suppressed the spontaneous apoptotic DNA fragmentation in a dose-dependent manner (Figure 4). The concentration of cycloheximide required for the inhibition of the DNA fragmentation was much the same as that of protein synthesis (14). These results indicate that the spontaneous apoptotic DNA fragmentation in cultured gastric mucosal cells is dependent on *de novo* protein synthesis.

A number of studies using specific inhibitors revealed that the caspase family plays an essential role in the various types of apoptosis (16, 17). Two members of this family, caspase 3 and 7, have been suggested to play a central role in the final step of the apoptosis pathway (16, 17). We then examined the effect of a caspase inhibitor on the spontaneous apoptotic DNA fragmentation in cultured gastric mucosal cells. We found that Z-VAD-fmk, a specific inhibitor of caspase 3 and 7 (16), inhibited the spontaneous apoptotic DNA fragmentation in a dose-dependent manner (Figure 5). The concentration of Z-VAD-fmk required for the inhibition of the DNA fragmentation was similar to that previously reported for other types of apoptosis (17, 18). Thus, the spontaneous apoptotic DNA fragmentation in cultured gastric mucosal cells appeared to depend on the function of caspase 3 or 7.

In this study, we reported that cultured gastric mucosal cells showed spontaneous apoptotic DNA fragmentation. Since mature gastric pit cells are eliminated by apoptosis *in vivo* (1), the spontaneous apoptosis of cultured gastric mucosal cells *in vitro* is considered to mimic the maturation-dependent apoptosis of gastric pit cells *in vivo*. Of course, our system *in vitro* did not have the special compartment that continuously supplies new epithelial cells and thus, we can not simply apply the present results to *in vivo* situations. However, this system may be very useful for studying the mechanism of the maturation and apoptosis of gastric pit cells. In this paper, we found that the apoptosis of gastric pit cells required *de novo* protein synthesis and caspase activities. We also examined whether the apoptosis was associated with the FCS-dependent proliferation and maturation of cultured gastric pit cells, using a serum-free culture system (12). In the serum-free culture, prepit cells were selectively attached to the dishes, and they did not replicate or differentiate at least for 48 hr (12). Spontaneous apoptosis did not occur in our serum-free conditions, supporting the idea that spontaneous apoptotic DNA fragmentation may represent the maturation-dependent cell death that occurs in normal gastric mucosa *in vivo*.

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Effects of Prostaglandin E_2 on Gastric Irritant-Induced Apoptosis

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We previously reported that various gastric irritants induced both apoptosis and necrosis in cultured gastric mucosal cells. In a continuation of this work, the present study has examined the effects of prostaglandin E_2 (PGE_2), a cytoprotective factor for gastric mucosa *in vivo*, on gastric irritant-induced apoptosis and necrosis *in vitro*. PGE_2 inhibited ethanol-induced apoptosis and increased cell viability in a dose-dependent manner in primary cultures of guinea pig gastric mucosal cells. PGE_2 also inhibited hydrogen peroxide-induced apoptosis. In contrast, PGE_2 showed no cytoprotective effects against ethanol-induced necrosis. Based on these results, we consider that the cytoprotective effects of PGE_2 on gastric mucosa *in vivo* can be partially explained by its inhibitory effect on gastric irritant-induced apoptosis.

KEY WORDS: apoptosis; necrosis; gastric mucosal cells; gastric irritants, prostaglandin E_2.

Prostaglandins (PGs), one of the major groups of chemical mediators in the mammalian body, are involved in numerous physiological reactions, such as inflammation and cellular differentiation (1). PGs, especially PGE_2, also have cytoprotective effects on gastric mucosa as a consequence of various physiological mechanisms that include increased epithelial mucus and bicarbonate secretion (2, 3), amelioration of mucosal blood flow (4), and inhibition of free-radical and enzyme release from neutrophils (5). It has also been suggested that PGs affect vascular, luminal, and/or extrinsic and intrinsic neural mechanisms, resulting in cytoprotective effects on gastric mucosa (6). It contrast, it is a matter of debate as to whether or not PGs are directly responsible for making gastric mucosal cells resistant to various gastric irritants, in other words, whether or not PGs have cytoprotective effects on gastric mucosal cells *in vitro*. Some previous reports suggested that in the presence of acetylsalicylic acid and ethanol, PGs do not have cytoprotective effects on gastric mucosal cells *in vitro* (7–9). On the other hand, other reports suggested that PGs do have cytoprotective effects against acetylsalicylic acid, taurocholate, and ethanol on gastric mucosal cells maintained *in vitro* (7, 10–13).

Various gastric irritants seem to damage gastric mucosa by inducing both apoptosis and necrosis *in vivo* (14–17). We recently reproduced such apoptosis and necrosis *in vitro* using primary cultures of guinea pig gastric mucosal cells (18–20). Various gastric irritants [nonsteroidal antiinflammatory drugs (NSAIDs), ethanol, hydrogen peroxide, and hydrochloric acid] induced apoptosis or necrosis when gastric mucosal cells were treated with low concentrations of these irritants for a long period or with high concentrations of them for a short period, respectively (18–21). We also found that these gastric irri-

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tants induced apoptosis through a common pathway in which mitochondrial dysfunction and caspase-8 activation play important roles (20). Heat-shock proteins, which serve as protective factors for gastric mucosa, showed inhibitory effects on both apoptosis and necrosis induced by these gastric irritants (21, 22). In this study, we examined the effect of PGE_2 on gastric irritant-induced apoptosis and necrosis *in vitro*. Results showed that PGE_2 inhibited the apoptosis but not the necrosis.

MATERIALS AND METHODS

Chemicals and Media. RPMI 1640 was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal calf serum (FCS) and trypsin solution were purchased from Gibco (Grand Island, New York, USA). Pronase E and type I collagenase were purchased from Kaken Pharmaceutical Co. (Kyoto, Japan) and Nitta Gelatin Co. (Osaka, Japan), respectively. Sodium-*N*-lauroylsarcosinate was from Wako Co. (Tokyo, Japan). PGE_2, proteinase K, Hoechst 33342 (Ho 342), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and RNase A were from Sigma Co. (Tokyo, Japan). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidaozolylcarbocyanine iodide (DiPsipher) was from Trevigen. Peptide for the assay of caspase-3 was from Peptide Institute, Inc.

The experiments and procedures described here were approved by the Animal Care Committee of Okayama University.

Preparation and Culture of Gastric Mucosal Cells. Male guinea pigs (4 weeks of age) were purchased from Shimizu Co. (Kyoto, Japan). Gastric mucosal cells were isolated from guinea pig fundic glands as described previously (23). Isolated gastric mucosal cells (1×10^6 cells/dish) were cultured for 48 hr in RPMI 1640 containing 0.3% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin in type-I collagen-coated plastic culture plates (Iwaki) under the conditions of 5% CO_2/95% air and 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 cell preparations cultured under these conditions have been previously characterized, with the majority (about 90%) of cells being identified as pit cells (23).

Treatment of Cells with Gastric Irritants and PGE_2. Cells were exposed to gastric irritants (ethanol, hydrogen peroxide, or hydrochloric acid) by replacement of the entire bathing medium with fresh medium containing one of the irritants. For some experiments, cells were preincubated with PGE_2 for 2 hr. After removal of these chemicals by changing the bathing medium, cells were further incubated with one of gastric irritants in the presence of PGE_2.

DNA Fragmentation Assay. Apoptotic DNA fragmentation was monitored on agarose gel electrophoresis. Cells (2×10^6) were collected with a rubber policeman and suspended in 20 μl 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 hr. RNase A was then added to a final concentration of 0.5 mg/ml and the lysate incubated at 50°C for 30 min. These samples were loaded on 2% agarose gel in the presence of 0.5 μg/ml ethidium bromide, run at 100 V for 1 hr, and monitored under UV illuminator.

Cell Viability Assay. Cell viability was examined by the trypan blue exclusion test or MTT assay.

For the trypan blue exclusion test; after treatment with gastric irritants, cells were treated with 1% trypsin and collected by centrifugation. Cells were resuspended in phosphate-buffered saline (PBS) containing 0.2% trypan blue dye and observed under a light microscope.

For the MTT assay; after the treatment with gastric irritants, cells were incubated with MTT solution at a final concentration of 1 mg/ml in PBS for 2 hr. 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 (24).

Caspase Activity Assay. The activity of caspase-3 was determined as described previously (25, 26). 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 $MgCl_2$, 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°C. The release of amino-methyl-cumarin (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.

Chromatin Condensation Assay. After gastric irritant treatment, cells were washed with PBS and fixed with 10% v/v formaldehyde for 4 hr. Cells were then washed with PBS, stained with 0.17 mM Ho 342, and observed under a fluorescence microscope as described previously (27).

Mitochondrial Membrane Potential Assay. Mitochondrial membrane potential was assayed using a fluorometric mitochondrial permeability assay kit (Trevigen) (28). Briefly, after gastric irritants treatment, cells were treated with DiPsipher (5 μg/ml) for 20 min at 37°C and observed under a fluorescence microscope with 590 nm for red emission and 530 nm for green emission.

Statistical Analysis. All values are expressed as the mean ± standard error (SEM). A Student's *t* test for paired results was performed for the evaluation of differences between the groups. Differences were considered to be significant at values of $P < 0.05$.

RESULTS

Effect of PGE_2 on the Ethanol-Induced Apoptosis in Cultured Gastric Mucosal Cells. We previously reported that treatment of guinea pig gastric mucosal cells in primary culture with 2–4% ethanol for 4 hr caused apoptosis. This conclusion was reached on the basis of results showing apoptotic DNA fragmentation, chromatin condensation, caspase activation, and mitochondrial dysfunction (20). We examined here

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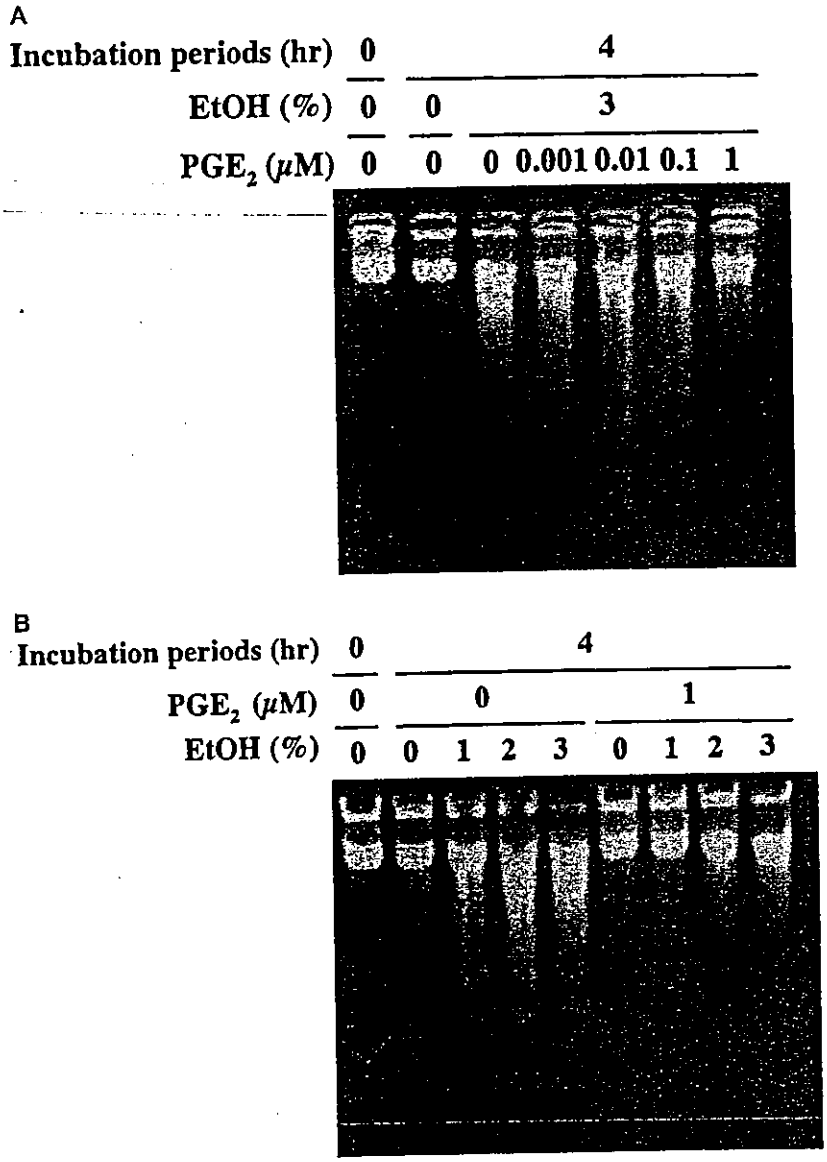

Fig 1. Effect of PGE_2 on ethanol-induced apoptotic DNA fragmentation. Cultured gastric mucosal cells were preincubated with the indicated concentrations (A) or 1 μM (B) of PGE_2 for 2 hr. Cells were further incubated for 4 hr with 3% (A) or indicated concentrations (B) of ethanol (EtOH) in the presence of the same concentrations of PGE_2 as that in the preincubation step. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis.

the effect of PGE_2 on such ethanol-induced apoptosis. Guinea pig gastric mucosal cells in primary culture were preincubated for 2 hr with various concentrations of PGE_2 and further incubated with 3% ethanol for 4 hr. As shown in Figure 1A, in the absence of PGE_2, the ethanol treatment caused apoptotic DNA fragmentation, which is in agreement with previously described results (20). We confirmed that the ethanol treatment induced chromatin condensation (Figure 2A), caspase-3 activation (Figure 3), and mitochondrial dysfunction (Figure 2B), in agreement with previously described results (20). When cells were pretreated with 1 μM PGE_2 for 2 hr and then exposed to 3% ethanol plus 1 μM PGE_2, the apoptotic DNA fragmentation seen with 3% ethanol (no added PGE_2) was clearly inhibited (Figure 1A). Treatment of cells with 1 μM PGE_2 also inhibited the apoptotic DNA fragmentation seen with 1 or 2%

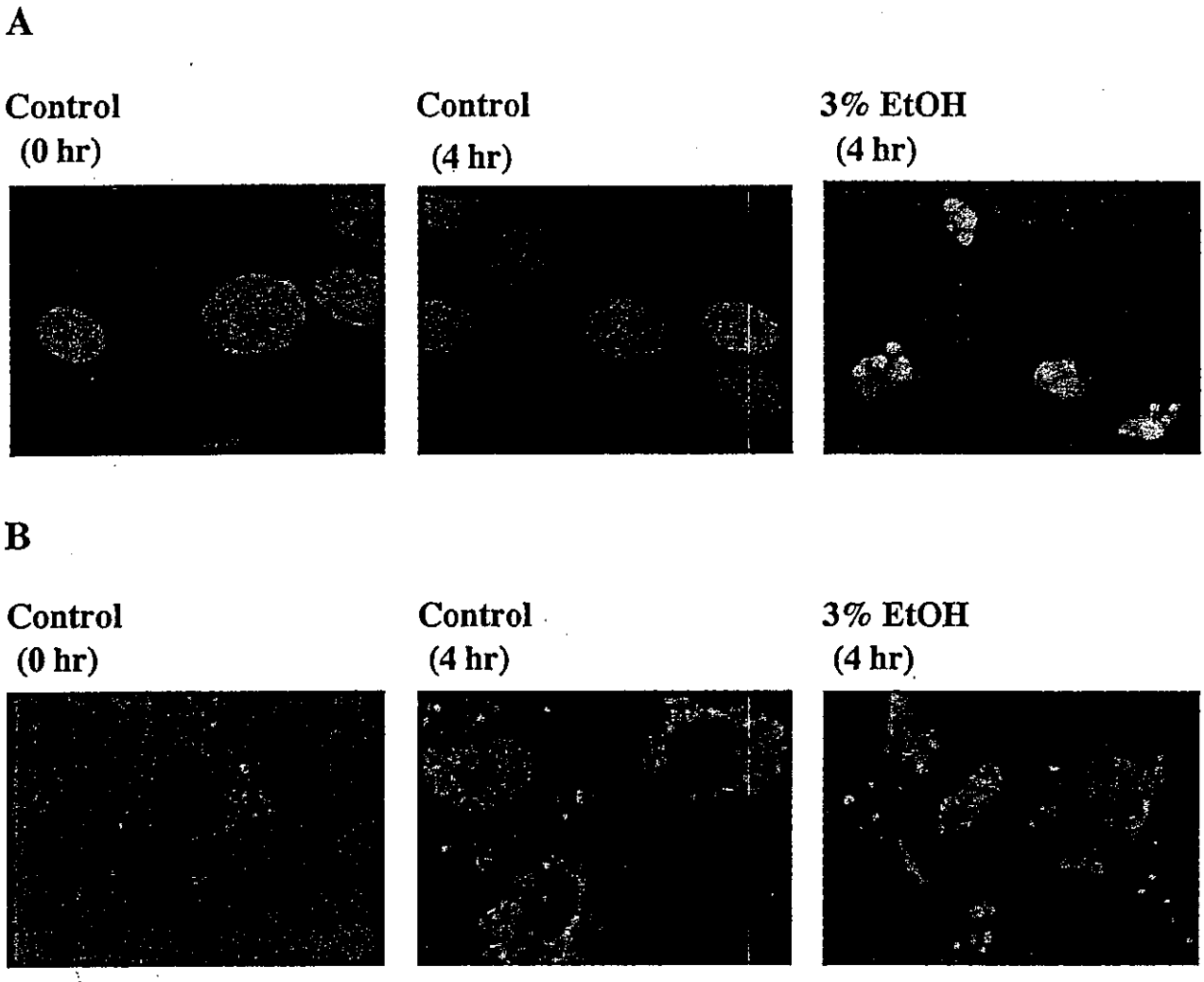

Fig 2. Chromatin condensation and dysfunction of mitochondrial membrane by ethanol. Cultured gastric mucosal cells were incubated for 4 hr with 3% ethanol (EtOH) (A, B). After staining with Ho 342, cells were observed under a fluorescence microscope (A). Cells were treated with DiPsipher (5 μg/ml), a mitochondrial dye, for 20 min at 37°C and then observed under a fluorescence microscope. This mitochondrial dye normally exists in solution as a monomer emitting green fluorescence (530 nm) and forms a dimer emitting red fluorescence (590 nm) in the reaction driven by changes in the mitochondrial membrane potential (28).

ethanol (Figure 1B). We also examined the effect of PGE_2 on caspase-3 activation by ethanol. As shown in Figure 3, treatment of cells with 1 μM PGE_2 partially inhibited the caspase-3 activation by ethanol.

The effect of PGE_2 on cell viability was also examined. Both the trypan blue exclusion test and MTT assay showed that PGE_2 increased the viability of gastric mucosal cells after treatment for 4 hr with 3% ethanol (conditions for inducing apoptosis, see Figure 1). A concentration of PGE_2 around 1 μM inhibited the ethanol-induced apoptosis (Figure 4), which corresponds to the concentration of PGE_2 required for its *in vitro* cytoprotective effect on cell viability as described in previous reports (10–13). Based on these observations, we concluded that PGE_2 is able to suppress apoptotic cell death induced by ethanol.

Requirement of Prior and Simultaneous Treatment of Cells with PGE_2 for Its Inhibitory Effect on Ethanol-Induced Apoptosis. Gastric mucosal cells were first incubated with PGE_2 (preincubation step) and then incubated with ethanol in the presence of the same concentrations of PGE_2 (incubation step) as for the experiments whose results are described in Figures 1–4. We subsequently examined whether both pre-incubation and incubation with PGE_2 were required for the inhibitory effect of PGE_2 on ethanol-induced apoptosis. When PGE_2 was omitted in the preincubation step, the inhibition of ethanol-induced

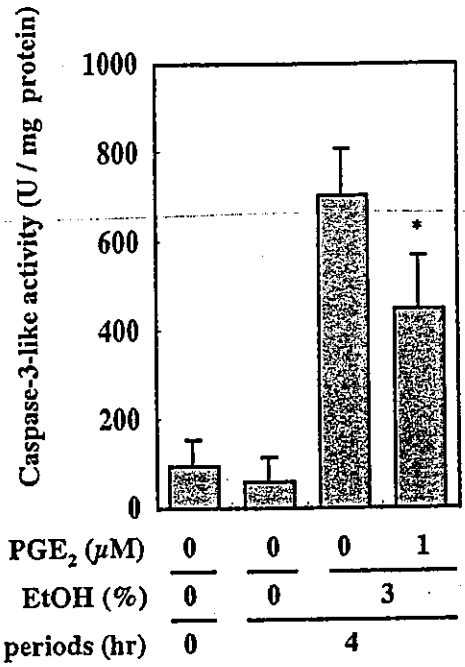

Fig 3. Effect of PGE_2 on caspase-3 activation by ethanol. Cultured gastric mucosal cells were preincubated with or without 1 μM of PGE_2 for 2 hr. Cells were further incubated for 4 hr with or without 3% ethanol (EtOH) in the presence of the same concentrations of PGE_2 as that in the preincubation step. Cell lysates were prepared, and the activity of caspase-3 was measured by a fluorometric assay using Ac-DEVD-MCA. One unit of protease activity was defined, as the amount of enzyme required to release 1 pmol AMC/min. Values shown are mean ± SD ($N = 3$). $^*P < 0.05$.

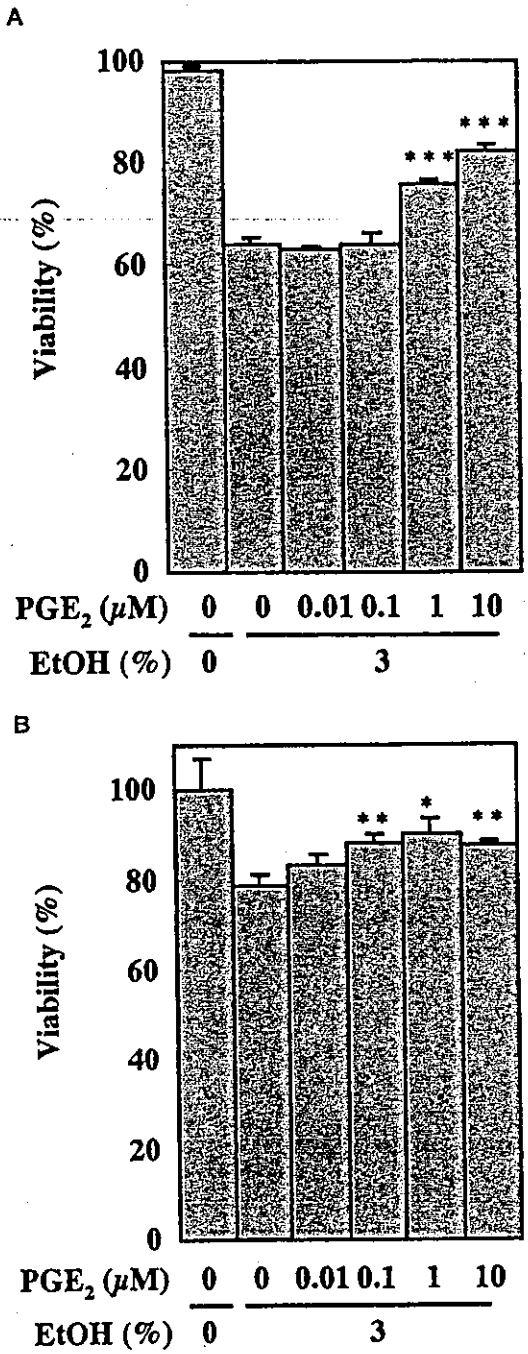

Fig 4. Effect of PGE_2 on apoptotic cell death of cultured gastric mucosal cells. Cultured gastric mucosal cells were preincubated in the presence of the indicated concentrations of PGE_2 for 2 hr. Cells were further incubated for 4 hr with 3% ethanol (EtOH) in the presence of the same concentrations of PGE_2 as that used in the preincubation step. Cell viability was determined by the trypan blue exclusion test (A) or MTT assay (B). Values are mean ± SD; n = 3; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

apoptosis was not as clear-cut (Figure 5, lane 4) as when PGE_2 was used in the preincubation and incubation steps. Moreover, when PGE_2 was omitted from the incubation step (but included in the preincubation step), the ethanol-induced apoptosis was not so clearly inhibited (Figure 5, lane 5) as when PGE_2 was included in both steps. Thus, it seems that PGE_2 must be continuously present in order to inhibit the ethanol-induced apoptosis.

Effect of PGE_2 on Ethanol-Induced Necrosis in Cultured Gastric Mucosal Cells. We previously reported that treatment of cultured gastric mucosal cells with 8–10% ethanol for 1 hr induced necrosis (21). This form of ethanol treatment decreased cell viability without causing DNA fragmentation and chromatin condensation (21). We subsequently examined the effect of PGE_2 on ethanol-induced necrotic cell death. Both the trypan blue exclusion test and MTT assay showed that treatment of cells with 8% ethanol for 1 hr caused a decrease in cell viability, as described previously (21). We confirmed here that the observed cell death was due to necrosis by showing that both DNA fragmentation and chromatin condensation were not seen under the experimental conditions employed (data not shown). PGE_2 showed no

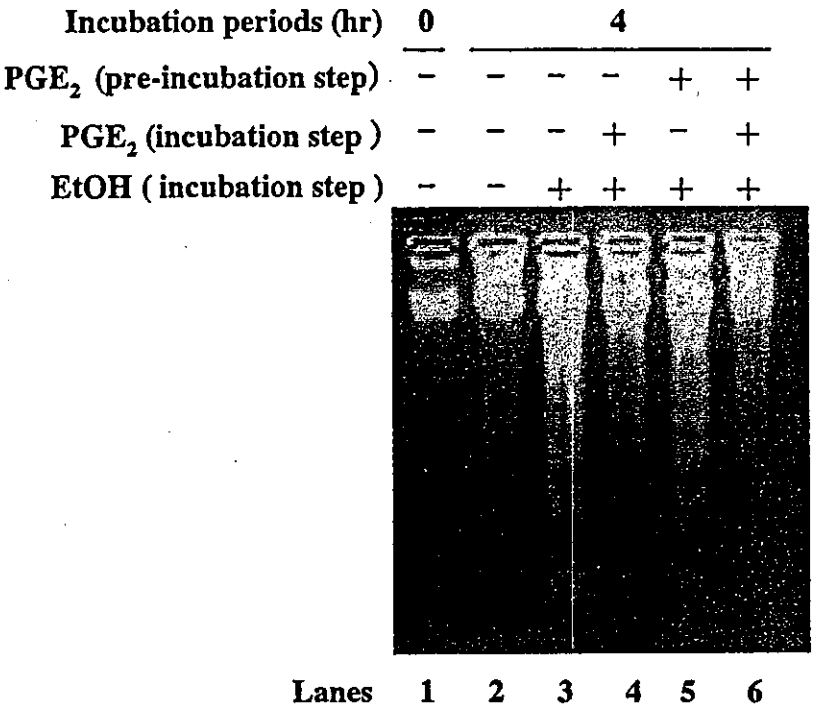

Fig 5. Requirement of the presence of PGE_2 in the preincubation and incubation steps for highlighting its inhibitory effects on ethanol-induced apoptosis. Cultured gastric mucosal cells were preincubated in the presence or absence of 1 μM PGE_2 for 2 hr (preincubation step). Cells were further incubated for 4 hr with or without 3% ethanol (EtOH) as indicated, and in the presence or absence of 1 μM PGE_2 (incubation step). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis.

protective effects against the ethanol (8%)-induced necrotic cell death at any concentrations of PGE_2 used (Figure 6). Moreover, PGE_2 did not show any protective effects against the necrotic cell death induced by 7, 9, or 10% ethanol (data not shown). Therefore, it seems that PGE_2 showed a cytoprotective effect on cultured gastric mucosal cells against apoptosis but not against necrosis.

Effect of PGE_2 on Hydrogen Peroxide- or Hydrochloric Acid-Induced Apoptosis in Cultured Gastric Mucosal Cells. The gastric mucosa *in vivo* is exposed to various irritants other than ethanol, and we previously showed that hydrogen peroxide or hydrochloric acid induced apoptosis in cultured gastric mucosal cells (20, 22). In further experiments we examined the effect of PGE_2 on apoptosis induced by hydrogen peroxide or hydrochloric acid. As shown in Figure 4, treatment of cells with hydrogen peroxide (0.3 or 0.5 mM) for 4 hr or with hydrochloric acid (15 or 17.5 mM) for 8 hr caused apoptotic DNA fragmentation, which is consistent with previously described results (20, 22). We confirmed that these treatments caused chromatin condensation, caspase activation, and mitochondrial dysfunction (data not shown), showing that apoptosis was induced under the conditions employed. PGE_2 completely inhibited the apoptotic DNA fragmentation induced by hydrogen peroxide (0.3 or 0.5 mM) (Figure 7A), suggesting that the inhibitory effect of PGE_2 on apoptosis is not specific for ethanol but observed for apoptosis induced by gastric irritants in general. It should be noted, however, that the apoptotic DNA fragmentation induced by hydrochloric acid (15 or 17.5 mM) was slightly inhibited by PGE_2 (Figure 7B). This may due to the fact that PGE_2 is stable only under conditions of physiological pH (29). As described above, the cytoprotective effect of PGE_2 on ethanol-induced apoptosis is the result of its action at both the preincubation and incubation steps (Figure 5). The low pH value in the presence of hydrochloric acid may inactivate PGE_2 at the incubation step resulting in only the partial inhibition of hydrochloric acid-induced apoptosis by PGE_2.

DISCUSSION

Several lines of clinical evidence have highlighted PGE_2 as being one of the most important protective

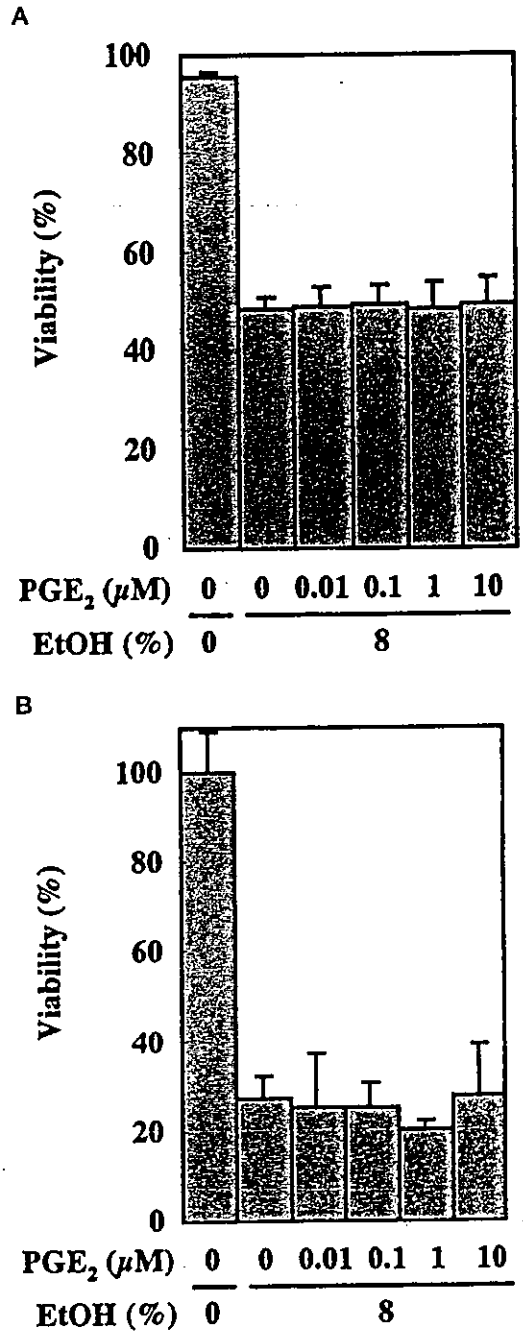

Fig 6. Effect of PGE_2 on necrotic cell death of cultured gastric mucosal cells. Cultured gastric mucosal cells were preincubated in the presence of the indicated concentrations of PGE_2 for 2 h. Cells were further incubated for 1 hr with 8% ethanol (EtOH) in the presence of the same concentrations of PGE_2 as that used in the preincubation step. Cell viability was determined by the trypan blue exclusion test (A) or MTT assay (B).

factors for gastric mucosa *in vivo*. A number of clinically used antiulcer drugs are related in their action to achieving increased intracellular levels of PGs. Furthermore, NSAIDs, one of the major causes of gastric ulcers, are thought to damage the gastric mucosa by inhibiting cyclooxygenase and decreasing the amount of PGs at the level of the gastric mucosa. However, in previous reports, there was a discrepancy about whether or not PGs are directly responsible for making gastric mucosal cells resistant to gastric irritants *in vitro* (see the introductory section). This paper reports the first attempt to examine the effects of PGE_2 on gastric irritant-induced cell death by distinguishing between apoptosis and necrosis in relation to the cell death. We found that PGE_2 showed cytoprotective effects on cultured gastric mucosal cells in relation to apoptosis but not with respect to necrosis. Therefore, it might be postulated that discrepancies in previous reports can explained on the basis of whether it was apoptosis or necrosis that was observed in the experiments described in each paper. Although no one has distinguished between apoptotic cell death and the necrotic cell death in previous reports, we are able to predict this point on the basis of the gastric irritant treatments employed in each paper. However, we could not find any relationship between the predicted type of cell death (apoptosis or necrosis) and the appearance of cytoprotective effects of PGE_2. We can only assume, therefore, that the discrepancies can be explained by differences in experimental conditions (such as types of cells and the presence of serum). Furthermore, since we have no good method for detection of necrosis *in vivo*, we cannot examine the effect of PGE_2 on necrosis and apoptosis by gastric irritants *in vivo*. Therefore, we cannot deny the possibility that our *in vitro* results in this study are not relevant for the *in vivo* situation.

In addition to gastric irritant-induced apoptosis, gastric mucosal cells undergo another type of apoptosis *in vivo*, that of spontaneous apoptosis. Gastric mucosal cells have a rapid rate of turnover *in vivo*, which is the result of rapid spontaneous apoptosis at the gastric surface (30). We previously reported that guinea pig gastric mucosal cells in primary culture underwent spontaneous apoptosis, which seems to mimic the rapid spontaneous apoptosis at the gastric surface *in vivo* (31, 32). Moreover, we recently found that PGE_2 inhibited spontaneous apoptosis *in vitro* (33). Thus, we consider that the protective effect of PGE_2 on gastric mucosa *in vivo* is partly due to an increase in the number of gastric mucosal cells which in turn are due to inhibition of both gastric irritant-induced apoptosis and spontaneous apoptosis.

The pathway for gastric irritant-induced apoptosis and spontaneous apoptosis seems to be different for

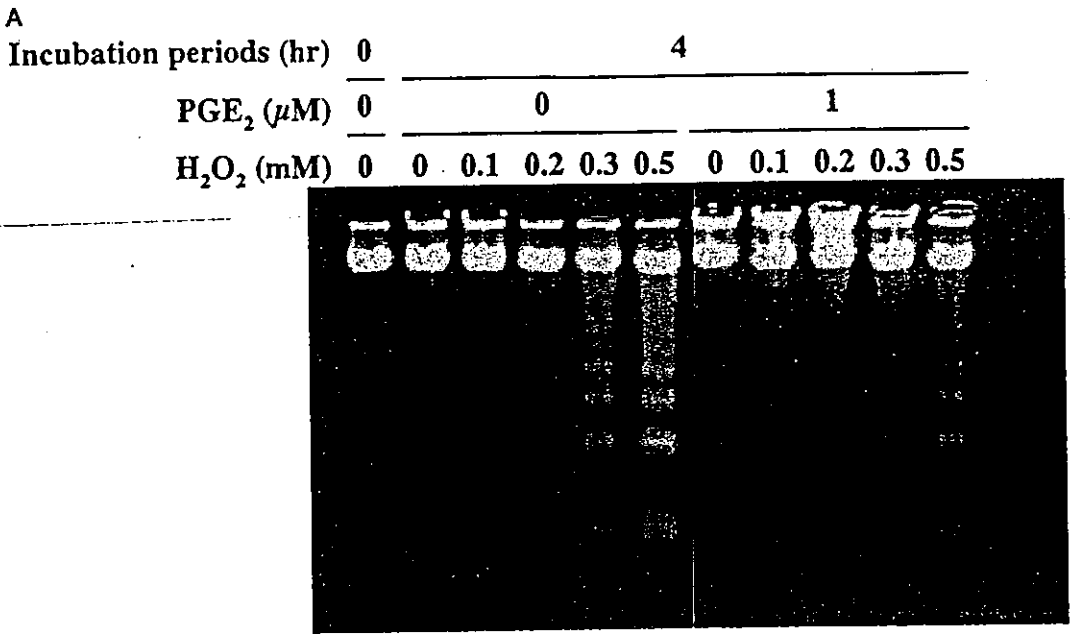

Fig 7. Effect of PGE_2 on apoptotic DNA fragmentation induced by hydrogen peroxide or hydrochloric acid. Cultured gastric mucosal cells were preincubated in the presence or absence of 1 μM PGE_2 for 2 hr. Cells were further incubated with the indicated concentrations of hydrogen peroxide (H_2O_2) for 4 hr (A) or hydrochloric acid (HCl) for 8 hr (B) in the presence of the same concentration of PGE_2 as that used in the preincubation step. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis.

each case: gastric irritants activate caspase-3, -8, and -9 (20), whereas only caspase-3 activation was observed in spontaneous apoptosis (34). Caspase-3 is located downstream in the apoptotic pathway and directly activates proteins responsible for DNA fragmentation and chromatin condensation (35, 36).

Caspase-8 and caspase-9 activate caspase-3 in some apoptotic pathways (37). Therefore, the target of PGE_2 as an inhibitor of apoptosis seems to be a protein, which is common to both pathways (such as caspase-3), if PGE_2 inhibits the gastric irritant-induced apoptosis and the spontaneous apoptosis by the same mechanism. On the other hand, since it was recently reported that the antiapoptotic effect of PGE_2 in human colon cancer cells is due to an increase in the expression of bcl-2 (an inhibitor of cytochrome *c* release from mitochondria) (38), it is also possible that PGE_2 inhibits the cytochrome *c*-dependent activation of caspase-9 through an increase in the expression of bcl-2, thereby inhibiting gastric irritant-induced apoptosis.

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Membrane permeabilization by non-steroidal anti-inflammatory drugs

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Abstract

The cytotoxicity of non-steroidal anti-inflammatory drugs (NSAIDs) is involved in the formation of NSAID-induced gastric lesions. The mechanism(s) behind these cytotoxic effects, however, is not well understood. We found here that several NSAIDs tested caused hemolysis when employed at concentrations similar to those that result in cytotoxicity. Moreover, these same NSAIDs were found to directly permeabilize the membranes of calcein-loaded liposomes. Given the similarity in NSAID concentrations for cytotoxic and membrane permeabilization effects, the cytotoxic action of these NSAIDs may be mediated through the permeabilization of biological membranes. Increase in the intracellular Ca^{2+} level can lead to cell death. We here found that all of NSAIDs tested increased the intracellular Ca^{2+} level at concentrations similar to those that result in cytotoxicity. Based on these results, we consider a possibility that membrane permeabilization by NSAIDs induces cell death through increase in the intracellular Ca^{2+} level.

Keywords: Membrane permeabilization; Cytotoxicity; Membrane fluidity; Gastric mucosal cells; NSAIDs; Intracellular Ca^{2+} level

Because of their efficacy in the treatment of pain, inflammation, and fever, non-steroidal 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 [1]. The action of NSAIDs is mediated via their capacity to inhibit cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong propensity for inducing inflammation. On the reverse side, NSAID use is associated with gastrointestinal complications, such as gastric lesions [2]. About 15–30% of chronic users of NSAIDs have 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 thought to be fully responsible for their gastrointestinal side effects [8]. This is because PGs have a strong cytoprotective effect on the gastrointestinal mucosa [9]. However, the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs are not always linked with each other [10,11], this would suggest that additional mechanisms are involved in the induction of gastrointestinal lesions by NSAIDs [12]. It is well known that NSAIDs have a direct cytotoxicity (topical irritant property) on gastric mucosal cells [12–14]. We recently demonstrated, using primary cultures of guinea pig gastric mucosal cells, that NSAIDs were able to induce both necrosis and apoptosis of cells [15]. Further to this, we also found that this direct cytotoxicity of NSAIDs is independent of the inhibition of COX activity and suggested that both the inhibition of COX activity and the direct cytotoxicity of NSAIDs are required for the induction

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of gastric lesions in vivo [16]. Therefore, the mechanism(s) governing the direct cytotoxicity of NSAIDs needs to be elucidated in order for the overall molecular mechanism of NSAID-induced gastric lesions to be understood and for safer NSAIDs to be developed. Previous reports suggested that various factors, such as bcl-2 family proteins and mitogen-activated protein kinases (MAPKs), are involved in NSAID-induced apoptosis [17,18]. However, the primary target of NSAIDs in relation to their direct cytotoxicity remains unknown. One such target candidate is the cell membrane. It has been reported that NSAIDs interact with phospholipids and that phospholipid liposomes reduce the direct cytotoxicity of NSAIDs in vivo [13,19–21]. In the experiments described here, we found that a range of NSAIDs have membrane permeabilization activity. Since the concentrations of NSAIDs required to induce apoptosis and necrosis in gastric mucosal cells were closely related with those required to increase membrane permeability, we propose that the primary target of these NSAIDs in relation to their direct cytotoxicity is the cell membrane.

Materials and methods

Chemicals, media, and animals. Fetal bovine serum (FBS) and trypsin were purchased from Gibco (Grand Island, New York). RPMI 1640 was obtained from Nissui Pharmaceutical (Tokyo, Japan). Pronase E and type I collagenase were purchased from Kaken Pharmaceutical (Kyoto, Japan) and Nitta Gelatin (Osaka, Japan), respectively. Nimesulide and flurbiprofen were from Cayman Chemical (Ann Arrbor, Michigan). Cholesterol, dicetyl phosphate (DCP), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), mefenamic acid, and flufenamic acid were from Sigma (Tokyo, Japan). Egg phosphatidylcholine (PC) was from Kanto Chemicals (Tokyo, Japan). Celecoxib was from LKT Laboratories (St. Paul, Minnesota). Fluo-3/acetoxymethyl ester (AM) and Pluronic F127 were from Dojindo Lab (Tokyo, Japan). Male guinea pigs (4 weeks of age) were purchased from Shimizu (Kyoto, Japan). All experiments and procedures described here were approved by the Animal Care Committee of Kumamoto University.

Preparation and culture of gastric mucosal cells. Gastric mucosal cells were isolated from guinea pig fundic glands as described previously [22–24]. Isolated gastric mucosal cells (3×10^6 cells/dish) were cultured for 12 h in RPMI 1640 medium containing 0.3% FBS, 100 U/ml penicillin, 2% BSA, and 100 μg/ml streptomycin in type-I collagen-coated plastic culture plates under the conditions of 5% CO_2/95% 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 [22,25].

Treatment of cells with NSAIDs. Cells were exposed to NSAIDs by replacement of the entire bathing medium with fresh medium containing the NSAID under investigation. NSAIDs were dissolved in DMSO and control experiments (without NSAIDs) were performed in the presence of same concentrations of DMSO. It was known that NSAIDs have high affinity for proteins, however, BSA and FBS in medium did not affect the cell death by NSAIDs in our system (data not shown).

For monitoring cell viability, cells were incubated for 2 h with MTT solution at a final concentration of 1 mg/ml. Isopropanol and hydrochloric acid were added to the culture medium at the 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 [26].

Assay for erythrocyte hemolysis and K^+ efflux. Hemolysis and K^+ efflux in erythrocytes were monitored as described [27,28] with some modifications. Human erythrocytes were washed twice with buffer A (5 mM Hepes/NaOH (pH 7.4) and 150 mM NaCl) and then suspended in fresh buffer A at a final concentration of 0.5% hematocrit (5×10^7 cells/ml). The concentration of phospholipids in this suspension was 30 μM in egg PC equivalent. After incubation with NSAIDs for 10 min at 30 °C, hemolysis was estimated by measuring the absorbance at 540 nm. K^+ efflux was measured with a K^+ ion-selective electrode.

Membrane permeability assay. Liposomes were prepared using reversed-phase evaporation method [29,30]. Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2, v/v) in the presence or absence of cholesterol (7.5 μmol, 2.9 mg) or DCP (1 μmol, 0.547 mg), dried, and dissolved in 1.5 ml diethyl ether. This was followed by the addition of 1 ml of 100 mM calcein–NaOH (pH 7.4). The mixture was sonicated to obtain a homogeneous emulsion. The diethyl ether solvent was removed using a conventional rotary evaporator under reduced pressure at 25 °C. The resulting suspension of liposome was centrifuged and washed twice with fresh buffer A to remove untrapped calcein. The final liposome precipitate was re-suspended in 5 ml buffer A. A 0.3 ml aliquot of this suspension was diluted with 19.7 ml buffer A, following which 500 μl of this suspension was incubated at 30 °C for 10 min in the presence of the NSAID under investigation. The final PC concentration was 30 μM. The release of calcein from liposomes was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm).

Fluorescence polarization. Membrane fluidity was measured by the fluorescence polarization technique [31]. Diphenylhexatriene (1 mol% of egg PC) was used as a fluorescence probe. Liposomes were prepared using reversed-phase evaporation method similar to membrane permeability experiments, except for the addition of 1 ml buffer A instead of 100 mM calcein–NaOH. The final PC concentration was 30 μM. Measurements were carried out using a Hitachi F-4500 fluorospectrophotometer. The degree of polarization (P) was calculated according to the following equation:

$$P = (I_{VV} - C_f I_{VH})/(I_{VV} + C_f I_{VH}),$$

where I is the fluorescence intensity, and subscripts V and H refer, respectively, to the vertical and horizontal orientations of the excitation (first) and emission (second) polarizers. C_f ($=I_{HV}/I_{HH}$) is a correction factor.

Measurement of the intracellular Ca^{2+} level. The intracellular Ca^{2+} level was monitored according to manufacturer's protocols (Dojindo Lab) [32]. Cells were detached by trypsin and washed with the assay buffer containing 115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM Hepes, and 13.8 mM glucose. Then cells were incubated with 4 μM fluo-3/AM in the assay buffer containing 0.1% BSA, 0.04% Pluronic F127, and 2 mM probenecid for 40 min at 37 °C. After washing twice with the assay buffer, cells were suspended with the assay buffer containing 2 mM probenecid. Fluo-3 fluorescence was measured in a water-jacketed cuvette (1.6×10^6 cells/cuvette) with a HITACHI F-2000 spectrofluorophotometer by recording excitation signals at 490 nm and emission signal at 530 nm at 1-s intervals. Maximum and minimum fluorescence values (F_{max} and F_{min}) were obtained by adding 10 μM ionomycin and 10 μM ionomycin plus 5 mM EGTA (in Ca^{2+} free medium), respectively. The intracellular Ca^{2+} level was calculated according to the equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the apparent dissociation constant (400 nM) of the fluorescence dye–Ca^{2+} complex [32].

Results and discussion

Induction of necrosis and apoptosis by NSAIDs in gastric mucosal cells

Fig. 1 shows structures of the five different NSAIDs that were used in this study. We previously reported that short-term (1 h) treatment of primary cultures of guinea pig gastric mucosal cells with relatively high concentrations of NSAIDs and long-term (16 h) treatment of these cells with relatively low concentrations of NSAIDs induced necrosis and apoptosis, respectively [15]. We first tested the ability of the different NSAIDs (Fig. 1) to induce necrosis and apoptosis, and found that cell viability was decreased following short-term (1 h) treatment with each NSAID tested (Fig. 2A). Since cell death in this manner was not associated with apoptotic DNA fragmentation and chromatin condensation (data not shown), it is likely to have been mediated by necrosis. In contrast, the decrease in cell viability with long-term (16 h) NSAID treatment (Fig. 2B) was associated with apoptotic DNA fragmentation and chromatin condensation (data not shown), suggesting that it is mediated by apoptosis. Higher concentrations of NSAIDs were required to induce necrosis compared to those required for apoptosis (Fig. 2), which is consistent with previous reports [15]. Among all of the NSAIDs tested, celecoxib showed the most potent necrosis- and apoptosis-inducing activity, followed by flufenamic acid (Fig. 2). The cytotoxicity of NSAIDs is not directly related to their potency to produce gastric lesions in vivo. This is because both the inhibition of COX activity and the direct cytotoxicity of NSAIDs are involved in the induction of gastric lesions in vivo [16]. Furthermore, various other factors, such as mucosal blood flow and gastric motility, are also involved in the induction of gastric lesions in vivo.

Two subtypes of COX, COX-1 and COX-2, are responsible for the majority of COX activity in gastric mucosal and inflammatory tissues, respectively, and recently a number of COX-2-selective NSAIDs were developed [33]. Among the NSAIDs whose data are graphed in Fig. 2, nimesulide and celecoxib have selectivity for COX-2. No relationship was evident between NSAID cytotoxicity and selectivity for COX-2, supporting the idea that the direct cytotoxicity of NSAIDs is independent of COX inhibition. We also confirmed that exogenously added PGE_2 (either native PGE_2 or 16,16-dimethyl-PGE_2) did not affect the extent of cell death by short-term and long-term treatment with NSAIDs even at a higher concentration of PGE_2 in the culture medium than is present endogenously (10^{-9} M) (data not shown).

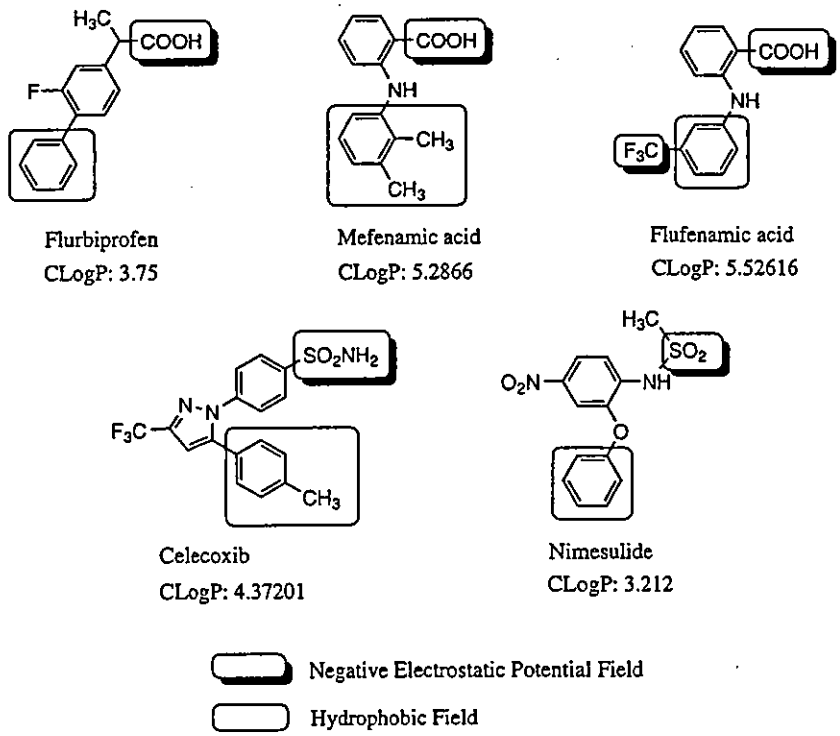

Fig. 1. Molecular structures and CLogP values of NSAIDs. Discriminative negative electrostatic potential fields are shown with bold-lined boxes, and discriminative hydrophobic fields (phenyl groups) are shown with normal-lined boxes. CLogP values were calculated with CLOGP3 program (Pomona MedChem Software 3.6).

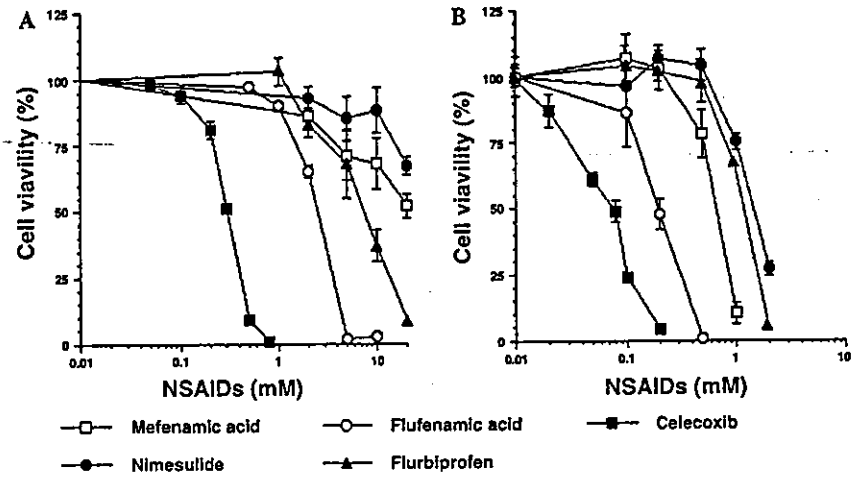

Fig. 2. Necrosis and apoptosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with NSAID under investigation for 1 h (A) or 16 h (B). Cell viability was determined by the MTT method. Values are means ± SEM ($n = 3$).

NSAID-induced hemolysis and K^+ efflux

Measurement of hemolysis is a standard method for testing the membrane permeabilization activities of drugs. As shown in Fig. 3A, all of the tested NSAIDs caused hemolysis of erythrocytes; a finding which strongly suggests that NSAIDs increase the permeability of cell membranes. The relative potency of each NSAID for hemolysis was approximately similar to that for cytotoxicity. For example, celecoxib showed the most potent activity for hemolysis, followed by flufenamic acid (Fig. 3A), which is in relative accordance with the cytotoxic potency of each NSAID (Fig. 2). Therefore, it would appear that NSAID-induced cell death (necrosis and apoptosis) is mediated by membrane permeabilization.

We also measured K^+ efflux from erythrocytes in the presence of each NSAID and found that most of them stimulated K^+ efflux (Fig. 3B) at similar concentrations to those required for inducing hemolysis (Fig. 3A). However, the increase in K^+ efflux induced by celecoxib was observed at a lower concentration than that at which hemolysis was observed (Fig. 3). This result suggests that, in a manner different from that of other NSAIDs, celecoxib causes initially the formation of small pores in the cytoplasmic membrane that are able to mediate the efflux of K^+ but not of hemoglobin.

Membrane permeabilization by NSAIDs

We next examined the ability of each NSAID to permeabilize calcein-loaded liposomes prepared from PC. Calcein fluoresces very weakly at high concentrations due to self-quenching. Thus, the addition of membrane permeabilizing drugs to a medium containing calcein-

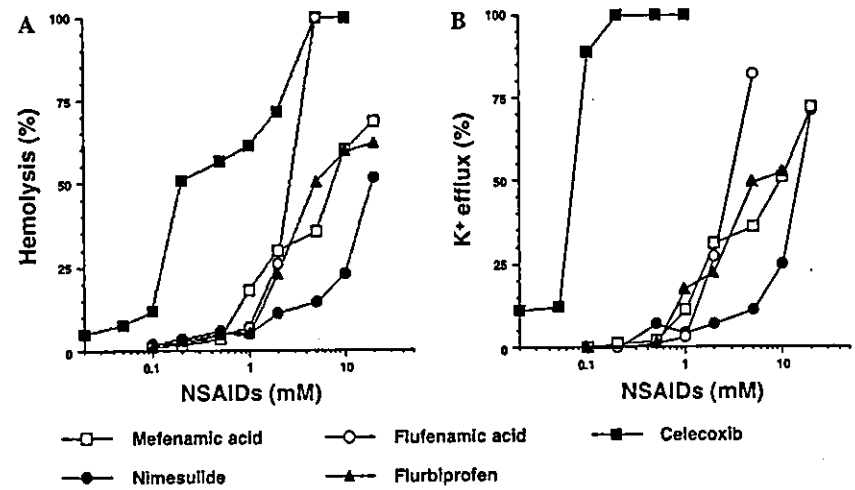

Fig. 3. NSAID-induced hemolysis and K^+ efflux from erythrocytes. Human erythrocytes were incubated in the presence of each NSAID for 10 min at 30 °C. Hemolysis was estimated by measuring the absorbance at 540 nm (A). The level of K^+ efflux was measured with a K^+ ion-selective electrode (B). Melittin (10 μM), a membrane permeabilizing reagent, was used to determine the 100% level of hemolysis and K^+ efflux [28].

loaded liposomes should cause an increase in fluorescence by releasing calcein trapped inside the liposomes [29,30]. Calcein fluorescence increased in the presence of each of all the NSAIDs tested, thereby showing that NSAIDs have membrane permeabilization effects on PC liposomes (Fig. 4). The target of NSAIDs in terms of their membrane permeabilization effects thus appears to be phospholipids. The relative potency of each NSAID for calcein release (Fig. 4) was approximately similar to that for cytotoxicity (Fig. 2). For example, celecoxib showed the most potent activity for calcein release, followed by flufenamic acid (Fig. 4). It would appear that the cytotoxic action of NSAIDs is mediated through their ability to permeabilize membranes.

As for the mechanism of membrane permeabilization-dependent cell death (necrosis and apoptosis), we considered the contribution of intracellular Ca^{2+} level, based on previous results; permeabilization of cytoplasmic membrane causes increase in intracellular Ca^{2+} level, increase in intracellular Ca^{2+} level can cause cell death through induction of both necrosis and apoptosis [34], and some NSAIDs increased the intracellular Ca^{2+} level [35–41]. Therefore, we examined the effect of each NSAID on the intracellular Ca^{2+} level by use of fluo-3/AM assay system. As shown in Fig. 5, each NSAID tested significantly increased the intracellular Ca^{2+} level at concentrations of ED_{50} value for apoptosis (concentrations required for inducing apoptosis in 50% cells), in other words, accompanying induction of apoptosis. This increase in the intracellular Ca^{2+} level by NSAIDs may contribute to their activity for inducing cell death.

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. We recently reported that exposure of cells to NSAID induced GRP78 that protects cells from ER stressor as well as CHOP, a transcription factor involved in apoptosis. Since NSAID-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, we proposed that ER stress response-related proteins, particularly CHOP, are involved in NSAID-induced apoptosis [42]. However, the upstream pathway for NSAID-induced ER stress response (induction of CHOP) remained unknown. In this study, we found that NSAID-induced apoptosis is related to their activity for membrane permeabilization and increase in the intracellular Ca^{2+} level. It is known that increase in intracellular Ca^{2+} level induces ER stress

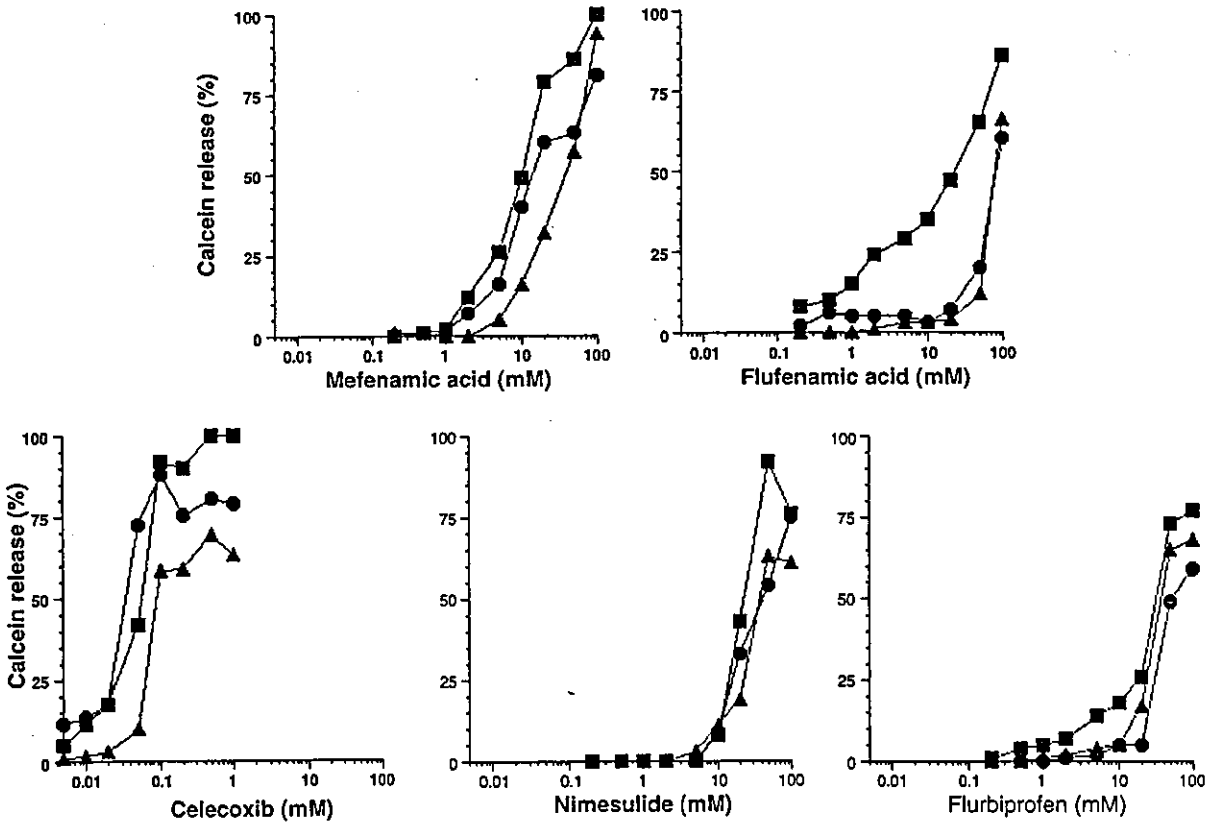

Fig. 4. Membrane permeabilization induced by NSAIDs. Calcein-loaded liposomes prepared from PC (squares), PC/cholesterol (circles), or PC/DCP (triangles) were incubated with each NSAID for 10 min at 30 °C. The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin (10 μM) was used to determine the 100% level of membrane permeabilization [28].

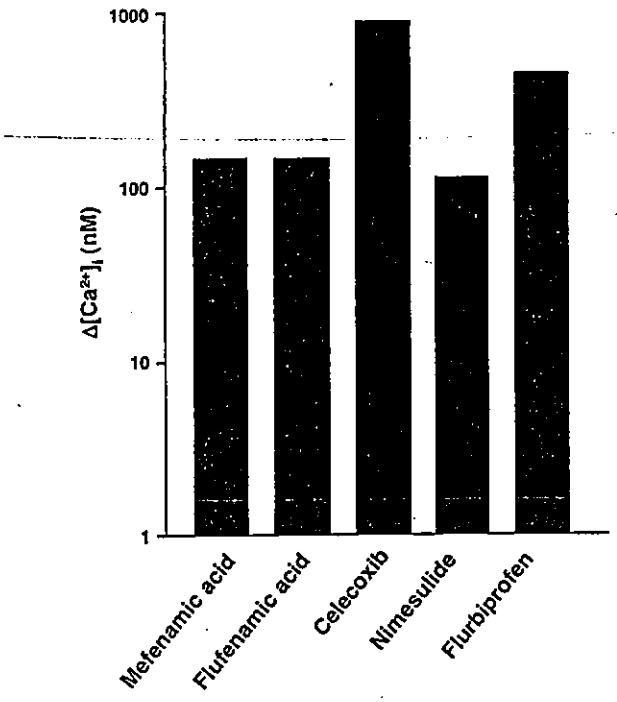

Fig. 5. Increase in the intracellular Ca^{2+} level by NSAIDs. The intracellular Ca^{2+} level was monitored by fluo-3/AM assay system as described in Materials and methods. NSAIDs were added to fluo-3/AM-loaded cells at concentrations of ED_{50} values for apoptosis (mefenamic acid, 0.7 mM; flufenamic acid, 0.2 mM; celecoxib, 0.08 mM; nimesulide, 1.6 mM; and flurbiprofen, 1.2 mM) and time course of fluo-3 fluorescence change was monitored. The maximum value for increase in the intracellular Ca^{2+} level ($\Delta[Ca^{2+}]_i$) of each NSAID was shown.

response; Ca^{2+} ionophore induces ER stress response [43,44]. Therefore, we assume that the increase in intracellular Ca^{2+} level is located in the upstream pathway of NSAID-induced ER stress response. In other words, permeabilization of cytoplasmic membrane by NSAIDs increases intracellular Ca^{2+} level, which in turn induced ER stress response (induction of CHOP), resulting in induction of apoptosis. Since celecoxib (but not other NSAIDs) was reported to inhibit sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [37], apoptosis by celecoxib may also involve this SERCA inhibition.

Although the chemical structures of the NSAIDs used in the experiments reported here are quite different, we attempted to identify a structure–activity relationship by focusing on common structural features between the various compounds. As shown in Fig. 1, all of the tested NSAIDs have hydrophobic field (phenyl groups) and negative electrostatic potential field (sulfonamide or carboxyl group). The partition coefficient is the equilibrium concentration of solute in a non-polar solvent divided by the concentration of the same species in a polar solvent. In this and most other applications, the polar solvent is water. The logarithm of the partition coefficient, log *P*, has been successfully used as a hydrophobic parameter in 'extrathermo-dynamic' Hammett methodology. 1-Octanol has much to recommend it as the choice for the non-polar phase and log *P* has been used successfully in quantitative structure–activity relationships (QSAR). By now many efficient methods of measurement of octanol/water partition coefficients have been developed, and the first attempt to reduce log *P* calculation to computer algorithm was done by Chou and Jurs [45]. It was called CLOGP. In this paper we calculated the CLogP (calculated log *P* by CLOGP program) values of the compounds, which indicate their hydrophobicity (the larger the CLogP value the higher the hydrophobicity) using CLOGP3 program (Pomona MedChem Software 3.6) (http://clogp.pomona.edu/medchem/chem/papers/14-clogp.html). This program is one of the efficient methods to calculate log *P* from structure by an additive-constitutive procedure. As can also be deduced from Fig. 1, there is a slight tendency for compounds with larger CLogP values to have higher cytotoxic (or membrane permeabilization) activity. For example, compounds with a CLogP value higher than 4.0 coincide with those with an ED_{50} value (for apoptosis induction) lower than 1.0 mM (Fig. 1). We consider that the common structural features described above and high CLogP values may be important if NSAIDs are to have potent cytotoxic (or membrane permeabilization) activity.

Mechanism of membrane permeabilization by NSAIDs

The effect of cholesterol on membrane permeabilization (calcein release) by NSAIDs was also examined in this study. As shown in Fig. 4, cholesterol made PC liposomes resistant to a NSAID (flufenamic acid) but not so evidently to others. We also examined the effect of anionic lipids (DCP) on membrane permeabilization (calcein release) by NSAIDs. DCP also made liposomes resistant to some NSAIDs (mefenamic acid, flufenamic acid, and celecoxib), but again not so clearly to others. These data suggest that the mechanism of membrane permeabilization by NSAIDs is different depending on the NSAID under investigation.

Some NSAIDs (for example, indomethacin and naproxen) are known to affect membrane fluidity [19]. We therefore examined the effect of each NSAID on membrane fluidity using the fluorescence polarization technique. In such experiments, the higher the calculated *P* value, the lower the membrane fluidity. As shown in Table 1, most of the NSAIDs tested (mefenamic acid, flufenamic acid, celecoxib, and nimesulide) decreased membrane fluidity. However, this effect of NSAIDs was not closely related with their cytotoxicity and membrane permeabilization activity. Flurbiprofen did not significantly affect membrane fluidity (Table 1). We also examined the effect of the different NSAIDs on the membrane fluidity of liposomes containing cholesterol. As shown in Table 1, cholesterol inhibited the decrease

Table 1
Effect of various NSAIDs on membrane fluidity

NSAID (mM)	Degree of polarization (*P*)	
	PC	PC + cholesterol
Control	0.095	0.306
Mefenamic acid		
0.1	0.170	0.298
Flufenamic acid		
0.1	0.134	0.302
1	0.259	0.335
10	0.304	0.341
Celecoxib		
0.01	0.106	0.273
0.1	0.117	0.236
1	0.391	0.288
Nimesulide		
0.1	0.209	0.316
1	0.196	
Flurbiprofen		
0.1	0.101	0.308
1	0.105	0.290
10	0.107	0.262

The degree of polarization (*P*) of PC or PC/cholesterol liposomes in the presence of various NSAIDs was measured as described under Materials and methods.

in membrane fluidity caused by NSAIDs as described above. Cholesterol, itself, decreased membrane fluidity in the absence of NSAIDs. All these data suggest that permeabilization activity of NSAIDs cannot be simply explained by a decrease in membrane fluidity.

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