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Figure Legends

Fig. 1. Influence of washing on the release of drug from the calcium carbonate nanoparticles (nano-$CaCO_3$) either incorporating or adsorbing erythropoietin. The abscissa represents the percentage of erythropoietin content compared with that measured before washing. Each column and horizontal bar represents the mean ±SEM of 3 experiments.

Fig. 2. *In vitro* release of granulocyte colony-stimulating factor (G-CSF) from calcium carbonate nanoparticles (nano-CaCO3) (closed circle) and inactivation of free G-CSF in buffer (open circle). Each symbol represents the mean of 3 experiments.

Fig. 3. Plasma concentration of betamethasone after the subcutaneous injection of calcium carbonate nanoparticles (nano-$CaCO_3$) incorporating betamethasone phosphate (BP) (closed circle) and inactivation of free G-CSF in buffer (open circle).

Each symbol represents the mean ± SEM of 3 experiments.

※ : significant difference ($p < 0.05$) between the 2 groups; † : significant difference ($p < 0.05$) in relation to 0 hour § : no significant difference ($p > 0.05$) in relation to 0 hour.

Table 1

Particle size of calcium carbonate nanoparticles (Nano-$CaCO_3$) loading betamethasone phosphate (BP) and their loading efficiency

Mixing speed	Particle size (nm)		Loading efficiency [a] (%)	
	Incorporation	Adsorption	Incorporation	Adsorption
Gentle ca. 650 rpm	140.7 ±21.4 (95.8 - 165.1)[b]	170.5 ±24.0 (139.5 - 209.5)	91.1 ±1.7	9.1 ±1.3
Vigorous ca. 1300 rpm	44.8 ±1.1 (35.3 - 56.8)	101.5 ±12.6 (80.9 - 131.7)	90.6 ±1.3	25.7 ±2.8

[a]Loading efficiency is the percentage of incorporated or adsorbed BP compared to the total BP used. Each value represents mean ±SEM of 3 experiments.

[b]Range of distribution in the particle size

Fig. 1. Mizushima

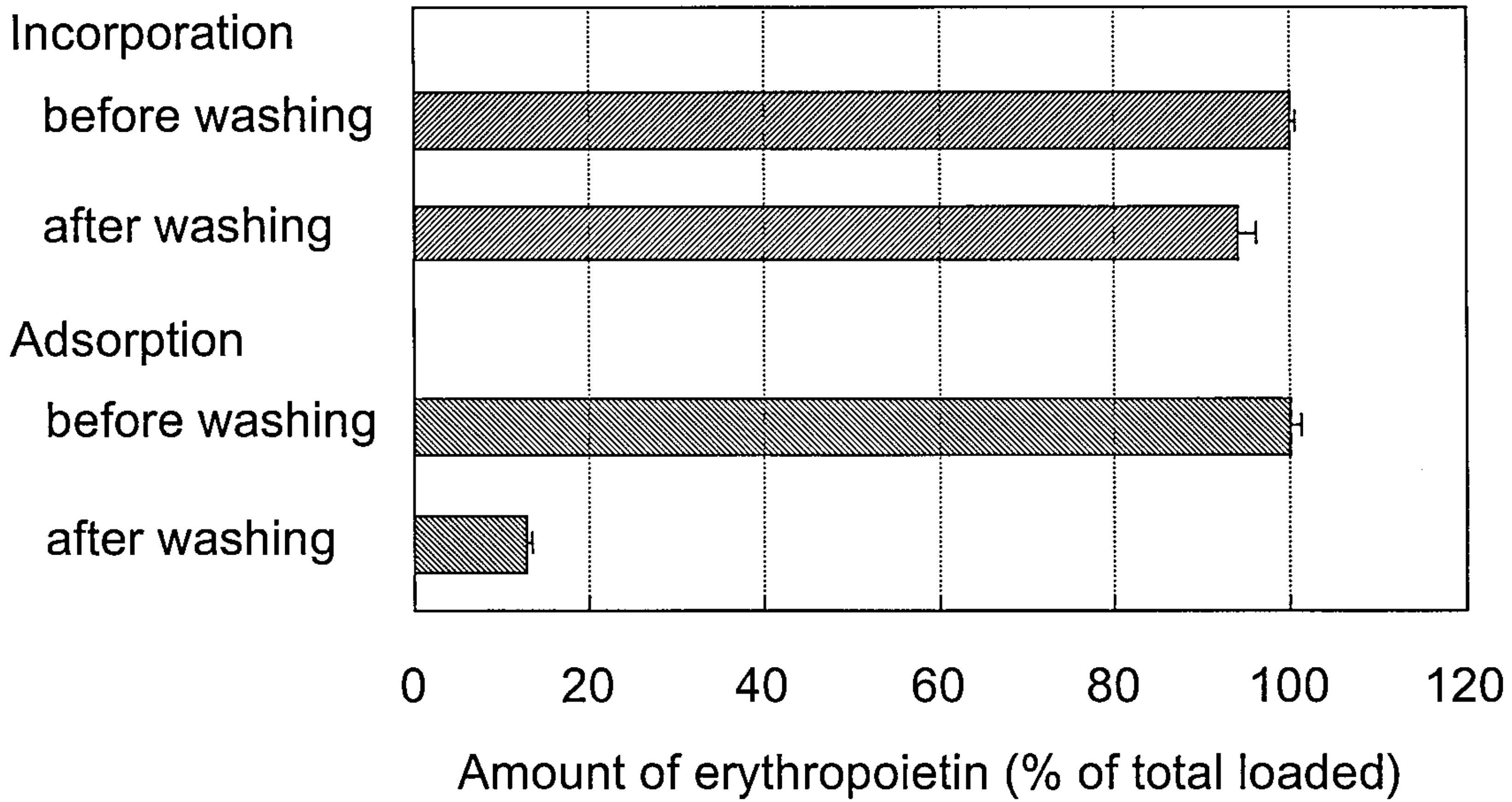


Fig. 2. Mizushima

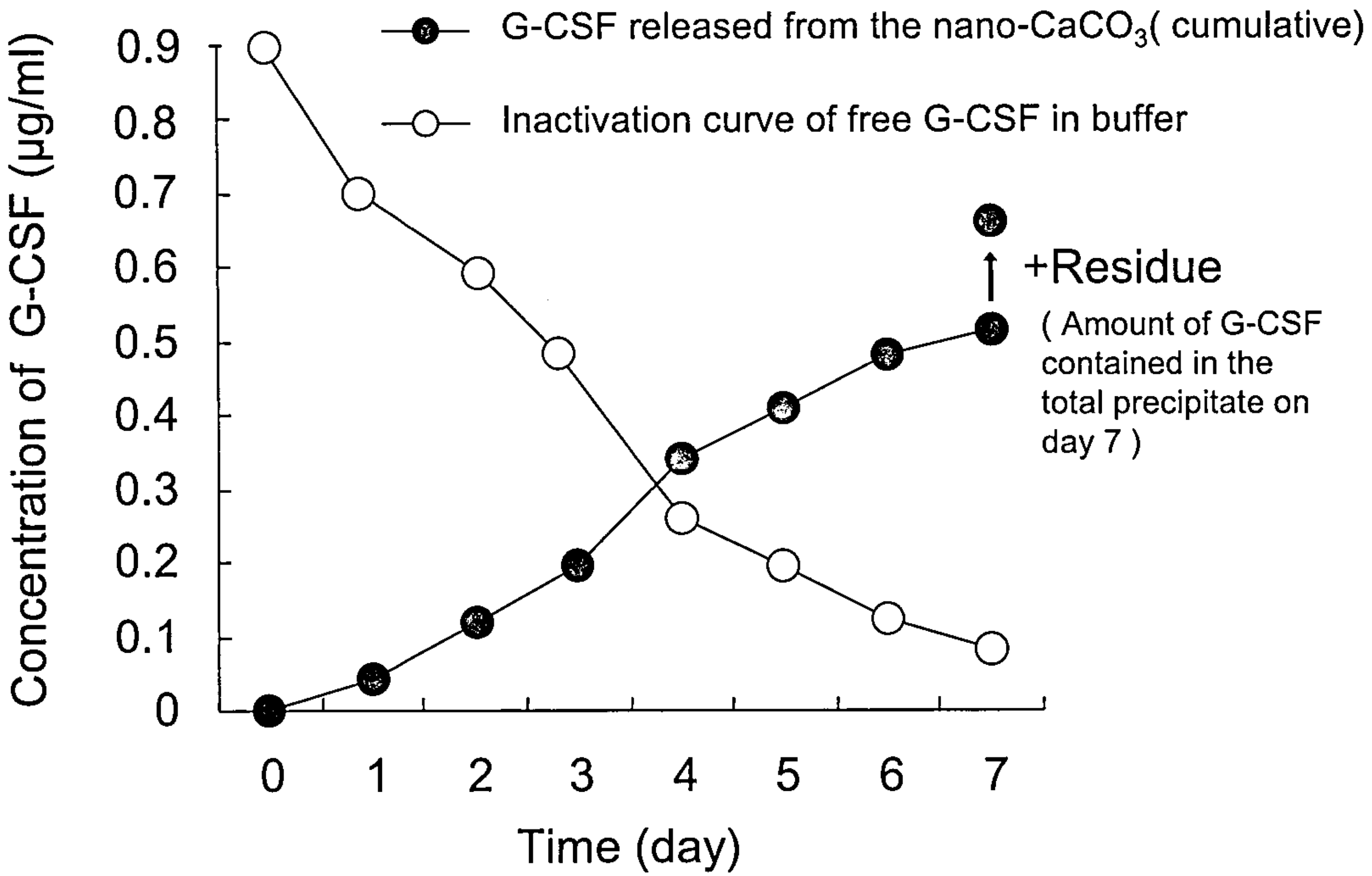
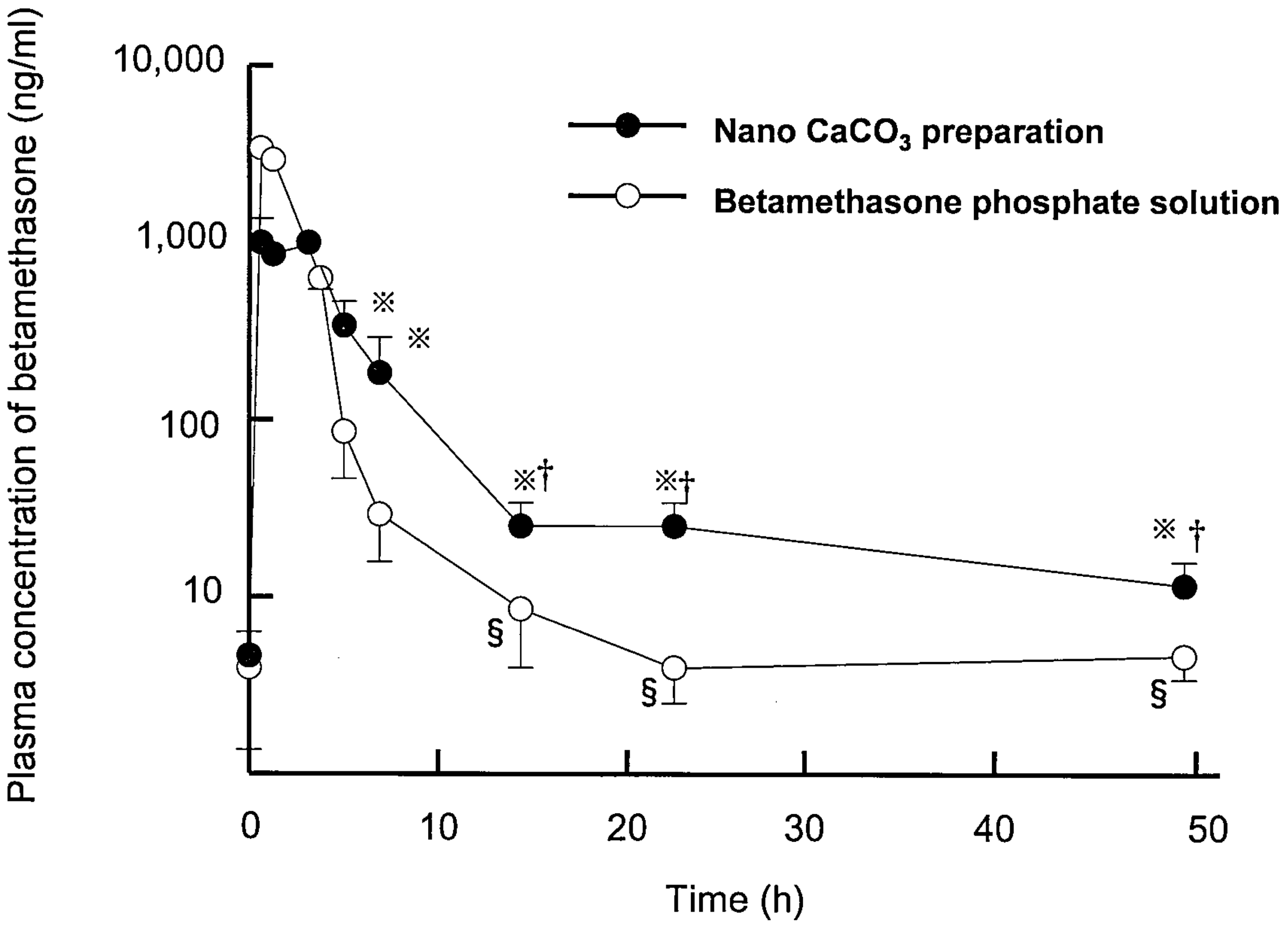


Fig.3. Mizushima



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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.
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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).

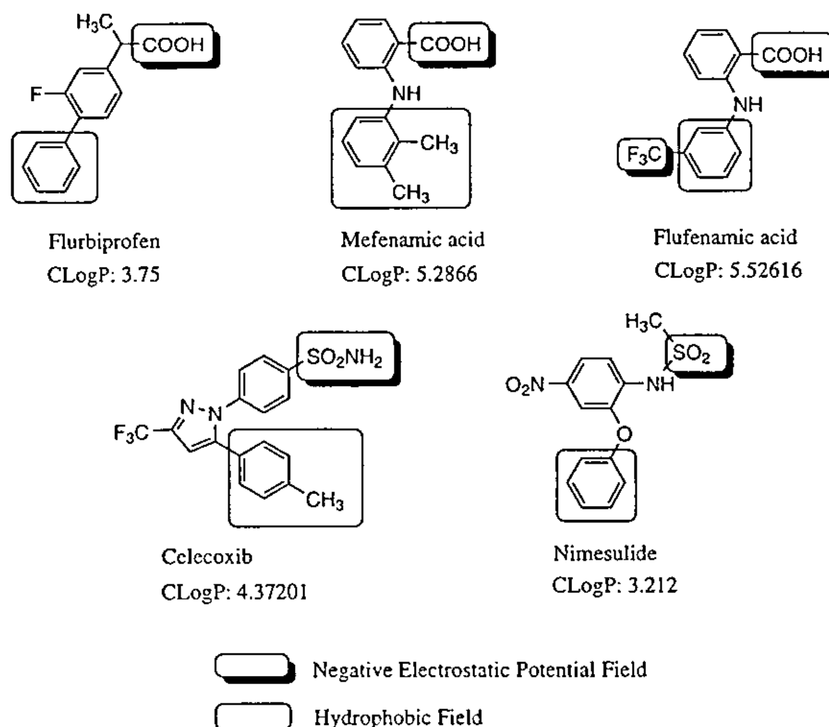

Fig. 1. Molecular structures and CLogP values of NSAIDs. Discriminative negative electrostatic potential fields are shown with bold-lined boxes, and discriminative hydrophobic fields (phenyl groups) are shown with normal-lined boxes. CLogP values were calculated with CLOGP3 program (Pomona MedChem Software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

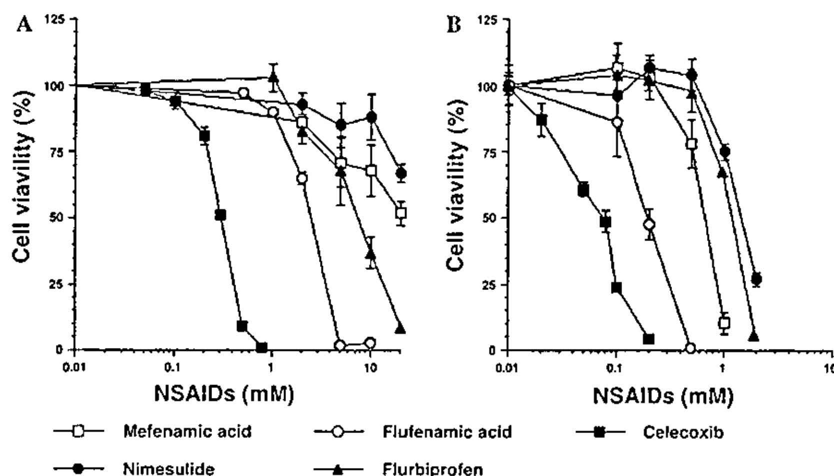

Fig. 2. Necrosis and apoptosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with NSAID under investigation for 1 h (A) or 16 h (B). Cell viability was determined by the MTT method. Values are means ± SEM ($n = 3$).

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-

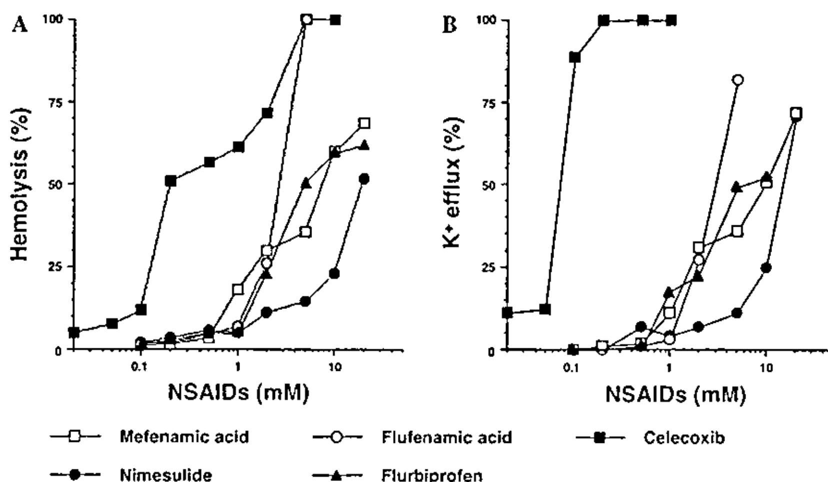

Fig. 3. NSAID-induced hemolysis and K^+ efflux from erythrocytes. Human erythrocytes were incubated in the presence of each NSAID for 10 min at 30 °C. Hemolysis was estimated by measuring the absorbance at 540 nm (A). The level of K^+ efflux was measured with a K^+ ion-selective electrode (B). Melittin (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

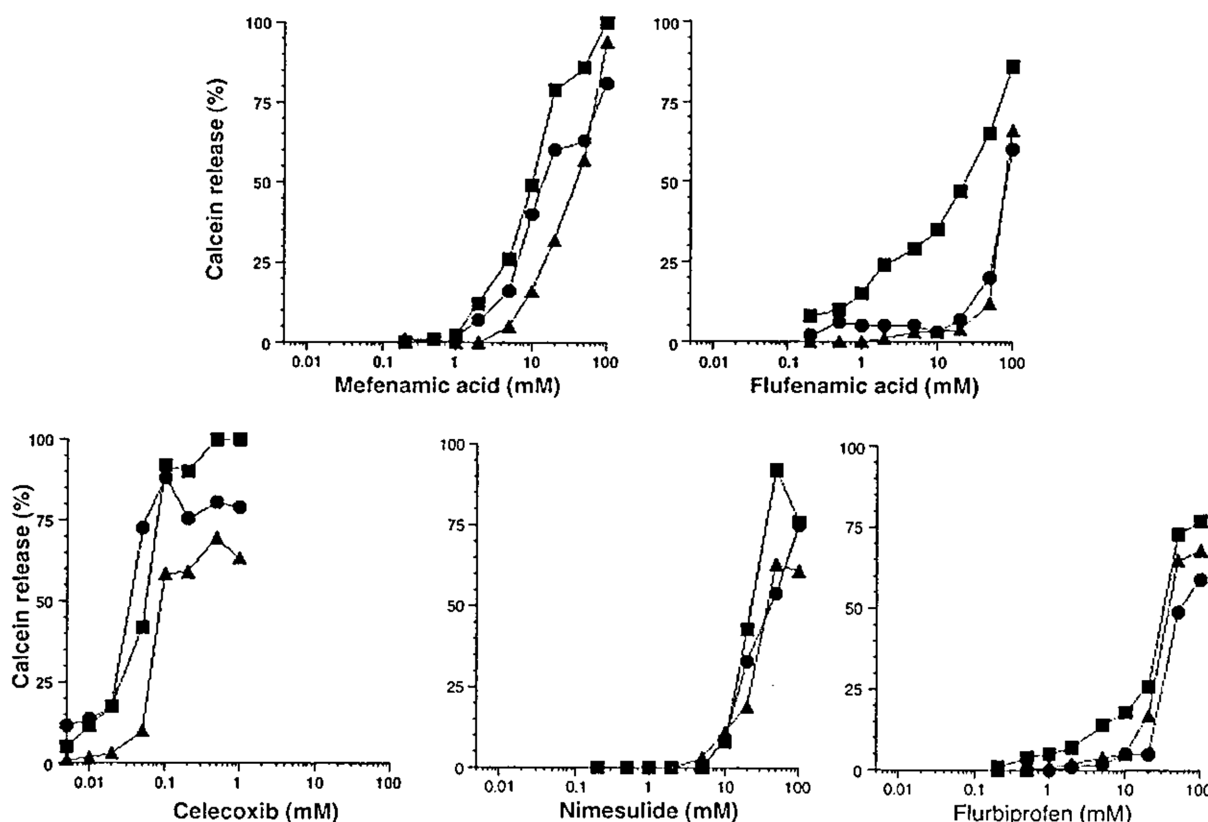

Fig. 4. Membrane permeabilization induced by NSAIDs. Calcein-loaded liposomes prepared from PC (squares), PC/cholesterol (circles), or PC/DCP (triangles) were incubated with each NSAID for 10 min at 30 °C. The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin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

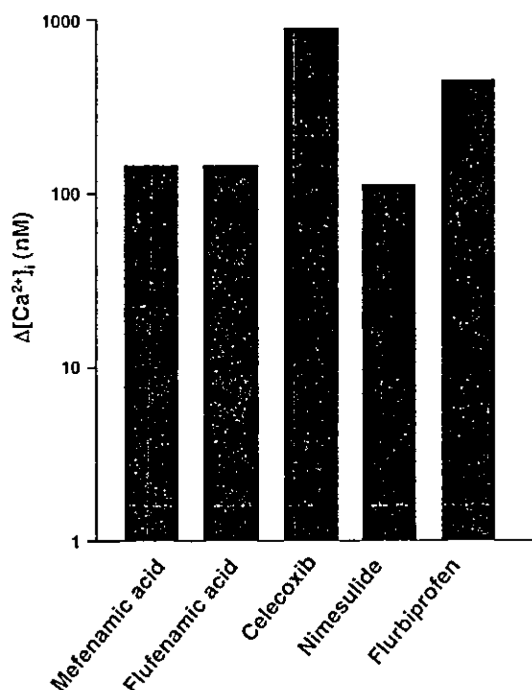

Fig. 5. Increase in the intracellular Ca^{2+} level by NSAIDs. The intracellular Ca^{2+} level was monitored by fluo-3/AM assay system as described in Materials and methods. NSAIDs were added to fluo-3/AM-loaded cells at concentrations of ED_{50} values for apoptosis (mefenamic acid, 0.7 mM; flufenamic 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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Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis

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Abstract

Apoptosis induced by nonsteroidal anti-inflammatory drugs (NSAIDs) is involved not only in the production of NSAID-induced gastric lesions but also in the antitumor activity of these drugs. The endoplasmic reticulum (ER) stress response is a cellular mechanism that aids in protecting the ER against ER stressors and is involved in ER stressor-induced apoptosis. Here, we examine the relationship between this response and NSAID-induced apoptosis in cultured guinea-pig gastric mucosal cells. Exposure of cells to indomethacin, a commonly used NSAID, induced GRP78 as well as CHOP, a transcription factor involved in apoptosis. Three factors that positively regulate CHOP expression (ATF6, ATF4 and XBP-1) were activated and/or induced by indomethacin. NSAIDs other than indomethacin (diclofenac, ibuprofen and celecoxib) also induced CHOP. Monitoring of the transcriptional activities of ATF6 and CHOP by luciferase assay revealed that both were stimulated in the presence of indomethacin. Furthermore, indomethacin-induced apoptosis was suppressed in cultured guinea-pig gastric mucosal cells by expression of the dominant-negative form of CHOP, or in peritoneal macrophages from CHOP-deficient mice. These results suggest that ER stress response-related proteins, particularly CHOP, are involved in NSAID-induced apoptosis.
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Abbreviations: ASK1, apoptosis signal-regulating kinase 1; ATF, activating transcription factor; CHOP, C/EBP homologous transcription factor; COX, cyclooxygenase; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; ERSE, ER stress response element; FBS, fetal bovine serum; GRP, glucose-regulated protein; IRE1, protein-kinase and site-specific endoribonuclease; JNK, c-jun NH_2-terminal kinase; MTT, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NSAIDs, nonsteroidal anti-inflammatory drugs; PEK, pancreatic eIF2 kinase; PERK, protein kinase R-like ER kinase; PG, prostaglandin; TRAF2, tumor necrosis factor receptor-associated factor; UPR, unfolded protein response; XBP-1, X box binding protein

Introduction

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

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

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

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

Results

Induction of GRP78 and CHOP by indomethacin associated with apoptosis

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

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

We also examined the effect of indomethacin on the expression of *GRP78* and *CHOP* mRNA by Northern blotting analysis (Figure 3). Similar to thapsigargin, indomethacin increased the amount of *GRP78* and *CHOP* mRNA, suggesting that these ER stress response-related proteins are induced at the level of transcription. Given that CHOP is a

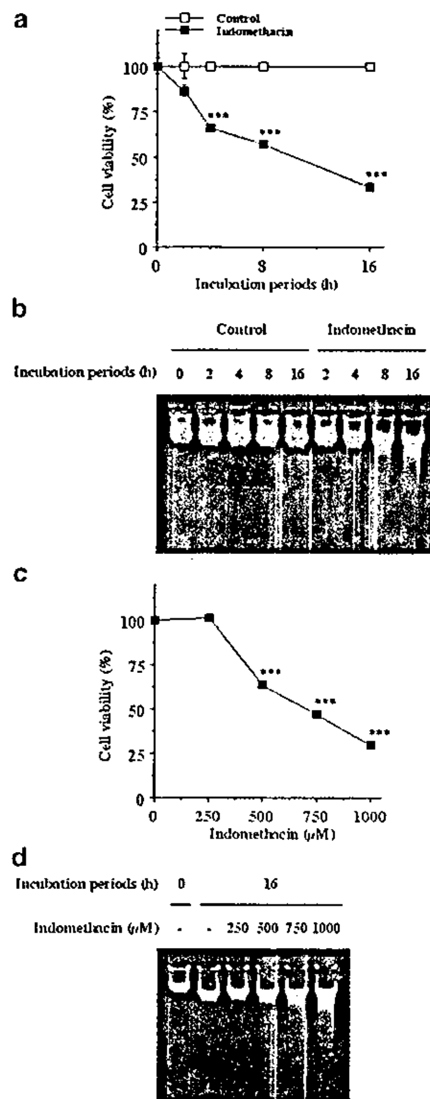

Figure 1 Apoptosis induced by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM (**a**, **b**) or the indicated concentrations (**c**, **d**) of indomethacin for the indicated periods (**a**, **b**) or 16 h (**c**, **d**). Cell viability was determined by the MTT method. Values are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

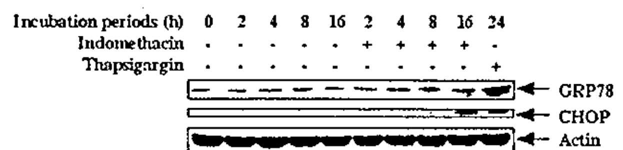

Figure 2 Immunoblotting analysis for induction of GRP78 and CHOP by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin or 2 μM thapsigargin (positive control) for the indicated periods. Whole cell extracts (20 μg protein for GRP78, 5 μg protein for actin and 30 μg protein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

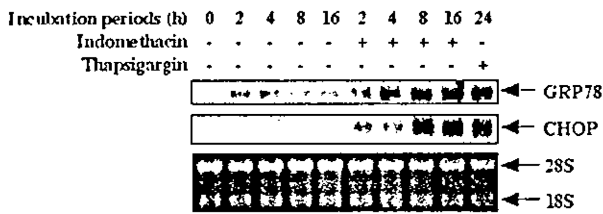

Figure 3 Northern blotting analysis for induction of *GRP78* and *CHOP* mRNA by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin or 2 μM thapsigargin (positive control) for the indicated periods. The level of the *GRP78* and *CHOP* mRNA was monitored by Northern blotting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

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

Induction of apoptosis is not specific for indomethacin, but is observed in response to NSAIDs in general. Given that we have previously found that aspirin, diclofenac, ibuprofen and celecoxib also induce apoptosis,[9] we examined the effect of some of these agents on *CHOP* mRNA induction. As well as indomethacin (1 mM), treatment of cells with diclofenac (1 mM), ibuprofen (2 mM) or celecoxib (80 μM) caused a decrease in cell viability and apoptotic DNA fragmentation, confirming that apoptosis is induced under these conditions (Figure 4a and b). These NSAIDs increased the amount of *CHOP* mRNA (Figure 4c). There are two subtypes of COX, these being COX-1 and COX-2, but COX activity in gastric mucosal cells is mainly derived from COX-1. With the exception of celecoxib, which is COX-2 specific, NSAIDs used in Figure 4 can inhibit both subtypes.[20] Therefore, if the induction of *CHOP* mRNA by indomethacin is caused by COX inhibition in gastric mucosal cells (in other words, inhibition of COX-1), higher concentrations of celecoxib would be required to reveal an effect. Given that the reverse was the case, it appears that CHOP induction by NSAIDs is not related to inhibition of COX and PG synthesis in gastric mucosal cells. Furthermore, we found that the addition of PGE_2 to the culture medium did not attenuate apoptosis and CHOP induction by indomethacin (data not shown).

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

Activation of ATF6 by indomethacin

ER stress response element (ERSE) has been identified in the promoter of ER chaperons and *CHOP* genes.[22] ER trans-

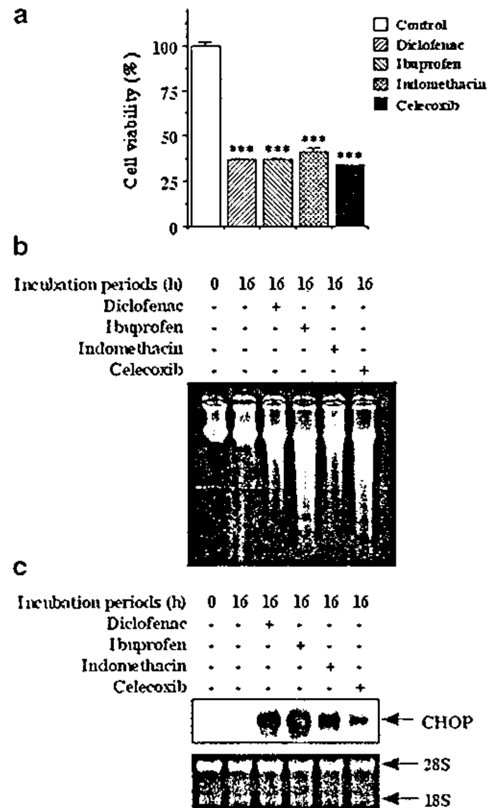

Figure 4 Induction of *CHOP* mRNA by NSAIDs other than indomethacin. Cultured gastric mucosal cells were incubated with 1 mM diclofenac, 2 mM ibuprofen, 1 mM indomethacin or 80 μM celecoxib for 16 h. Cell viability was determined by the MTT method. Values are expressed as mean $\pm$ S.E.M. ($n = 4$).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

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

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

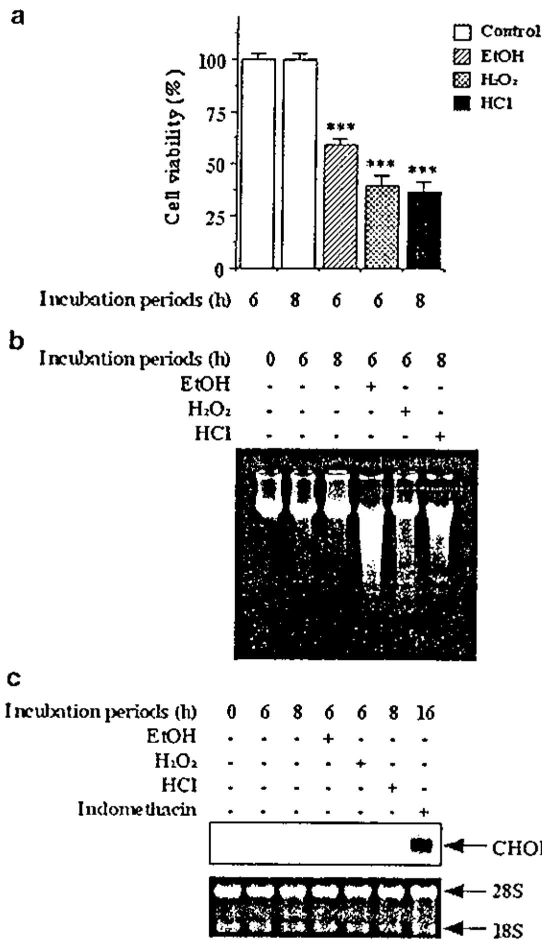

Figure 5 Induction of *CHOP* mRNA by gastric stressors other than NSAIDs. Cultured gastric mucosal cells were incubated with 4% ethanol (EtOH) for 6 h, 300 μM hydrogen peroxide (H_2O_2) for 6 h, 20 mM hydrochloric acid (HCl) for 8 h or 1 mM indomethacin for 16 h. Cell viability was determined by the MTT method. Values are mean ± S.E.M. ($n=4$). $^{***}P<0.001$ (a). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (b). The level of *CHOP* mRNA was monitored by Northern blotting analysis. Bands of ribosomal RNA (28S and 18S) stained with ethidium bromide are shown (c)

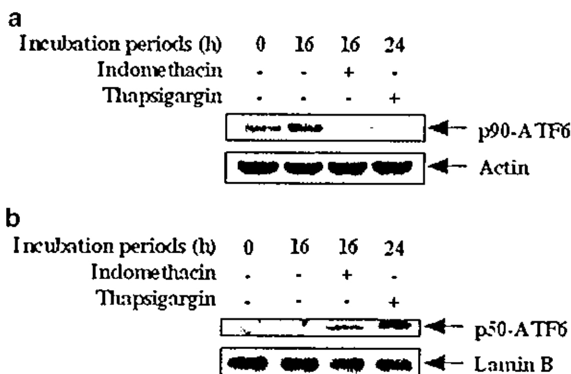

Figure 6 Activation of ATF6 by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin for 16 h or 2 μM thapsigargin for 24 h (positive control). Whole cell extracts (100 μg protein for ATF6 and 5 μg protein for actin) (a) or nuclear extracts (30 μg protein for ATF6 and 5 μg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

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

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

Induction of ATF4 and XBP-1 by indomethacin

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

Activation of XBP-1 and JNK by indomethacin

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

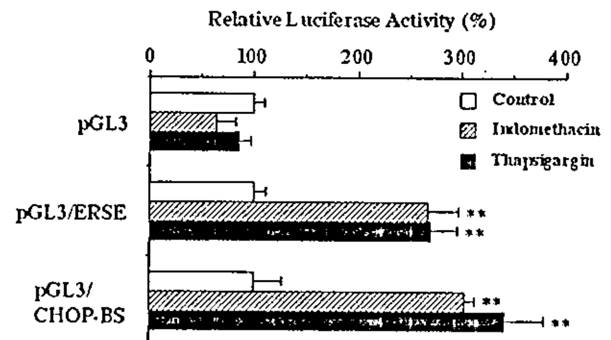

Figure 7 Activation by indomethacin of transcription from ERSE and the CHOP binding site. Cultured gastric mucosal cells were cotransfected with pRL-SV40 (internal control plasmid with the *Renilla reniformis* luciferase gene) and pGL3 (*Photinus pyralis* luciferase gene with SV40 promoter), pGL3/ERSE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

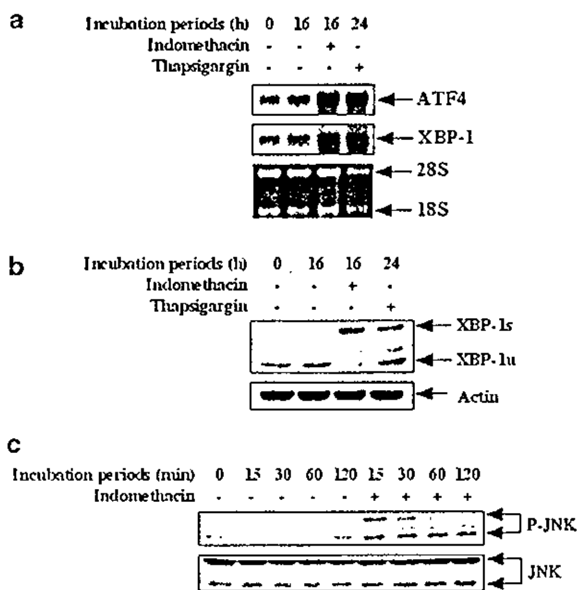

Figure 8 Induction and/or activation of ATF4, XBP-1 and JNK by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin or 2 μM thapsigargin (positive control) for indicated periods. The level of *ATF4* or *XBP-1* mRNA was monitored by Northern blotting analysis. Bands of ribosomal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

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

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

ER stressors activate ASK1 through IRE1 and activated ASK1 induces apoptosis through activation (phosphorylation) of c-Jun NH_2-terminal kinase (JNK). As shown in Figure 8c, phosphorylation of JNK was strongly induced by treatment of cells with indomethacin. Therefore, the ASK1 pathway seems to be activated by indomethacin.

Involvement of CHOP in indomethacin-induced apoptosis

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

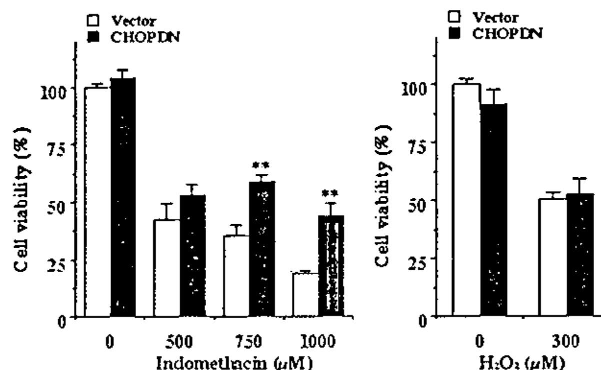

Figure 9 Prevention of indomethacin-induced apoptosis by the expression of the dominant-negative form of CHOP. Cultured gastric mucosal cells were cotransfected with 3 μg of pEGFP-C1 (EFGP expression plasmid) and 10 μg of pOPRSVI-L133/L140ACHOP (expression plasmid for dominant-negative form of CHOP, CHOPDN) or vector. After 24 h, the cells were incubated with the indicated concentrations of indomethacin for 12 h or with 300 μM hydrogen peroxide (H_2O_2) for 6 h. Cells were observed by fluorescence microscope and EGFP-positive cells were counted and expressed relative to numbers of vector-transfected cells without stress. Values are expressed as mean $\pm$ S.E.M. ($n = 3$). $^{**}P<0.01$

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

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

Discussion

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