub> synthesis by ethanol is responsible for the adaptive cytoprotection against NSAIDs in AGS cells, we examined the effect of indomethacin (an inhibitor of PG synthesis) on the process. As shown in Figure 5B, pretreatment of cells with indomethacin did not prevent the adaptive cytoprotection from occurring. Since we confirmed that indomethacin (0.5 mM) inhibited the stimulation of PG synthesis caused by 3% ethanol treatment (data not shown), these results suggest that the ethanol-induced adaptive cytoprotection against NSAIDs is not mediated by the stimulation of PG synthesis. The mechanism of adaptive cytoprotection induced by ethanol against NSAIDs thus seems to be different from that against ethanol.

Low concentrations of ethanol have been reported to induce adaptive cytoprotection against ethanol in primary cultures of rat gastric mucosal cells (12). These authors pointed out the importance of mucin secretion in the appearance of adaptive cytoprotection by showing that changing the culture medium after the pretreatment with low concentrations of ethanol diminished the degree to which the adaptive cytoprotection was manifested (12). Although AGS cells produce mucin (32), changing the culture medium after pretreatment with 3% ethanol did not affect the extent to which adaptive cytoprotection against NSAIDs was seen in this cell type (data not shown). Furthermore, we examined here the effect of 3% ethanol on mucin synthesis by measuring the incorporation of [<sup>3</sup>H]glucosamine, a substrate of mucin synthesis, into cells. As shown in Figure 6A, exposure of cells to 3% ethanol slightly decreased [<sup>3</sup>H]glucosamine uptake, suggesting that mucin synthesis was partially inhibited by ethanol. We also determined the amount of mucin in culture media after 3% ethanol treatment by using the enzyme-linked lectin-binding assay (20, 21). As shown in Figure 6B, treatment of cells with 3% ethanol did not affect the amount of mucin in culture media, suggesting that the secretion of mucin from cells was not affected by treatment with 3% ethanol. Therefore, it seems that mucin synthesis and secretion are not involved in the ethanol-induced adaptive cytoprotection against NSAIDs seen in AGS cells.

**Adaptive Cytoprotection Against NSAIDs Induced by Ethanol *In Vivo*.** As described in the introductory section, evidence of adaptive cytoprotection is also observed *in vivo*. For example, preadministration of a low dose of ethanol reduced the production of gastric lesions caused by subsequent administration of a high dose of ethanol

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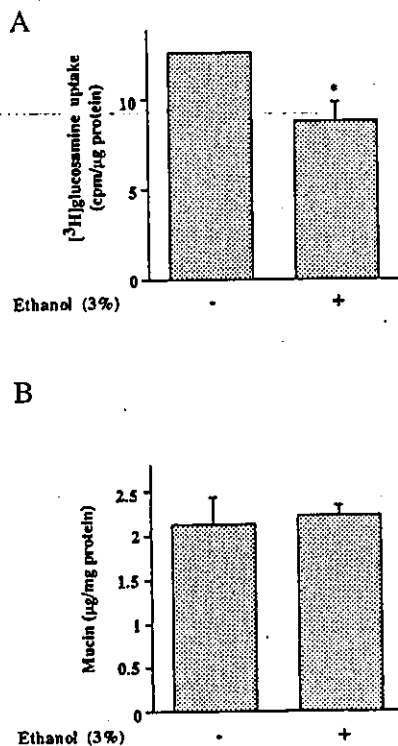


Fig 6. Effect of ethanol on synthesis and secretion of mucin. AGS cells were incubated with [<sup>3</sup>H]glucosamine in the presence or absence of 3% ethanol for 1 hr in a culture dish (diameter, 6 cm). Incorporated radioactivities were determined with a liquid scintillation counter (A). AGS cells were incubated with or without 3% ethanol for 1 hr in a culture dish (diameter, 6 cm) and the amount of mucin in the culture medium was determined by the enzyme-linked lectin-binding assay. Values are mean ± SE (n = 3). \*P < 0.05.

(10, 33). However, as for the adaptive cytoprotection against NSAIDs induced by ethanol *in vivo*, just one paper has reported that preadministration of 20% ethanol did not affect the production of gastric lesions caused by subsequent administration of indomethacin (34). We therefore examined here the effect of preadministration of various doses of ethanol on the production of gastric lesions induced by subsequent administration of NSAIDs. We first examined the production of gastric lesions by ethanol alone and found that more than 80% ethanol is required for it alone to produce gastric lesions (data not shown). We then sought to examine the effect of preadministration of lower doses of ethanol on the production of gastric lesions resulting from the subsequent administration of indomethacin. As shown in Figure 7, oral preadministration of 40% ethanol significantly reduced the production of gastric lesions caused by subsequent administration of indomethacin. Preadministration of 20% ethanol did not reduce the production of gastric lesions, which is consistent with the previous report (34). We also found that oral

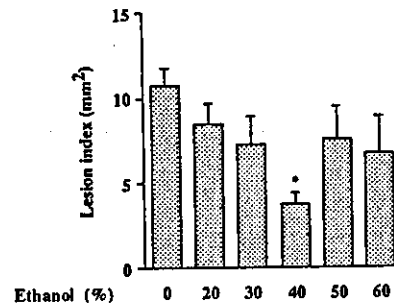


Fig 7. Effect of ethanol preadministration on production of gastric lesions by indomethacin in rats. Rats were orally preadministered the indicated concentrations of ethanol. After 0.5 hr, animals were orally administered 30 mg/kg indomethacin. After 3 hr the stomach was removed and scored for hemorrhagic damage. Values are mean ± SE (n = 10–14). \*P < 0.05.

preadministration of 40% ethanol significantly reduced the production of gastric lesions caused by subsequent administration of diclofenac or ibuprofen (Figure 8). Therefore, it was concluded that preadministration of low doses of ethanol reduces the production of gastric lesions caused by subsequent administration of NSAIDs.

In this study, we identified the occurrence of ethanol-induced adaptive cytoprotection against NSAIDs in AGS cells. We also found that this process takes place *in vivo*,

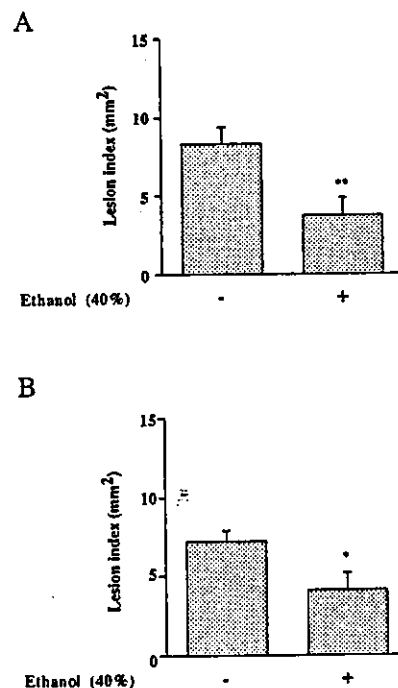


Fig 8. Effect of ethanol preadministration on production of gastric lesions by NSAIDs in rats. Rats were orally preadministered 40% ethanol. After 0.5 hr, animals were orally administered 40 mg/kg diclofenac (A) or 100 mg/kg ibuprofen (B). After 3 hr the stomach was removed and scored for hemorrhagic damage. Values are mean ± SE (n = 6). \*\*P < 0.01; \*P < 0.05.

with preadministration of low doses of ethanol reducing the production of gastric lesions caused by subsequent administration of NSAIDs. Because the *in vivo* system is complicated by the involvement of neural input, mucosal blood flow, salivary factors, and the immune system, we are unable to state definitively that the adaptive cytoprotection *in vivo* is related to the adaptive cytoprotection *in vitro*.

While we attempted to reveal the molecular mechanism governing the process of ethanol-induced adaptive cytoprotection against NSAIDs in AGS cells by using inhibitors of protein synthesis or PG synthesis and by measuring mucin synthesis and secretion, we can only say that the induction of distinct proteins and compounds that protect cells from NSAIDs (such as HSP induction, PG synthesis, and mucin synthesis and secretion) is not responsible for the adaptive cytoprotection. We consider that a protein synthesis-independent signal transduction, such as protein phosphorylation, is involved in this process. Identification of the molecular mechanism governing the ethanol-induced adaptive cytoprotection against NSAIDs may be important to establish a clinical protocol for the treatment of NSAID-induced gastric lesions.

#### ACKNOWLEDGMENT

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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