

**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>16,29</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% polypeptone (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  $P < 0.05$ .

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## References

- Smalley WE, Ray WA, Daugherty JR and Griffin MR (1995) Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. *Am. J. Epidemiol.* 141: 539–545
- Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS,

- Hampel H, Hull M, Landreth G, Lue L, Mraz R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strommeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegryniak B, Wenk G and Wyss-Coray T (2000) Inflammation and Alzheimer's disease. *Neurobiol. Aging* 21: 383-421
3. Levy GN (1997) Prostaglandin H synthases, nonsteroidal anti-inflammatory drugs, and colon cancer. *FASEB J.* 11: 234-247
  4. Rogers J, Webster S, Lue LF, Brachova L, Civin WH, Emmerling M, Shivers B, Walker D and McGeer P (1996) Inflammation and Alzheimer's disease pathogenesis. *Neurobiol. Aging* 17: 681-686
  5. Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE and Koo EH (2001) A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. *Nature* 414: 212-216
  6. Rigas B and Shiff SJ (2000) Is inhibition of cyclooxygenase required for the chemopreventive effect of NSAIDs in colon cancer. A model reconciling the current contradiction. *Med. Hypotheses* 54: 210-215
  7. Hawkey CJ (2000) Nonsteroidal anti-inflammatory drug gastropathy. *Gastroenterology* 119: 521-535
  8. Singh G (1998) Recent considerations in nonsteroidal anti-inflammatory drug gastropathy. *Am. J. Med.* 105: 31S-38S
  9. Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, Mio M, Tsuchiya T and Mizushima T (2004) Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. *Biochem. Pharmacol.* 67: 575-585
  10. Tomisato W, Tsutsumi S, Rokutan K, Tsuchiya T and Mizushima T (2001) NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. *Am. J. Physiol. Gastrointest. Liver Physiol.* 281: G1092-G1100
  11. Kaufman RJ (2002) Orchestrating the unfolded protein response in health and disease. *J. Clin. Invest.* 110: 1389-1398
  12. Ron D (2002) Translational control in the endoplasmic reticulum stress response. *J. Clin. Invest.* 110: 1383-1388
  13. Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M and Mori K (2000) ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Mol. Cell. Biol.* 20: 6755-6767
  14. Kaufman RJ (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 13: 1211-1233
  15. Mori K (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 101: 451-454
  16. Liu H, Bowes III RC, van de Water B, Sillence C, Nagelkerke JF and Stevens JL (1997) Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca<sup>2+</sup> disturbances, and cell death in renal epithelial cells. *J. Biol. Chem.* 272: 21751-21759
  17. Reddy RK, Lu J and Lee AS (1999) The endoplasmic reticulum chaperone glycoprotein GRP94 with Ca(2+)-binding and antiapoptotic properties is a novel proteolytic target of calpain during etoposide-induced apoptosis. *J. Biol. Chem.* 274: 28476-28483
  18. Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL and Ron D (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* 12: 982-995
  19. Mengesdorf T, Althausen S, Oberndorfer I and Paschen W (2001) Response of neurons to an irreversible inhibition of endoplasmic reticulum Ca(2+)-ATPase: relationship between global protein synthesis and expression and translation of individual genes. *Biochem. J.* 356 (Part 3): 805-812
  20. Kawai S, Nishida S, Kato M, Furumaya Y, Okamoto R, Koshino T and Mizushima Y (1998) Comparison of cyclooxygenase-1 and -2 inhibitory activities of various nonsteroidal anti-inflammatory drugs using human platelets and synovial cells. *Eur. J. Pharmacol.* 347: 87-94
  21. Tsutsumi S, Tomisato W, Takano T, Rokutan K, Tsuchiya T and Mizushima T (2002) Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. *Biochim. Biophys. Acta* 1589: 168-180
  22. Yoshida H, Haze K, Yanagi H, Yura T and Mori K (1999) Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J. Biol. Chem.* 273: 33741-33749
  23. Gotoh T, Oyadomari S, Mori K and Mori M (2002) Nitric oxide-induced apoptosis in RAW 264.7 macrophages is mediated by endoplasmic reticulum stress pathway involving ATF6 and CHOP. *J. Biol. Chem.* 277: 12343-12350
  24. Yoshida H, Matsui T, Yamamoto A, Okada T and Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 107: 881-891
  25. Ma Y, Brewer JW, Diehl JA and Hendershot LM (2002) Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J. Mol. Biol.* 318: 1351-1365
  26. Yoneda T, Imaizumi K, Oono K, Yui D, Gomi F, Katayama T and Tohyama M (2001) Activation of caspase-12, an endoplasmic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. *J. Biol. Chem.* 276: 13935-13940
  27. Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, Yoshida H, Mori K and Kaufman RJ (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev.* 16: 452-466
  28. Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A and Ichijo H (2002) ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev.* 16: 1345-1355
  29. Sugawara S, Takeda K, Lee A and Dennert G (1993) Suppression of stress protein GRP78 induction in tumor B/C10ME eliminates resistance to cell mediated cytotoxicity. *Cancer Res.* 53: 6001-6005
  30. Takano T, Tsutsumi S, Tomisato W, Hoshino T, Tsuchiya T and Mizushima T (2002) Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced apoptosis. *Dig. Dis. Sci.* 47: 1546-1553
  31. Fernandez PM, Tabbara SO, Jacobs LK, Manning FC, Tsangaris TN, Schwartz AM, Kennedy KA and Patierno SR (2000) Overexpression of the glucose-regulated stress gene GRP78 in malignant but not benign human breast lesions. *Breast Cancer Res. Treat.* 59: 15-26
  32. Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautsch SA and Cole GM (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *J. Neurosci.* 20: 5709-5714
  33. Takahashi Y, Hayashi I, Tominari Y, Rikimaru K, Morohashi Y, Kan T, Natsugari H, Fukuyama T, Tomita T and Iwatsubo T (2003) Sulindac sulfide is a noncompetitive gamma-secretase inhibitor that preferentially reduces Abeta 42 generation. *J. Biol. Chem.* 278: 18664-18670
  34. Yang Y, Turner RS and Gaut JR (1998) The chaperone BiP/GRP78 binds to amyloid precursor protein and decreases Abeta40 and Abeta42 secretion. *J. Biol. Chem.* 273: 25552-25555
  35. Matsumoto M, Minami M, Takeda K, Sakao Y and Akira S (1996) Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. *FEBS Lett.* 395: 143-147
  36. Hirakawa T, Rokutan K, Nikawa T and Kishi K (1996) Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* 111: 345-357
  37. Tomisato W, Hoshino T, Tsutsumi S, Tsuchiya T and Mizushima T (2002) Maturation-associated increase in sensitivity of cultured guinea pig gastric pit cells to hydrogen peroxide. *Dig. Dis. Sci.* 47: 2125-2133
  38. Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T and Mizushima T (2003) Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. *J. Biol. Chem.* 278: 12752-12758
  39. Schreiber E, Matthias P, Muller MM and Schaffner W (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* 17: 6419
  40. Haas IG and Meo T (1988) cDNA cloning of the immunoglobulin heavy chain binding protein. *Proc. Natl. Acad. Sci. USA* 85: 2250-2254
  41. Carninci P and Hayashizaki Y (1999) High-efficiency full-length cDNA cloning. *Methods Enzymol.* 303: 19-44

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

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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

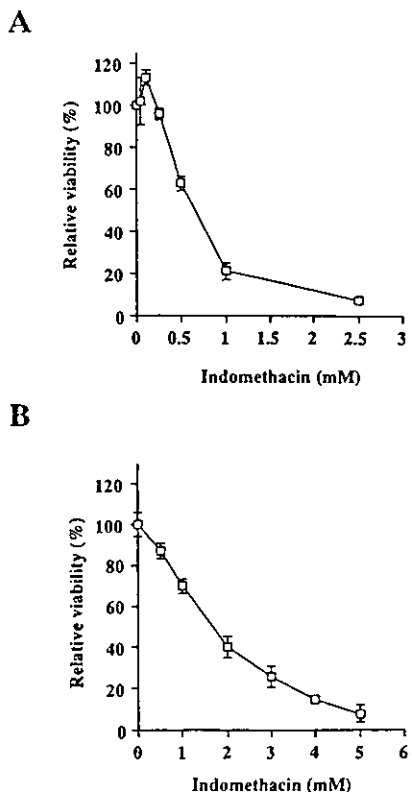


Fig. 1. Cell Death Induced by Exposure to Indomethacin  
 Cultured gastric mucosal cells were treated with indicated concentrations of indomethacin for 24 h (A) or for 1 h (B). Cell viability was determined by the MTT method. Values are mean ± S.E.M. (n=3).

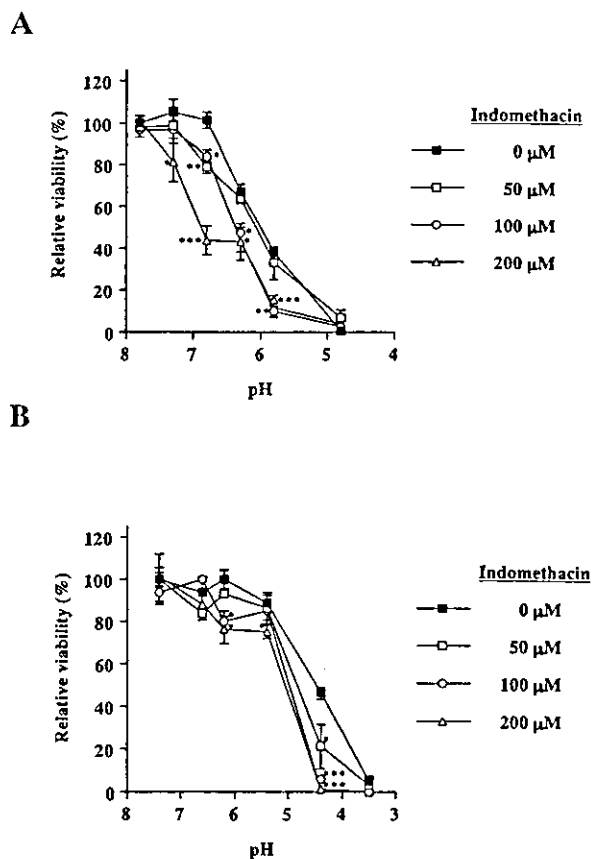


Fig. 2. Cell Death Induced by the Combined Use of Indomethacin and Hydrochloric Acid  
 Cultured gastric mucosal cells were treated with different concentrations of hydrochloric acid (0, 10, 15, 18, 20, 22 mM) in the presence of indicated concentrations of indomethacin for 24 h (A) or for 1 h (B). The 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

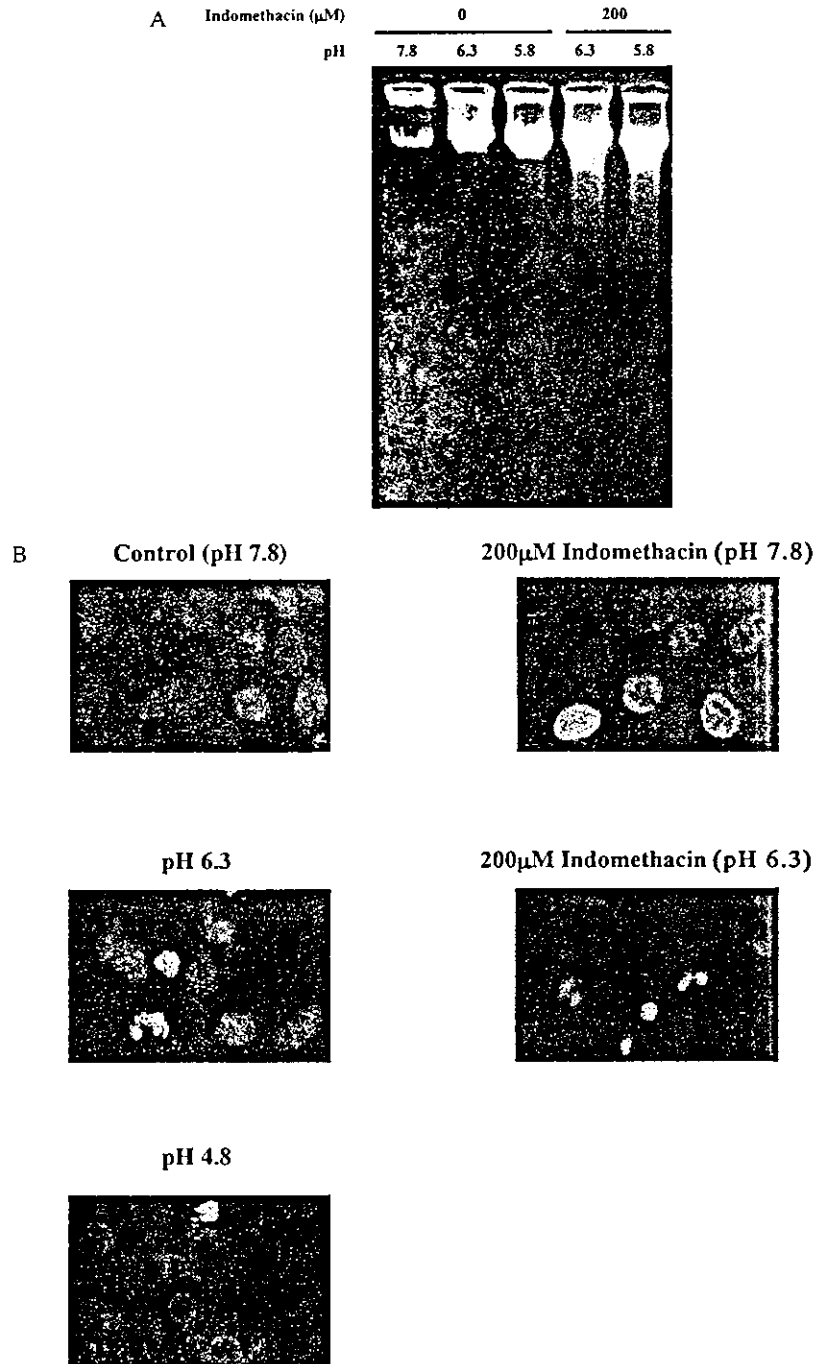


Fig. 3. Apoptotic DNA Fragmentation and Chromatin Condensation by a Combination between Indomethacin and Hydrochloric Acid

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

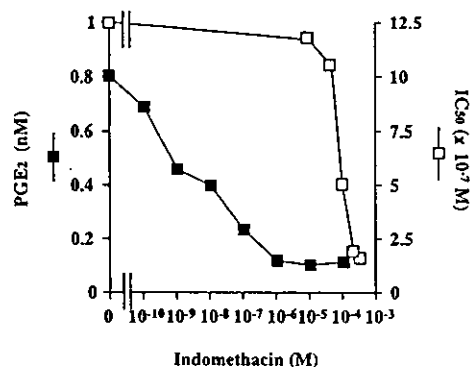


Fig. 4. Relationship between Inhibition of PG Synthesis and the Synergistic Response by Indomethacin

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.

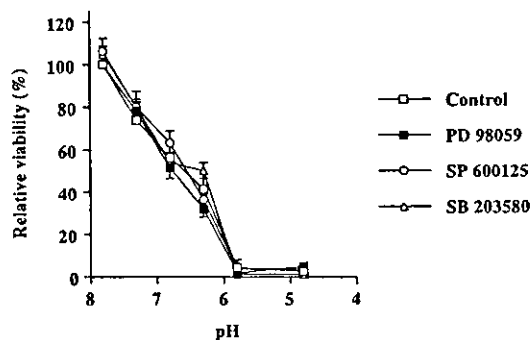


Fig. 5. Effect of Inhibitors of MAPKs on the Cytotoxic Synergy between Indomethacin and Hydrochloric Acid

Cultured gastric mucosal cells were pre-treated for 1 h with 10  $\mu$ M 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  $\mu$ M indomethacin. The pH value of the culture medium was determined and plotted. Cell viability was determined by the MTT method. Values are mean  $\pm$  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.

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**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

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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.

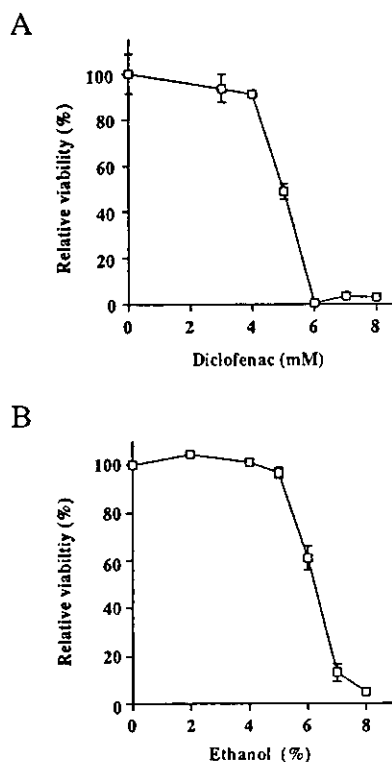
**Determination of Mucin Levels in Culture Media.** Preparation of mucin fractions from culture media was performed as described previously (20, 21). Mucin fractions were purified and concentrated by the freeze-dry method and ethanol precipitation. The amount of mucin in samples was determined by the 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

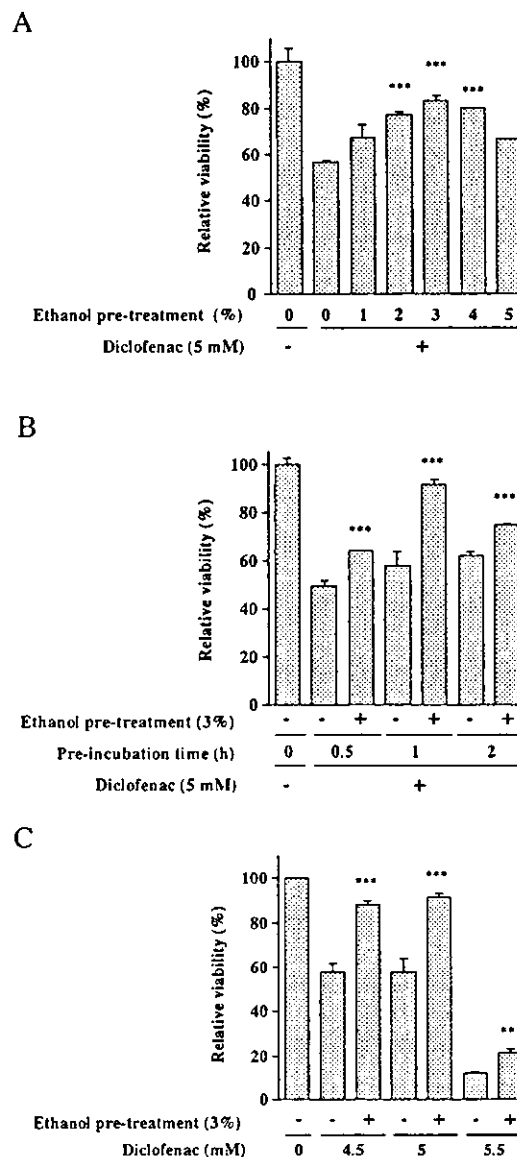
**Adaptive Cytoprotection Against NSAIDs Induced by Ethanol.** We first measured the sensitivity of AGS cells to diclofenac, one of the NSAIDs. Treatment of cells with diclofenac (5 mM) for 2 hr decreased cell viability to about 50% of the control (Figure 1A). We also measured the sensitivity of AGS 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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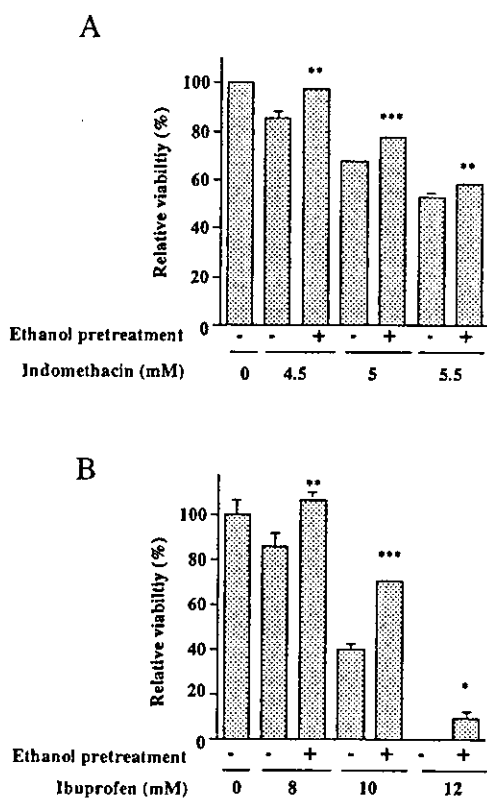


Fig 3. Ethanol-induced adaptive cytoprotection against NSAIDs other than diclofenac. AGS cells were pretreated with 3% ethanol for 1 hr. Cells were further treated with the indicated concentrations of indomethacin (A) or ibuprofen (B) for 2 hr. Cell viability was determined by the MTT method. Values 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

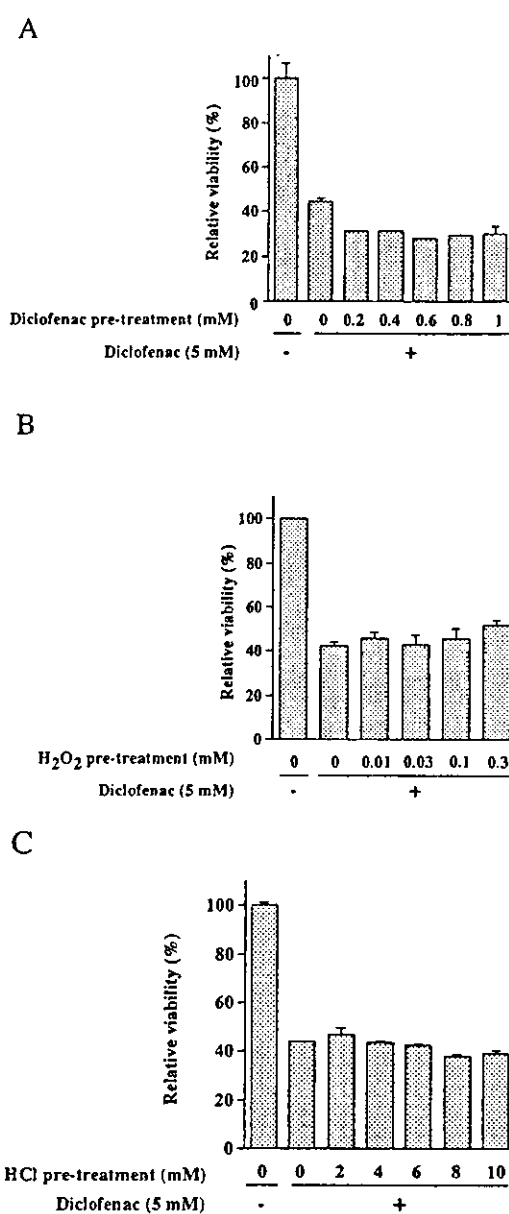


Fig 4. Adaptive cytoprotection against diclofenac induced by various irritants. AGS cells were pretreated with the indicated concentrations of diclofenac (A), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (B), or hydrochloric acid (HCl) for 1 hr. Cells were further treated with diclofenac (5 mM) for 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

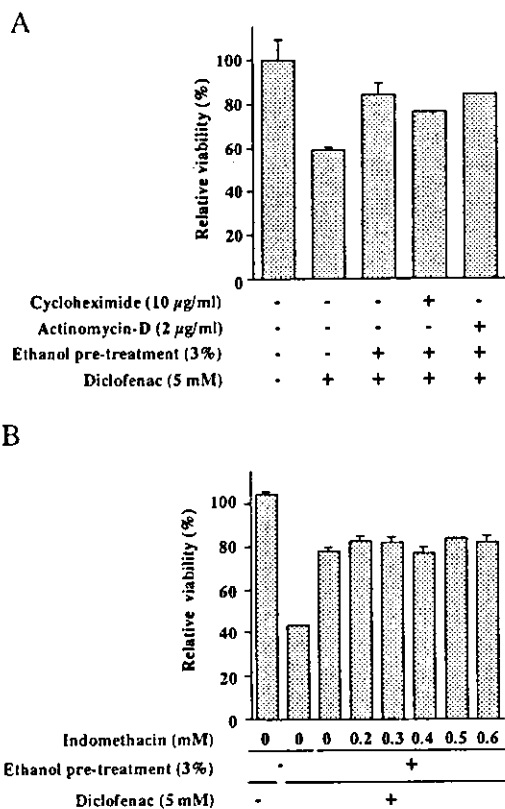


Fig 5. Effect of an inhibitor of protein synthesis or PG synthesis on ethanol-induced adaptive cytoprotection. AGS cells were initially treated with cycloheximide (CHX; 10 µg/ml) or actinomycin-D (Act-D; 2 µg/ml) for 0.5 hr (A) or indicated concentrations of indomethacin for 1 hr (B), then with 3% ethanol 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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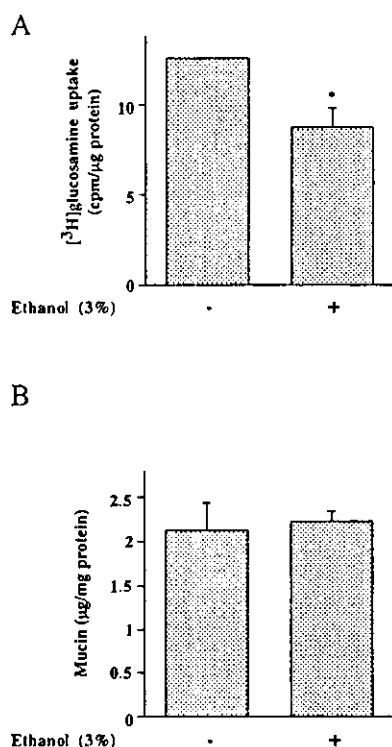


Fig 6. Effect of ethanol on synthesis and secretion of mucin. AGS cells were incubated with [<sup>3</sup>H]glucosamine in the presence or absence of 3% ethanol for 1 hr in a culture dish (diameter, 6 cm). Incorporated radioactivities were determined with a liquid scintillation counter (A). AGS cells 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

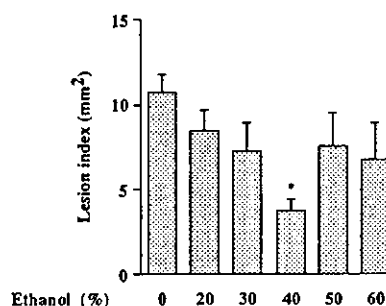


Fig 7. Effect of ethanol preadministration on production of gastric lesions by indomethacin in rats. Rats were orally preadministered the indicated concentrations of ethanol. After 0.5 hr, animals were orally administered 30 mg/kg indomethacin. After 3 hr the stomach was removed and scored for hemorrhagic 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*,

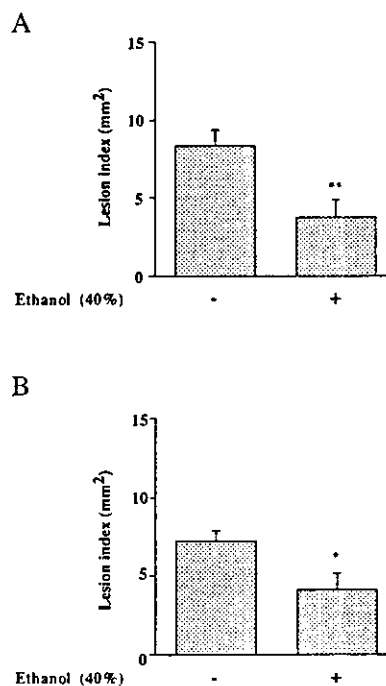


Fig 8. Effect of ethanol preadministration on production of gastric lesions by NSAIDs in rats. Rats were orally preadministered 40% ethanol. After 0.5 hr, animals were orally administered 40 mg/kg diclofenac (A) or 100 mg/kg ibuprofen (B). After 3 hr, the stomach was removed and scored for hemorrhagic 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.

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#### REFERENCES

- Smalley WE, Ray WA, Daugherty JR, Griffin MR: Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. *Am J Epidemiol* 141:539-545, 1995
- Hawkey CJ: Nonsteroidal anti-inflammatory drug gastropathy. *Gastroenterology* 119:521-535, 2000
- Barrier CH, Hirschowitz BI: Controversies in the detection and management of nonsteroidal anti-inflammatory drug-induced side effects of the upper gastrointestinal tract. *Arth Rheum* 32:926-932, 1989
- Gabriel SE, Jaakkimainen L, Bombardier C: Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs. A meta-analysis. *Ann Intern Med* 115:787-796, 1991
- Fries JF, Miller SR, Spitz PW, Williams CA, Hubert HB, Bloch DA: Toward an epidemiology of gastropathy associated with nonsteroidal anti-inflammatory drug use. *Gastroenterology* 96:647-655, 1989
- Kurata JH, Abbey DE: The effect of chronic aspirin use on duodenal and gastric ulcer hospitalizations. *J Clin Gastroenterol* 12:260-266, 1990
- Singh G: Recent considerations in nonsteroidal anti-inflammatory drug gastropathy. *Am J Med* 105:31S-38S, 1998
- Vane JR, Botting RM: Mechanism of action of anti-inflammatory drugs. *Scand J Rheumatol Suppl* 102:9-21, 1996
- Miller TA: Protective effects of prostaglandins against gastric mucosal damage: Current knowledge and proposed mechanisms. *Am J Physiol* 245:G601-G623, 1983
- Robert A, Nezamis JE, Lancaster C, Davis JP, Field SO, Hanchar AJ: Mild irritants prevent gastric necrosis through "adaptive cytoprotection" mediated by prostaglandins. *Am J Physiol* 245:G113-G121, 1983
- Kokoska ER, Smith GS, Rieckenberg CL, Deshpande Y, Banan A, Miller TA: Adaptive cytoprotection against deoxycholate-induced injury in human gastric cells in vitro: Is there a role for endogenous prostaglandins? *Dig Dis Sci* 43:806-815, 1998
- Mutoh H, Ota S, Hiraishi H, Ivey KJ, Terano A, Sugimoto T: Adaptive cytoprotection in cultured rat gastric mucus-producing cells. Role of mucus and prostaglandin synthesis. *Dig Dis Sci* 40:872-878, 1995
- Kokoska ER, Smith GS, Deshpande Y, Rieckenberg CL, Miller TA: Adaptive cytoprotection induced by ethanol in human intestinal cells: Role of prostaglandins and calcium homeostasis. *Ann Surg* 228:123-130, 1998
- Kokoska ER, Smith GS, Wolff AB, Deshpande Y, Rieckenberg CL, Banan A, Miller TA: Role of calcium in adaptive cytoprotection and cell injury induced by deoxycholate in human gastric cells. *Am J Physiol* 275:G322-G330, 1998
- Sharma SA, Tummuru MK, Miller GG, Blaser MJ: Interleukin-8 response of gastric epithelial cell lines to *Helicobacter pylori* stimulation in vitro. *Infect Immun* 63:1681-1687, 1995
- Nilius M, Bode G, Buchler M, Malfertheiner P: Adhesion of *Helicobacter pylori* and *Escherichia coli* to human and bovine surface mucus cells in vitro. *Eur J Clin Invest* 24:454-459, 1994
- Tsutsumi S, Mima S, Tomisato W, Hoshino T, Tsuchiya T, Mizushima T: Molecular mechanism of adaptive cytoprotection induced by ethanol in human gastric cells. *Exp Biol Med* 228:1089-1095, 2003
- Wolf BB, Green DR: Suicidal tendencies: Apoptotic cell death by caspase family proteinases. *J Biol Chem* 274:20049-20052, 1999
- Tomisato W, Tsutsumi S, Rokutan K, Tsuchiya T, Mizushima T: NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. *Am J Physiol Gastrointest Liver Physiol* 281:G1092-G1100, 2001
- Watanabe T, Ohara S, Miyazawa S, Saigenji K, Hotta K: Augmentative effects of L-cysteine and methylmethionine sulfonium chloride on mucin secretion in rabbit gastric mucous cells. *J Gastroenterol Hepatol* 15:45-52, 2000
- Ohara S, Watanabe T, Hotta K: Comparative study of carbohydrate portion of gastrointestinal mucins using enzyme-linked lectin-binding assay (ELLA). *Comp Biochem Physiol B Biochem Mol Biol* 116:167-172, 1997
- Tomisato W, Tsutsumi S, Tsuchiya T, Mizushima T: Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced necrosis by induction of heat-shock proteins. *Biol Pharm Bull* 24:887-891, 2001
- Mizushima T, Tsutsumi S, Rokutan K, Tsuchiya T: Suppression of ethanol-induced apoptotic DNA fragmentation by geranylgeranylacetone in cultured guinea pig gastric mucosal cells. *Dig Dis Sci* 44:510-514, 1999
- Takano T, Tsutsumi S, Tomisato W, Hoshino T, Tsuchiya T, Mizushima T: Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced apoptosis. *Dig Dis Sci* 47:1546-1553, 2002
- Nakamura K, Rokutan K, Marui N, Aoike A, Kawai K: Induction of heat shock proteins and their implication in protection against ethanol-induced damage in cultured guinea pig gastric mucosal cells. *Gastroenterology* 101:161-166, 1991
- Tomisato W, Takahashi N, Komoto C, Rokutan K, Tsuchiya T, Mizushima T: Geranylgeranylacetone protects cultured guinea pig

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- gastric mucosal cells from indomethacin. *Dig Dis Sci* 45:1674-1679, 2000
27. Hirakawa T, Rokutan K, Nikawa T, Kishi K: Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* 111:345-357, 1996
  28. Kauffman GL Jr, Reeve JJ Jr, Grossman MI: Gastric bicarbonate secretion: Effect of topical and intravenous 16,16-dimethyl prostaglandin E2. *Am J Physiol* 239:G44-G48, 1980
  29. Keogh JP, Allen A, Garner A: Relationship between gastric mucus synthesis, secretion and surface gel erosion measured in amphibian stomach in vitro. *Clin Exp Pharmacol Physiol* 24:844-849, 1997
  30. Hoshino T, Takano T, Tsutsumi S, Tomisato W, Tsuchiya T, Mizushima T: Effects of prostaglandin E2 on gastric irritant-induced apoptosis. *Dig Dis Sci* 47:2370-2379, 2002
  31. Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, Mizushima T: Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. *J Biol Chem* 278:12752-12758, 2003
  32. Basque JR, Chenard M, Chailier P, Menard D: Gastric cancer cell lines as models to study human digestive functions. *J Cell Biochem* 81:241-251, 2001
  33. Glavin GB, Lockhart LK, Rockman GE, Hall AM, Kiernan KM: Evidence of "cross-stressor"-induced adaptive gastric cytoprotection. *Life Sci* 41:2223-2227, 1987
  34. Ligumsky M, Sestieri M, Karmeli F, Rachmilewitz D: Protection by mild irritants against indomethacin-induced gastric mucosal damage in the rat: Role of prostaglandin synthesis. *Isr J Med Sci* 22:807-811, 1986



## Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions

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### Abstract

A major clinical problem encountered with the use of non-steroidal anti-inflammatory drugs (NSAIDs), is gastrointestinal complications. We previously reported that NSAIDs induce both necrosis and apoptosis *in vitro*. We here examined the cyclooxygenase (COX) dependency of this cytotoxic effect of NSAIDs and its involvement in NSAID-induced gastric lesions. Necrosis and apoptosis by NSAIDs was observed with all selective COX-2 inhibitors except rofecoxib and was not inhibited by exogenously added prostaglandin E<sub>2</sub>, suggesting that cytotoxicity of NSAIDs seems to be independent of the inhibition of COX. Intravenously administered indomethacin, which completely inhibited COX activity at gastric mucosa, did not produce gastric lesions. Orally administered selective COX-2 inhibitors, which did not inhibit COX at gastric mucosa, also did not produce gastric lesions. Interestingly, a combination of the oral administration of each of all selective COX-2 inhibitors except rofecoxib with the intravenous administration of indomethacin clearly produced gastric lesions. These results suggest that in addition to COX inhibition by NSAIDs, direct cytotoxicity of NSAIDs may be involved in NSAID-induced gastric lesions.

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**Keywords:** Direct cytotoxicity; Cyclooxygenase; NSAIDs; Gastric lesions; Prostaglandin E<sub>2</sub>; Selective cyclooxygenase-2 inhibitors

### 1. Introduction

NSAIDs are one of the most frequently used classes of medicines in the world and account for nearly 5% of all prescribed medications [1]. However, NSAID administration is associated with gastrointestinal complications, such as gastric ulcers and bleeding, which sometimes become life-threatening diseases [2]. About 15–30% of chronic users of NSAIDs have gastrointestinal ulcers and bleeding [3–6]. In the United States, about 16,500 people per year die as a result of NSAID-associated gastrointestinal complications [7]. Therefore, the molecular mechanism governing NSAID-induced gastrointestinal damage needs to

be elucidated in order to develop new NSAIDs that do not have these side effects.

Inhibition of COX by NSAIDs, which is responsible for their anti-inflammatory activity was previously thought to be fully responsible for their gastrointestinal side effects [8]. This is because COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong cytoprotective effect on the gastrointestinal mucosa [9]. There are at least two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastric mucosa and tissues with inflammation, respectively [10,11]. Therefore, it is reasonable to speculate that selective COX-2 inhibitors have anti-inflammatory activity without gastrointestinal side effects [10]. In fact, a greatly reduced incidence of gastroduodenal lesions was reported for selective COX-2 inhibitors (such as celecoxib and rofecoxib) both in animal and clinical data [12–15], however, recently published paper showed no difference in serious gastrointestinal complications between celecoxib and two non-selective NSAIDs [16]. Two lines of evidence have worked against the idea that the gastrointestinal side

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**Abbreviations:** NSAID, non-steroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; FBS, fetal bovine serum; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PGI<sub>2</sub>, prostacyclin.



effects of NSAIDs are caused only by the inhibition of COX-1. The first is that a selective COX-1 inhibitor (SC-560) induced no gastric injury even at high dosages, and COX-1 knockout mice showed no detectable gastric ulcers [17–20]. The second line of evidence is that the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs are not always linked with each other. For example, higher doses of NSAIDs were required for producing gastric lesions than were required for inhibiting COX at the gastric mucosa [21,22]. The former contradiction can be explained by the recent proposal that inhibition of both COX-1 and COX-2 was necessary for NSAID-induced gastrointestinal lesions [17]. However, the latter contradiction cannot be explained by this idea, suggesting therefore that NSAID-induced gastrointestinal lesions involve additional mechanisms [23]. Understanding the additional mechanisms is necessary in order to establish an alternative method for development of gastro-intestinally safe NSAIDs other than simply increasing their COX-2 selectivity. This new class of NSAIDs may be clinically beneficial because clinical disadvantages (i.e. risk of cardiovascular thrombotic disease) of selective COX-2 inhibitors were recently suggested [24,25].

In addition to various possibilities proposed for this additional mechanism (such as reduced blood flow, hypermotility, and activation of neutrophils) [26–28], a direct cytotoxic effect of NSAIDs (topical irritant property) on gastric mucosal cells was also proposed to be involved in NSAID-induced gastric lesions [23,29]. As for this direct cytotoxic effect, we previously reported that NSAIDs (indomethacin and aspirin) induced both necrosis and apoptosis in primary cultures of guinea pig gastric mucosal cells [30]. In this study, we suggested that the direct cytotoxic effect of NSAIDs is independent of the inhibition of COX and suggest that in addition to COX inhibition by NSAIDs, direct cytotoxic effect of NSAIDs is involved in NSAID-induced gastric lesions *in vivo* by use of a combination of the oral administration of selective COX-2 inhibitors with the intravenous administration of non-selective NSAIDs.

## 2. Materials and methods

### 2.1. Chemicals, media, and animals

FBS and trypan blue were from Gibco Co. Indomethacin, aspirin, and NS-398 were from Wako Co. Ibuprofen, diclofenac, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma Co. Celecoxib was from LKT Laboratories Inc. Rofecoxib was synthesized in our laboratory. We confirmed that this rofecoxib has bioequivalence with that made by Merck, by measuring their inhibitory effect on inflammatory PG synthesis. Etodolac was gift kindly provided by Nippon Shinyaku Co. The ELISA kits for PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>

quantitation were from Cayman Chemical Co. Male Wistar rats weighing 160–190 g and male guinea pigs weighing 200–300 g were purchased from Shimizu Co. 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.

### 2.2. *In vitro* assay of cytotoxicity and DNA fragmentation

Gastric mucosal cells were isolated from guinea pig fundic glands, as described previously [31,32]. Isolated gastric mucosal cells were cultured for 12 hr 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 under the conditions of 5% CO<sub>2</sub>/95% air and 37°. After removing non-adherent 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 were previously characterized, with the majority (about 90%) of cells being identified as pit cells [31]. 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 changing the entire bathing medium. Cell viability was determined by the trypan blue exclusion test or the MTT method as previously described [33].

Apoptotic DNA fragmentation was monitored as previously described [34]. 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° for 2 hr. RNase A was then added to a final concentration of 0.5 mg/mL and incubated at 50° for 30 min. These samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5  $\mu$ g/mL ethidium bromide.

### 2.3. Gastric damage assay

Rats (24 hr fasted) were orally administered with selective COX-2 inhibitors or non-selective NSAIDs with 1% methylcellulose in a volume of 5 mL/kg. Control rats received an equal volume of the vehicle (1% methylcellulose). In some experiments, 1 hr before the oral administration, indomethacin (dissolved in PBS), aspirin (dissolved in PBS), or vehicle (PBS) was administered intravenously *via* the tail vein. Six hours after the oral administration, the rats were anesthetized and the stomach was 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. Determination of PGE<sub>2</sub> levels at the gastric mucosa was done by ELISA as previously described [35].

2.4. Caspase activity assay

The activity of caspase 3 was determined as described previously [30]. Briefly, cells were collected by centrifugation and suspended in extraction buffer (50 mM PIPES

(pH 7.0), 50 mM KCl, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, and 1 mM DTT). Suspensions were sonicated and centrifuged, after which the supernatants were incubated with fluorogenic peptide substrate (Ac-DEVD-MCA) in reaction buffer (100 mM HEPES-KOH (pH 7.5), 10% sucrose, 0.1% CHAPS, and 1 mg/mL BSA) for 15 min at 37°. The release of AMC was determined using a fluorescence spectrophotometer. One unit of protease activity was defined, as the amount of enzyme required to release 1 pmol AMC/min.

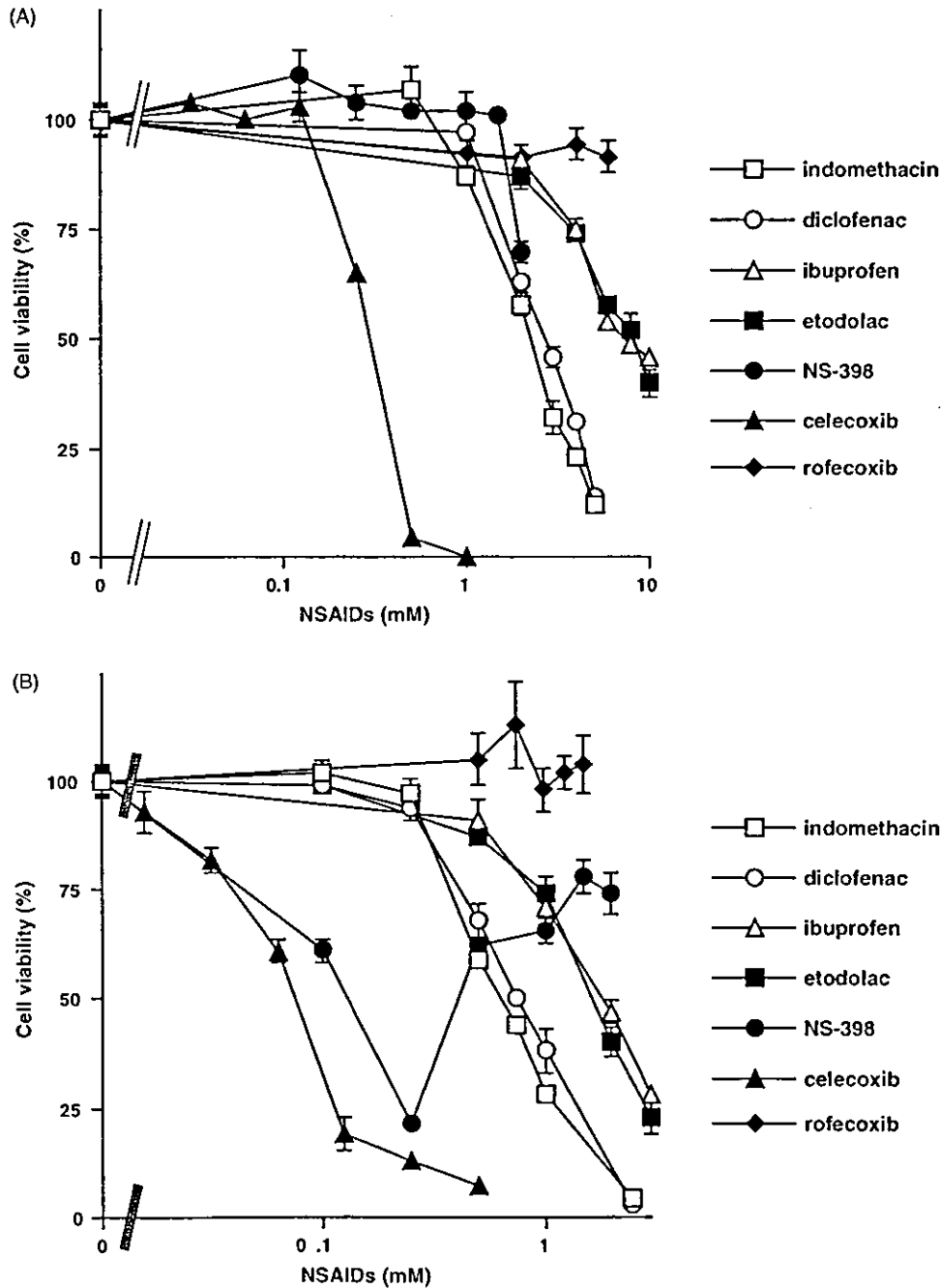


Fig. 1. Necrosis and apoptosis induced by various NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of various NSAIDs for 1 hr (A, C) or 16 hr (B, C). Cell viability was determined by the MTT method (A, B). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (C). Values are mean ± SEM (N = 3).

(C)

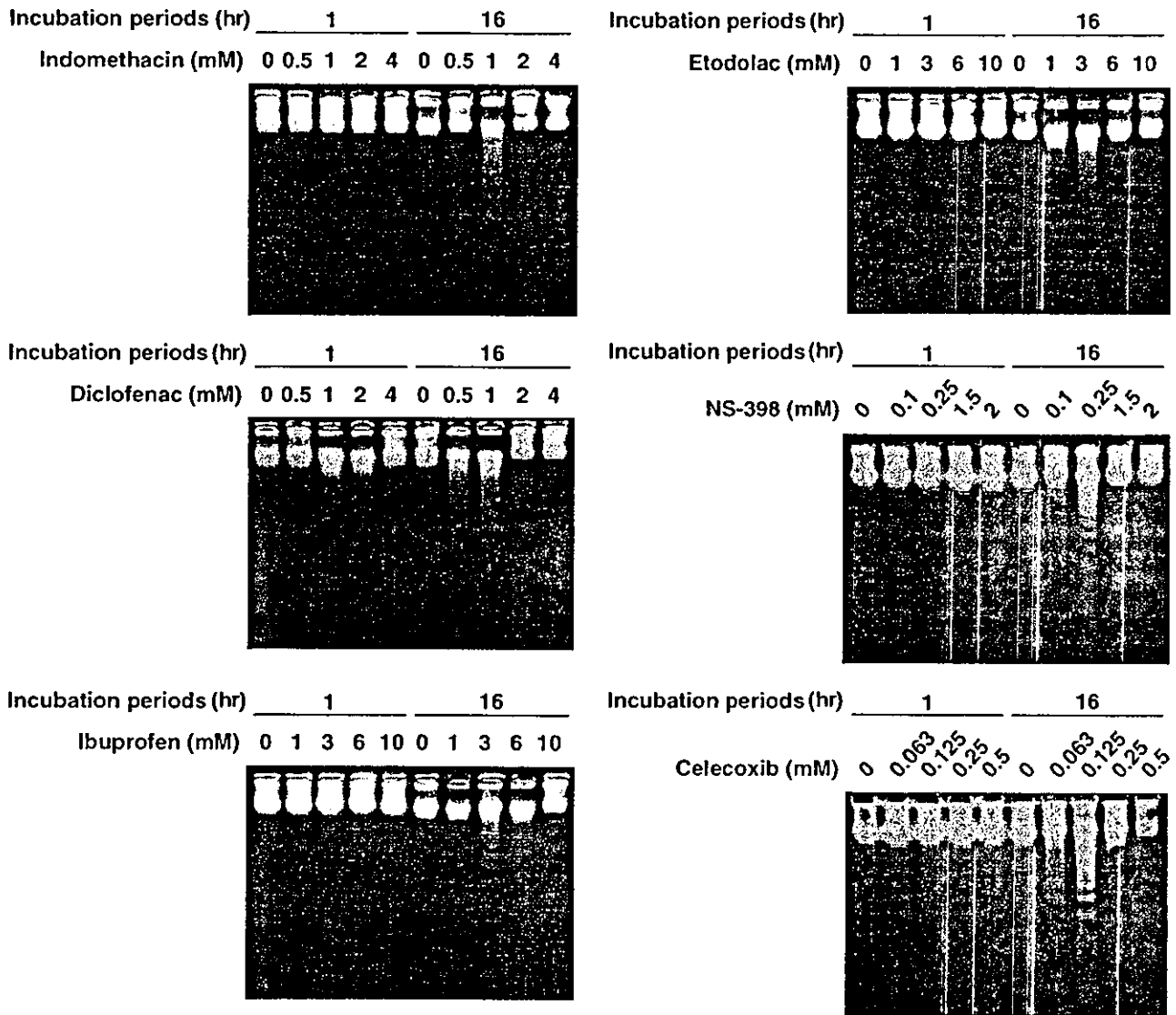


Fig. 1. (Continued).

### 2.5. Statistical analysis

All values are expressed as the mean  $\pm$  standard error (SEM). One-way 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 for the evaluation of differences between two groups. Differences were considered to be significant for values of  $P < 0.05$ .

## 3. Results

### 3.1. *In vitro* necrosis and apoptosis induced by various NSAIDs, and their relationship with COX inhibition

We previously reported that short-term (1 hr) treatment of primary cultures of guinea pig gastric mucosal cells with

high concentrations of NSAIDs (indomethacin, 2.5 mM) and long-term (16 hr) treatment of these cells with low concentrations of NSAIDs (indomethacin, 1 mM) induced necrosis and apoptosis, respectively [30]. In the present study, selective COX-2 inhibitors (etodolac, NS-398, celecoxib, and rofecoxib) and non-selective NSAIDs (or slightly selective NSAIDs for COX-1 or COX-2) (indomethacin, diclofenac, and ibuprofen) were tested for their ability to induce necrosis and apoptosis *in vitro*. Necrosis and apoptosis were assessed on the basis of the presence and absence, respectively, of apoptotic DNA fragmentation and caspase 3 activation. The decrease in cell viability with short-term (1 hr) NSAIDs treatment (Fig. 1A) is not associated with apoptotic DNA fragmentation (Fig. 1C) and caspase 3 activation (Table 1), suggesting that it is mediated by necrosis. To confirm this finding, we carried out double-staining experiments with propidium iodide and Hoechst 33342. Since necrotic cells lose

Table 1  
Activation of caspase 3 by NSAIDs

Incubation (hr)	NSAIDs	Caspase 3-like activity (U/mg protein)
1	Control	10 ± 3
	4 mM indomethacin	11 ± 1
	4 mM diclofenac	13 ± 6
	10 mM ibuprofen	13 ± 2
	10 mM etodolac	10 ± 3
	2 mM NS-398	11 ± 5
	0.5 mM celecoxib	10 ± 5
	6 mM rofecoxib	10 ± 4
16	Control	14 ± 4
	1 mM indomethacin	525 ± 23***
	1 mM diclofenac	564 ± 50***
	3 mM ibuprofen	591 ± 32***
	3 mM etodolac	455 ± 29***
	0.25 mM NS-398	473 ± 33***
	0.125 mM celecoxib	637 ± 61***
	3 mM rofecoxib	29 ± 8

Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of various NSAIDs for 1 or 16 hr. Activities of caspase 3 were examined by the use of specific fluorogenic peptide substrates (Ac-DEVD-MCA). Values are mean ± SEM (N = 3).

\*\*\*P < 0.001.

their membrane integrity, propidium iodide staining causes pink nuclear staining in necrotic cells, whereas living cells and apoptotic cells are not stained with propidium iodide. We previously reported that 1 hr treatment with 2.5 mM indomethacin caused pink nuclear staining [30]. We here performed the same type of experiment for other NSAIDs used in Fig. 1 and found that 1 hr treatment with 4 mM indomethacin, 4 mM diclofenac, 10 mM ibuprofen, 10 mM etodolac, 2 mM NS-398, or 0.5 mM celecoxib caused pink nuclear staining (data not shown), strongly

suggesting that cell death shown in Fig. 1A is mediated through necrosis. In contrast, the decrease in cell viability with long-term (16 hr) NSAIDs treatment (Fig. 1B) is associated with apoptotic DNA fragmentation (Fig. 1C) and caspase 3 activation (Table 1), suggesting that it is mediated by apoptosis. Among all of NSAIDs tested here, only rofecoxib induced neither necrosis nor apoptosis (Fig. 1). Interestingly, apoptosis induced by NS-398 was greatest at a concentration of 0.25 mM and the higher concentrations of NS-398 caused the less induction. We have no explanation for this phenomenon at present. We consider that the concentrations of NSAIDs required for necrosis and apoptosis *in vitro* are possible *in vivo* associating with gastric ulceration in animal models, as discussed in our previous paper [30]. However, it is unclear whether gastric mucosal cells can be exposed to NSAIDs as long as 16 hr in animal models. Furthermore, it is also unclear whether these concentrations are relevant for clinical use of NSAIDs. There seemed to be no direct relationship between the cytotoxicity (concentrations of NSAIDs required for necrosis and apoptosis) and the selectivity for COX-2 of NSAIDs.

The level of PGE<sub>2</sub> in the medium upon treatment of cells with various NSAIDs was measured. Compared to non-selective NSAIDs, higher concentrations of selective COX-2 inhibitors were required for inhibiting PGE<sub>2</sub> synthesis, being consistent with the idea that the majority of COX activity is derived from COX-1 activity in gastric mucosal cells [10,11]. Comparing results (Figs. 1 and 2), it is clear that there is no relationship between them in terms of their cytotoxicity and their ability to inhibit PGE<sub>2</sub> synthesis. For example, celecoxib was the strongest compound in terms of cytotoxicity, but the weakest for inhibiting PGE<sub>2</sub> synthesis. Therefore, it seems that the cytotoxic

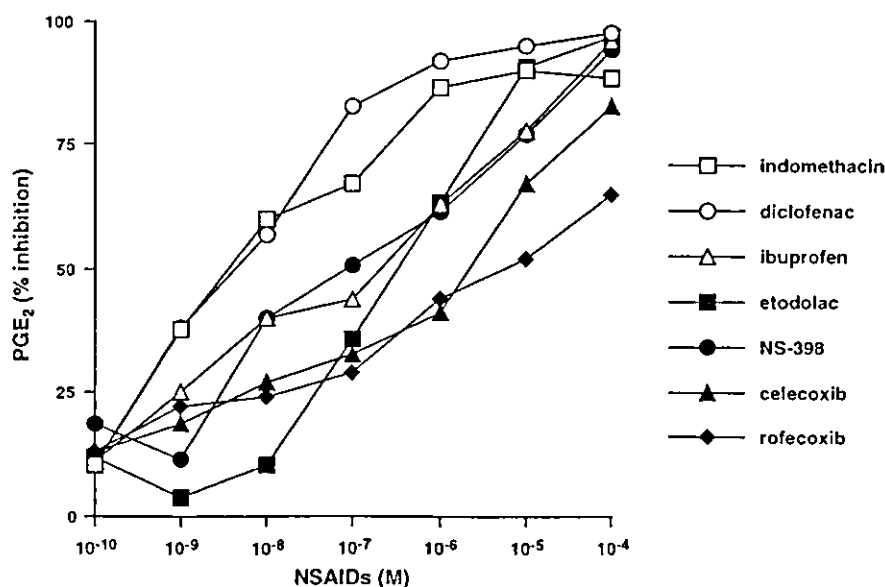


Fig. 2. Inhibition of PGE<sub>2</sub> synthesis by exposure to various NSAIDs in cultured cells. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of various NSAIDs for 30 min, following which the levels of PGE<sub>2</sub> in the media were determined by ELISA. Values are expressed as relative to control (without NSAIDs) ( $2 \times 10^{-9}$  M).