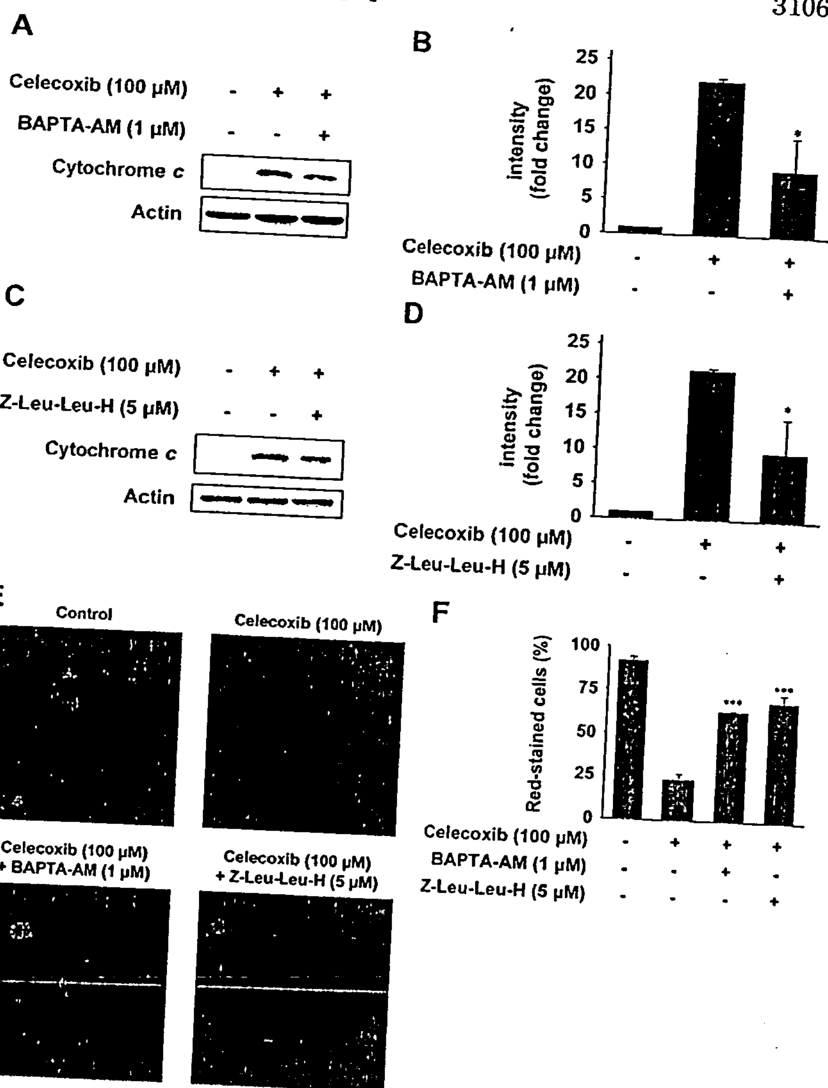

FIG. 7. **Effect of an intracellular Ca^{2+} chelator or an inhibitor of calpain on celecoxib-dependent mitochondrial dysfunction.** Cultured guinea pig gastric mucosal cells were preincubated with indicated concentrations of BAPTA-AM (*A*, *B*, *E*, and *F*) or Z-Leu-Leu-H (*C–F*) for 1 h. Cells were further incubated with or without 100 μM celecoxib for 16 h under the same conditions as the preincubation step. After subcellular fractionation, cytosolic fractions were analyzed by immunoblotting with an antibody against cytochrome *c* or actin (*A* and *C*). After normalization by the use of actin bands, the amounts of cytochrome *c* relative to control (without celecoxib and BAPTA-AM or Z-Leu-Leu-H) were determined and expressed as intensity. Values are mean ± S.E. (n = 3). *, $p < 0.05$ (*B* and *D*). Mitochondrial membrane potential was analyzed as described under "Experimental Procedures" (*E*). The numbers of *red*-stained cells with DePsipher were counted from representative photomicrographs and are expressed as a percentage of total cell number (n = 100). Values are mean ± S.E. (n = 3). ***, $p < 0.001$ (*F*).

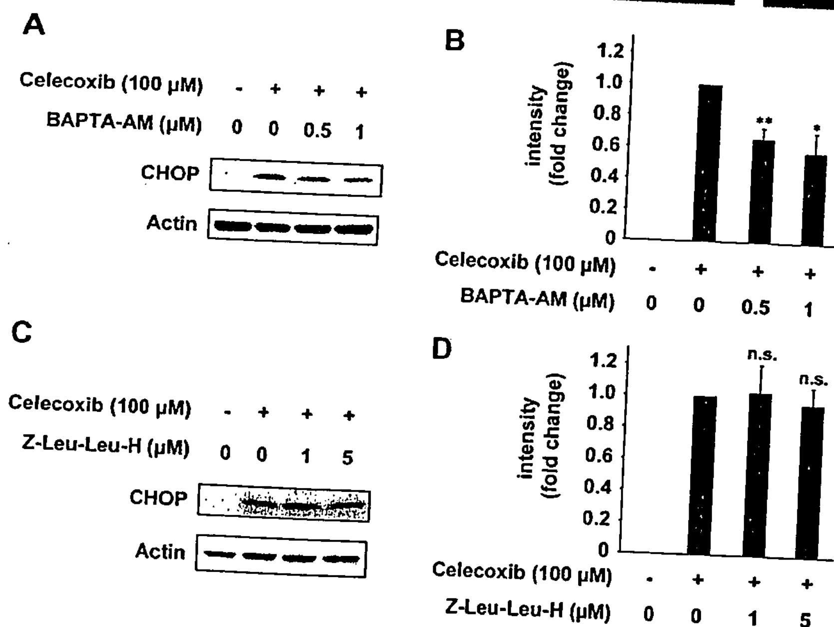

FIG. 8. **Effect of an intracellular Ca^{2+} chelator or an inhibitor of calpain on celecoxib-dependent CHOP induction.** Cultured guinea pig gastric mucosal cells were preincubated with indicated concentrations of BAPTA-AM (*A* and *B*) or Z-Leu-Leu-H (*C* and *D*) for 1 h. Cells were further incubated with or without 100 μM celecoxib under the same conditions as the preincubation step. Whole cell extracts were analyzed by immunoblotting with an antibody against CHOP or actin (*A* and *C*). The intensity of the actin bands was used to correct for differences in sample loading, and the relative amounts of CHOP to control (with celecoxib but without BAPTA-AM or Z-Leu-Leu-H) are expressed as intensity. Values are mean ± S.E. (n = 3). **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant (*B* and *D*).

contrast to BAPTA-AM, addition of Z-Leu-Leu-H did not significantly affect CHOP induction in the presence of celecoxib (Fig. 8, *C* and *D*), suggesting that induction of CHOP by celecoxib is independent of calpain.

DISCUSSION

In this study, we aimed to identify the pathway for celecoxib-induced apoptosis using primary cultures of gastric mucosal cells. First, using 10 different NSAIDs, we demonstrated the

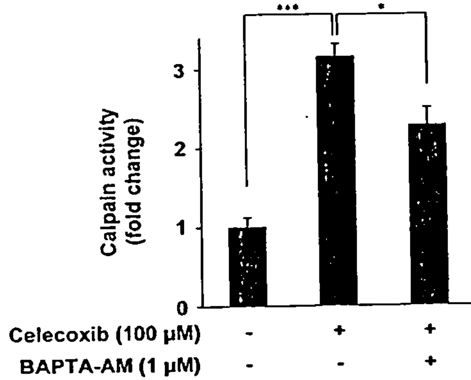

FIG. 9. **Activation of calpain by celecoxib.** Cultured guinea pig gastric mucosal cells were preincubated with or without 1 μM BAPTA-AM for 1 h. Cells were further incubated with or without 100 μM celecoxib for 16 h under the same conditions as the preincubation step. Calpain activity was measured using fluorogenic peptide substrates (succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methylcoumaryl-7-amide). Values are mean ± S.E. ($n = 3$). ***, $p < 0.001$; *, $p < 0.05$.

close relationship between their apoptosis-inducing and membrane permeabilization activities. Therefore, it seems that membrane phospholipids are the primary target of NSAIDs for the induction of apoptosis. To investigate the mechanism by which the membrane permeabilization activity of NSAIDs causes apoptosis, we focused on the intracellular Ca^{2+} level, because permeabilization of cytoplasmic membranes increases the intracellular Ca^{2+} level by stimulating Ca^{2+} influx, and the intracellular Ca^{2+} level is a key regulator of apoptosis. We have concluded that intracellular Ca^{2+} levels are involved in celecoxib-induced apoptosis, since each of the NSAIDs that we tested increased the intracellular Ca^{2+} level, this Ca^{2+}-increasing activity of NSAIDs (except etodolac) correlated with their membrane permeabilization activity, and an intracellular Ca^{2+} chelator (BAPTA-AM) partially inhibited celecoxib-dependent apoptosis and mitochondrial dysfunction. To elucidate the mechanism by which the increase in the intracellular Ca^{2+} level causes apoptosis, we focused on calpain, because Ca^{2+} activates this enzyme, and activation of this enzyme can induce apoptosis. We have demonstrated the involvement of calpain in celecoxib-induced apoptosis by showing that an inhibitor of calpain (Z-Leu-Leu-H) partially inhibited celecoxib-dependent apoptosis and mitochondrial dysfunction. Cleavage by calpain activates and inhibits Bax and Bid and Bcl-2 and Bcl-X_L, respectively. Since Bax and Bid cause mitochondrial membrane permeabilization (stimulate release of cytochrome *c* from the mitochondria and decrease the mitochondrial membrane potential) (25, 30–32), whereas Bcl-2 and Bcl-X_L protect mitochondrial membrane from permeabilization, activation of calpain by increasing the intracellular Ca^{2+} level seems to play an important role in celecoxib-induced mitochondrial dysfunction. Calpain cleaves and thereby activates procaspase-12 and apoptosis-inducing factor and cleaves and thereby inhibits the X-linked inhibitor of apoptosis (26, 27, 41). These events may be involved in celecoxib-induced apoptosis.

Suppression of celecoxib-induced apoptosis by BAPTA-AM was partial, suggesting that a Ca^{2+}-independent pathway is also involved in this process. One candidate is the death receptor signaling pathway. It was recently reported that celecoxib induces expression of death receptors, especially DR5, and small interfering RNA for DR5 partially suppresses celecoxib-induced apoptosis in lung cancer cells (42). Induction of death receptors causes activation of caspase-8, and it has been shown that celecoxib activates caspase-8 and that an inhibitor of caspase-8 partially suppresses celecoxib-dependent apoptosis (42). We previously reported that various NSAIDs activate caspase-8 in cultured gastric mucosal cells (11). This DR5-dependent pathway may be involved in celecoxib-induced apoptosis in cultured gastric mucosal cells. As described in the Introduction, inactivation of phosphatidylinositol 3-kinase/3-phosphoinositide-dependent kinase-1/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase signal transduction systems may also be involved in NSAID-induced apoptosis (20–22). However, at present, it is not clear whether these pathways are Ca^{2+}-dependent or Ca^{2+}-independent, because upstream signals for these systems have not yet been revealed.

Suppression of celecoxib-induced apoptosis by Z-Leu-Leu-H was also partial, and this implies that a calpain-independent pathway is involved in this process. Of course, logically, the Ca^{2+}-independent pathways discussed above may be responsible for the calpain-independent pathway. However, we have considered the contribution of Ca^{2+}-dependent but calpain-independent pathways to celecoxib-induced apoptosis. One candidate for such a pathway is the ER stress response, in particular the induction of CHOP. We recently reported that NSAID-induced apoptosis is suppressed in cultured guinea pig gastric mucosal cells by expression of the dominant negative form of CHOP and in peritoneal macrophages from CHOP-deficient mice (19). In the current study, we have shown that celecoxib-dependent CHOP induction is suppressed by BAPTA-AM but not by Z-Leu-Leu-H, suggesting that in the celecoxib-induced apoptosis pathway, CHOP induction is located downstream of the increase in the intracellular Ca^{2+} level but not of calpain activation. Furthermore, various Ca^{2+}-dependent but calpain-independent signal pathways other than CHOP induction may be involved in celecoxib-induced apoptosis. For example, Ca^{2+}-dependent activation of protein kinase Cδ, a protein kinase C isoform, induces release of cytochrome *c* from mitochondria (43). Calcineurin, a Ca^{2+}-dependent serine-threonine phosphatase, dephosphorylates BAD (a proapoptotic member of the Bcl-2 family) and stimulates apoptosis (44). Opening of permeability transition pores in mitochondria and stimulation of reactive oxygen species synthesis are also possible Ca^{2+}-dependent but calpain-independent pathways for celecoxib-induced apoptosis (26–29).

We recently proposed that both COX inhibition at gastric mucosa and direct gastric mucosal cell damage (such as induction of apoptosis) by NSAIDs are required for the production of gastric lesions *in vivo*. Gastric lesions develop in a manner that depends on both an intravenously administered low dose of indomethacin (inhibition of COX activity at the gastric mucosa without direct gastric mucosal cell damage) and an orally administered COX-2-selective NSAID, such as celecoxib (causes direct gastric mucosal cell damage without inhibition of COX at gastric mucosa) (12). Therefore, based on the results in this study, it may be suggested that NSAIDs without membrane-permeabilizing activity and intracellular Ca^{2+} level-increasing activity may have reduced gastrointestinal side effects compared with other NSAIDs. An issue that was recently raised concerning the use of COX-2-selective NSAIDs, a class of gastrointestinally safe NSAIDs, is their potential risk for promoting cardiovascular thrombotic events (45). Prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 in vascular endothelial cells, whereas thromboxane A_2, a potent aggregator of platelets and a vasoconstrictor, is mainly produced by COX-1 in platelets (46, 47). Until recently, rofecoxib and celecoxib were leading COX-2-selective NSAIDs in the market. Rofecoxib was withdrawn from the market due to the risk of its promoting cardiovascular thrombotic events, and the United States Food and Drug Administration advised physicians to consider alternatives to

celecoxib due to the risk of its causing cardiovascular thrombotic events (48, 49). Based on our findings, NSAIDs that do not exhibit membrane permeabilization activity may be safe for the gastrointestinal tract even if they are not highly selective for COX-2. This type of NSAID may be of clinical benefit, because they are predicted to be safe for both the gastrointestinal tract and cardiovascular system.

On the other hand, much attention has also been given to the direct cytotoxicity of NSAIDs in relation to cancer therapy. Epidemiological studies have shown that prolonged use of aspirin or other NSAIDs reduces the risk of cancer (50, 51). Furthermore, several NSAIDs are presently under clinical development as anti-cancer drugs. A number of *in vivo* and *in vitro* studies have revealed that NSAIDs cause apoptosis (or stimulate apoptosis by anti-cancer reagents) in cancer cells and that these activities of NSAIDs are involved in their anticancer activity (52). PGs, such as PGE_2, inhibit apoptosis (53). Furthermore, overexpression of COX-2 has been reported in various tumor cells and tissues (54, 55). Therefore, the inhibition of COX by NSAIDs was previously thought to be solely responsible for their chemopreventive effect. However, several lines of evidence suggest that chemoprevention by NSAIDs also involves COX-independent mechanisms. Sulindac sulfone, a derivative of the NSAID sulindac, does not inhibit COX activity and has been shown to display anti-tumor activity *in vivo* as well as induce apoptosis and inhibit cell growth in tumor cells *in vitro* (56, 57). Moreover, NSAID induction of apoptosis and inhibition of cell growth in COX-null fibroblasts and tumor cells in which COX expression was absent have been reported (58, 59). Therefore, an understanding of COX-independent mechanisms for the induction of apoptosis by NSAIDs is very important in order to develop more effective NSAIDs for cancer therapy. If the results presented here can be applied to the case of cancer cells, NSAIDs with more potent membrane-permeabilizing and Ca^{2+}-increasing activity may have more potent anticancer effects than other NSAIDs.

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Geranylgeranylacetone Protects Membranes against Nonsteroidal Anti-Inflammatory Drugs

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ABSTRACT

Direct gastric mucosal cell damage mediated by nonsteroidal anti-inflammatory drugs (NSAIDs) is involved in the formation of NSAID-induced gastric lesions. We recently suggested that this direct cytotoxicity of NSAIDs is caused by their membrane-permeabilization activity. Geranylgeranylacetone (GGA), a clinically used antiulcer drug, can protect gastric mucosa against lesion formation mediated by NSAIDs. However, the mechanism by which this occurs is not fully understood. In this study, we show that GGA acts to stabilize membranes against NSAIDs. GGA suppressed NSAID-induced permeabilization of calcein-loaded liposomes and NSAID-induced stimulation of K^+-efflux across the cytoplasmic membrane in cells. GGA was effective even when coadministered with NSAIDs and was also able to restore membrane fluidity that had been compromised by NSAIDs. This mechanism seems to play an important role in the antiulcer activity of GGA.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are of significant clinical value, accounting for nearly 5% of all prescribed medications (Smalley et al., 1995). Nonetheless, NSAID use is often associated with gastrointestinal complications (Hawkey, 2000), with approximately 15 to 30% of long-term users experiencing gastrointestinal ulcers and bleeding (Barrier and Hirschowitz, 1989; Fries et al., 1989; Kurata and Abbey, 1990; Gabriel et al., 1991). In the United States alone, approximately 16,500 people per year die as a result of these complications (Singh, 1998). Therefore, in general, antiulcer drugs are prescribed in combination with NSAIDs to prevent the NSAID-induced side effects.

Geranylgeranylacetone (GGA) was developed in Japan and has become the leading antiulcer drug on the Japanese market (Murakami et al., 1981). In both preclinical and clinical studies, it has been shown to protect the gastric mucosa against the development of lesions induced by various irritants, including NSAIDs, without affecting gastric acid secretion (Murakami et al., 1981; Terano et al., 1986; Pappas et al., 1987). Various mechanisms have been proposed for this protective effect of GGA. First, it stimulates the synthesis of mucus (Terano et al., 1986; Bilski et al., 1987; Rokutan et al., 2000) and increases mucosal blood flow, an important factor in maintaining the integrity of the mucosa (Kunisaki and Sugiyama, 1992). It has also been reported recently that GGA induces heat shock proteins (HSPs), a novel activity of GGA that has been shown to be involved in its ability to protect the gastric mucosa against NSAIDs (Hirakawa et al., 1996; Mizushima et al., 1999; Tomisato et al., 2001b; Takano et al., 2002). However, the rapid anti-ulcer activity of GGA against NSAIDs observed in clinical situations cannot be fully explained by these indirect actions of GGA, given that this ameliorating effect is observed even when GGA is coadministered with NSAIDs. Therefore, GGA is also believed to have unknown direct actions.

The anti-inflammatory action of NSAIDs is mediated through their inhibitory effect on cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. The inhibition of COX was believed to be the sole explanation for the gastric complications of NSAIDs, given that PGs exert a strong protective effect on gastric mucosa (Miller, 1983; Vane and Botting, 1996). However, it is now believed that the induction of gastric lesions by NSAIDs involves additional mechanisms, because the increased inci-

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; GGA, geranylgeranylacetone; HSP, heat shock protein; COX, cyclooxygenase; PG, prostaglandin; PC, phosphatidylcholine.

dence of gastric lesions and the decrease in PG levels induced by NSAIDs do not always occur in parallel (Ligumsky et al., 1983, 1990). We have demonstrated previously that NSAIDs induce in vitro cell death (apoptosis and necrosis) independent of COX inhibition and have suggested that both COX inhibition and NSAID-induced cell death are required to produce gastric lesions in vivo (Tomisato et al., 2001a, 2004b). Furthermore, we have shown recently that all of the NSAIDs tested have membrane-permeabilization activity, which seems to be responsible for the NSAID-induced apoptosis and necrosis (Tomisato et al., 2004a). In this study, we have found that GGA protects membranes from permeabilization by NSAIDs. This is the first report showing that a clinically used antiulcer drug has membrane-stabilization activity in the presence of NSAIDs. We have also demonstrated that GGA restores the membrane fluidity that is compromised by NSAIDs.

Materials and Methods

Chemicals, Media, and Animals. Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA). RPMI 1640 medium was obtained from Nissui (Tokyo, Japan). Indomethacin was purchased from Wako Pure Chemicals (Tokyo, Japan), whereas ibuprofen, diclofenac, mefenamic acid, flufenamic acid, and ketoprofen came from Sigma-Aldrich (Tokyo, Japan). Nimesulide and flurbiprofen were obtained from Cayman Chemical (Ann Arbor, MI), and egg phosphatidylcholine (PC) was from Kanto Chemicals Co. (Tokyo, Japan). GGA was kindly provided by Eisai Co. (Tokyo, Japan). Celecoxib was purchased from LKT Laboratories, Inc. (St. Paul, MN). Etodolac was a gift, kindly provided by Nippon Shinyaku Co. (Kyoto, Japan).

Treatment of Cells with NSAIDs. Human gastric carcinoma (AGS) cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. Cells were exposed to NSAIDs by replacement of the entire bathing medium with fresh medium containing the NSAID under investigation. NSAIDs were dissolved in dimethyl sulfoxide; control experiments (without NSAIDs) were performed in the presence of the same concentration of dimethyl sulfoxide.

Membrane Permeability Assay. Permeabilization of calcein-loaded liposomes was assayed as described previously (Tomisato et al., 2004a), with some modifications. Liposomes were prepared using the reversed-phase evaporation method. Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2; v/v), dried, dissolved in 1.5 ml of diethyl ether, and added to 1 ml of 100 mM calcein-NaOH, pH 7.4. The mixture was then sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed, and the resulting suspension of liposomes was centrifuged and washed twice with fresh buffer A [10 mM phosphate buffer (Na_2HPO_4-NaH_2PO_4, pH 6.8) containing 150 mM NaCl] to remove untrapped calcein. The final liposome precipitate was resuspended in 5 ml of buffer A. A 30-μl aliquot of this suspension was diluted with buffer A up to 20 ml, and 400 μl of this diluted suspension was then incubated at 30°C for 10 min in the presence of the NSAID under investigation. T release of calcein from liposomes was determined by measuri fluorescence intensity at 520 nm (excitation at 490 nm).

Assay for K^+ Efflux from Cells. K^+ efflux from cells was mo itored as described previously (Katsu et al., 1987), with some moc fications. Cells were washed twice with buffer A and then suspende in fresh buffer A (2.4 × 10^6 cells/ml). After incubation with NSAIL for 10 min at 37°C, K^+ efflux from the cells was measured with a K ion-selective electrode.

Fluorescence Polarization. Membrane fluidity was measure using the fluorescence polarization technique (Makise et al., 2002 Diphenylhexatriene (1%, mol/mol, of egg PC) was used as a fluores cence probe. Liposomes were prepared using a reversed-phase evap oration method similar to that used in the membrane-permeabilit experiments, except for the addition of 1 ml of buffer A instead of 10(mM calcein-NaOH. Measurements were carried out using a Hitach F-4500 fluorospectrophotometer (Hitachi Software Engineering Yokohama, Japan). 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.

Statistical Analyses. All results were expressed as the mean ± S.E.M. One-way analysis of variance followed by Scheffe's multiple comparison was used for the evaluation of differences between the groups. A Student's t test for unpaired results was performed to evaluate differences between two groups. Differences were considered significant for values of $P < 0.05$.

Results

GGA Suppresses NSAID-Induced Membrane Permeabilization. We have reported recently that some NSAIDs (celecoxib, mefenamic acid, flufenamic acid, nimesulide, and flurbiprofen) cause membrane permeabilization in calcein-loaded liposomes (Tomisato et al., 2004a). In this study, we first confirmed the membrane-permeabilization activity of a number of NSAIDs using the same assay. Calcein fluoresces very weakly at high concentrations caused by self-quenching, so the addition of membrane-permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by diluting the calcein (Tomisato et al., 2004a). As shown in Fig. 1, each of the NSAIDs tested increased the calcein fluorescence in a dose-dependent manner, indicating that they have membrane-permeabilization activity. Results for some NSAIDs were consistent with our previous reports (Tomisato et al., 2004a). Indomethacin, diclofenac, and celecoxib were selected for further study because their membrane-permeabilization activity was higher than that of the other NSAIDs.

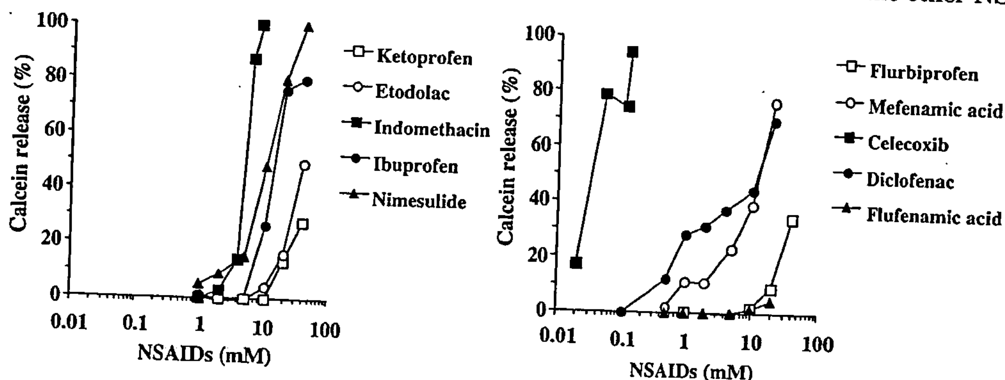

Fig. 1. Membrane permeabilization by NSAIDs. Calcein-loaded liposomes were incubated for 10 min with varying concentrations of each NSAID. The release of calcein from the liposomes was then determined by measuring fluorescence intensity. Melittin (10 μM) was used to establish the 100% level of membrane permeabilization (Katsu et al., 1987).

The effect of GGA on indomethacin-induced membrane permeabilization is illustrated in Fig. 2A. GGA decreased the calcein fluorescence in a dose-dependent manner in the presence of 6 or 8 mM indomethacin. Treatment with GGA had no effect on fluorescence when calcein-loaded liposomes were studied in the absence of indomethacin (data not shown). Furthermore, GGA did not directly affect calcein fluorescence (data not shown). These results suggested that GGA was protecting the liposome membranes from permeabilization by indomethacin. As shown in Fig. 2, B and C, GGA also protected liposome membranes against diclofenac and celecoxib, although relatively higher concentrations of GGA (greater than 10^{-5} M) were required in the case of celecoxib.

In vivo, gastric mucosa can be exposed not only to NSAIDs but also to various other lesion-inducing irritants (such as ethanol, gastric acid, and reactive oxygen species) against which GGA provides protection. We therefore examined the membrane-permeabilization activity of these irritants using the same assay. Ethanol, but not hydrochloric acid or hydrogen peroxide, showed membrane-permeabilization activity under our assay conditions (data not shown). As shown in Fig. 2D, GGA protected liposome membranes from permeabilization by 10 or 20% ethanol, suggesting that the effect of GGA is nonspecific.

GGA Protects Membranes against NSAIDs even when Coadministered. As for the experiments described above, calcein-loaded liposomes were preincubated with GGA and were subsequently treated with various NSAIDs in the presence of the same concentration of GGA, as in the preincubation step. As shown in Fig. 3A, GGA suppressed the indomethacin-induced membrane permeabilization under these conditions ("pretreated" in Fig. 3A). However, a similar result was obtained even when GGA was added simultaneously with indomethacin ("cotreated" in Fig. 3A). Treatment with GGA and either diclofenac or celecoxib (Fig. 3, B and C) also produced a similar outcome. These results showed that GGA very rapidly protects liposome membranes against NSAIDs. In contrast, GGA did not significantly affect the calcein fluorescence when it was added after NSAID-treatment ("post-treated" in Fig. 3), again supporting the notion that the activity of GGA in this paradigm cannot be explained by its direct effect on calcein fluorescence.

GGA Protects Cell Membranes from NSAID-Mediated K^+ Efflux. We next examined whether GGA can protect cell membranes against NSAIDs. Permeabilization of cytoplasmic membranes should stimulate K^+ efflux from cells. Here we examined the effect of various NSAIDs on K^+ efflux from AGS cells. The K^+ concentration in the medium increased depending on the dose of not only celecoxib but also indomethacin or diclofenac (Fig. 4), showing that each of these NSAIDs stimulated K^+ efflux from the cells or, in other words, permeabilized the cytoplasmic membranes. As shown in Fig. 5, the increase in K^+ concentration in the medium was not as great in the presence of GGA. GGA alone had no effect (data not shown). These findings suggest that GGA protects the cytoplasmic membrane from permeabilization by NSAIDs.

GGA Increases Membrane Fluidity. We have recently reported that some NSAIDs (celecoxib, mefenamic acid, flufenamic acid, nimesulide, and flurbiprofen) decrease membrane fluidity (Tomisato et al., 2004a). Here, we examined the effect of GGA on membrane fluidity in the presence or

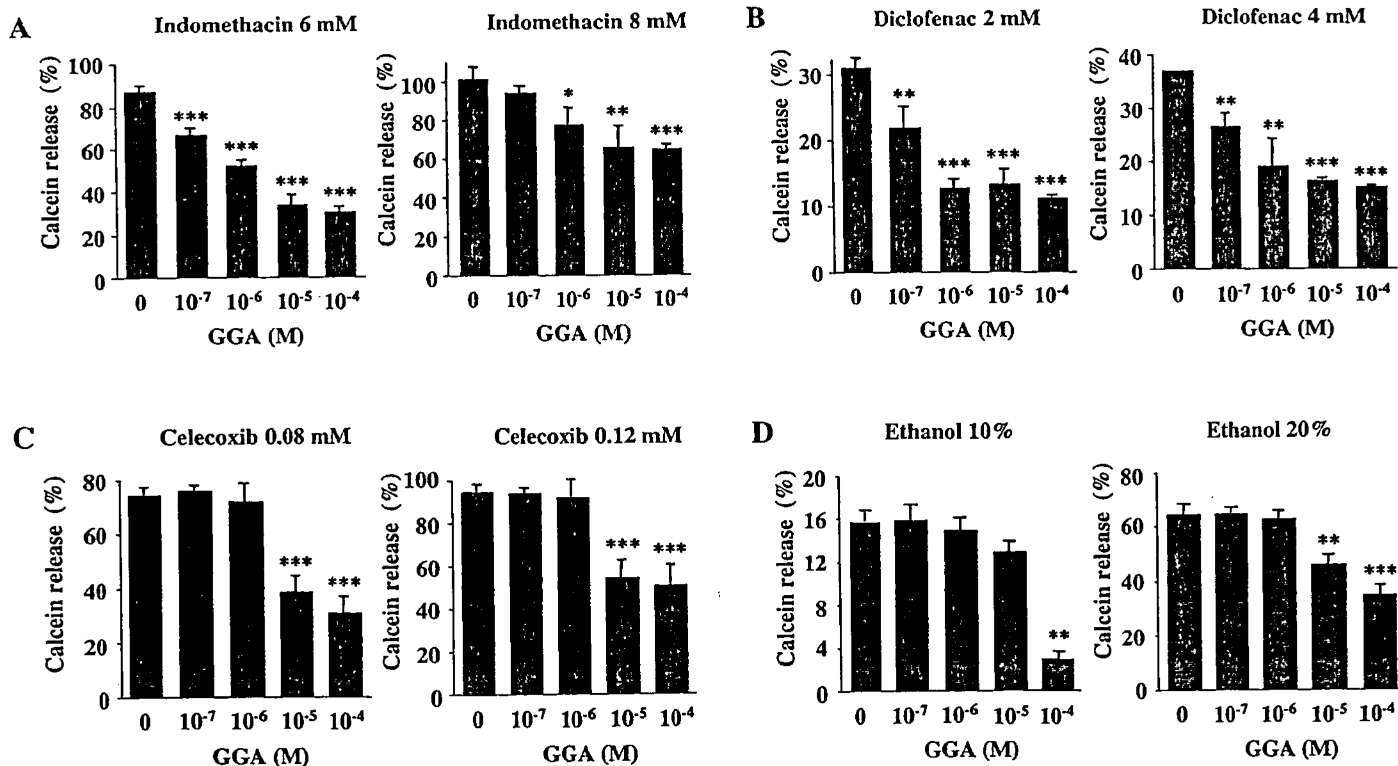

Fig. 2. Effect of GGA on membrane permeabilization. Calcein-loaded liposomes were preincubated with varying concentrations of GGA for 10 min and then treated with NSAID (A–C) or ethanol (D) in the presence of the same concentration of GGA. The release of calcein from liposomes was determined and expressed as described in the legend of Fig. 1. Values shown are mean ± S.E.M. (n = 3). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

absence of NSAIDs using the fluorescence polarization technique. In such experiments, the higher the calculated *P* value, the lower the membrane fluidity. We first examined the effect of various NSAIDs on the membrane fluidity of PC liposomes. As described previously (Tomisato et al., 2004a), celecoxib increased the *P* value (i.e., decreased the membrane fluidity) (Table 1). Indomethacin and diclofenac had a similar effect, although the extent of the decrease differed between NSAIDs (Table 1). In contrast, GGA decreased the *P* value in a dose-dependent manner, reflecting an increase in membrane fluidity (Table 1).

We next examined the effect of GGA on membrane fluidity in the presence of NSAIDs. Membrane fluidity in the presence of various concentrations of celecoxib was restored by GGA in a dose-dependent manner (Table 1). GGA (10^{-4} M) also partially restored membrane fluidity in the presence of 2 mM indomethacin or diclofenac but had no effect in the presence of 1 mM indomethacin or 5 or 10 mM diclofenac.

Discussion

In this study, we have shown that GGA suppresses NSAID-induced K^+ efflux from cells, suggesting that GGA protects the cytoplasmic membranes from permeabilization. Because a similar effect was observed in calcein-loaded liposomes, which consist only of phospholipids (without membrane proteins), the membrane stabilization activity of GGA seems to be mediated by its direct interaction with phospholipids, a conclusion supported by the observation that GGA increases the membrane fluidity of PC liposomes. This is the first report that a clinically used antiulcer drug protects membranes from permeabilization by NSAIDs and other gastric irritants (ethanol). GGA is clinically used at 150 mg/day. The maximum serum concentration under this administration is approximately 5 μM (5×10^{-6} M) in patients (data from interview form from the manufacturer). The maximum concentration of GGA at gastric mucosa should be higher,

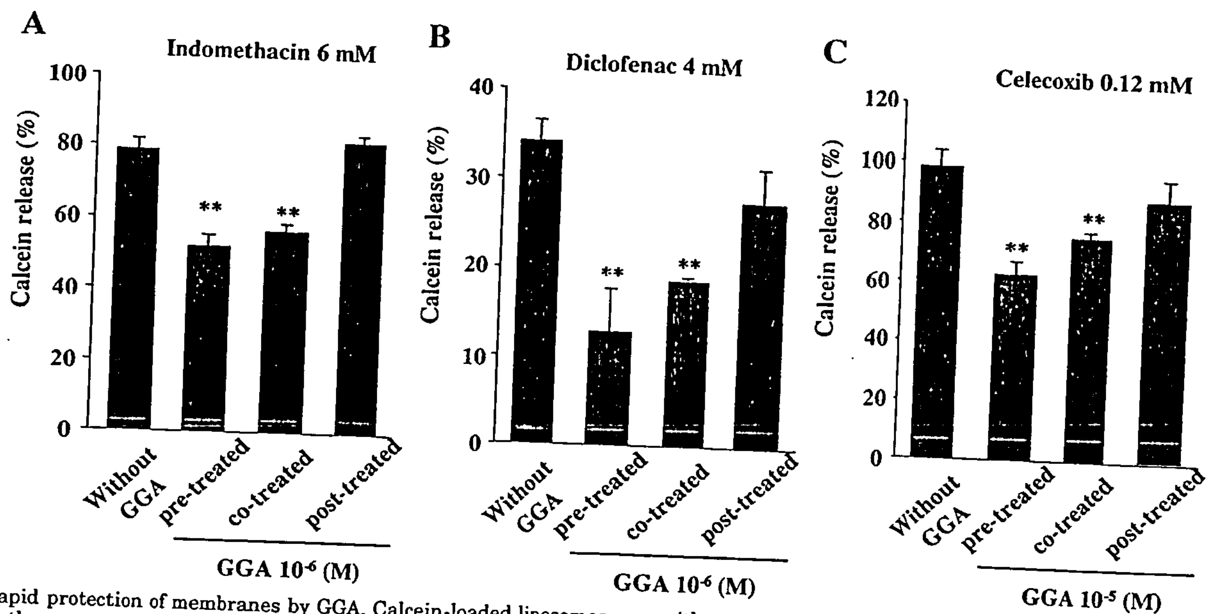

Fig. 3. Rapid protection of membranes by GGA. Calcein-loaded liposomes were either preincubated with GGA for 10 min and then incubated with NSAID in the presence of GGA (pretreated), simultaneously incubated with GGA and NSAID (cotreated), or preincubated with NSAID for 10 min and then treated with GGA in the presence of NSAID (post-treated). The release of calcein from liposomes was determined and expressed as described in the legend to Fig. 1. Values shown are mean ± S.E.M. ($n = 3$). **, $P < 0.01$.

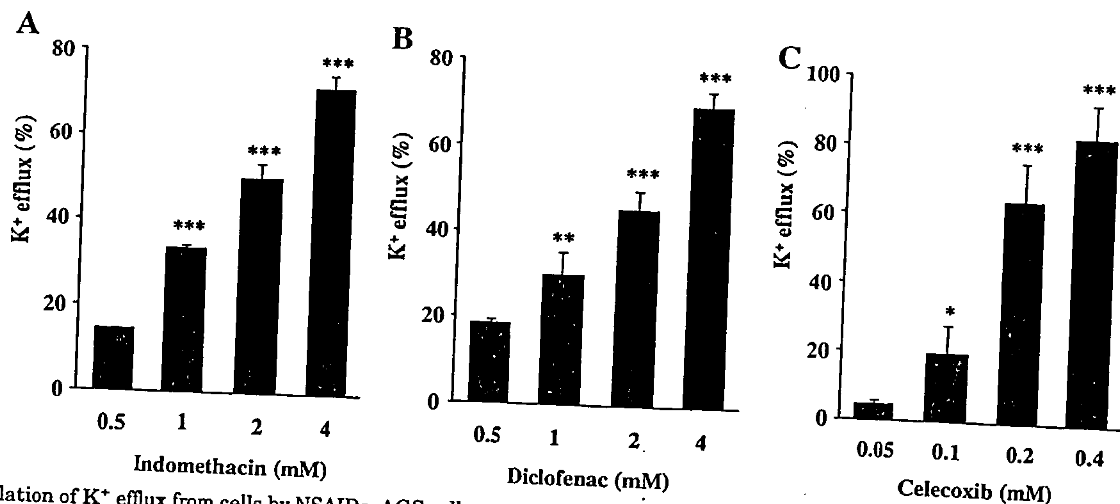

Fig. 4. Stimulation of K^+ efflux from cells by NSAIDs. AGS cells were incubated with varying concentrations of each NSAID for 10 min, and the level of K^+-efflux was measured using a K^+ ion-selective electrode. Melittin (10 μM) was used to establish the 100% level of K^+ efflux (Katsu et al., 1987). Values shown are mean ± S.E.M. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

suggesting that concentrations of GGA used in this study are clinically significant.

From our previous studies (see below), we consider that this novel activity of GGA is involved in its antiulcer activity against NSAIDs. We recently proposed that both COX inhibition at the gastric mucosa and direct gastric mucosal cell damage (necrosis and apoptosis in gastric mucosal cells) are required for the production of gastric lesions by NSAIDs in vivo; in this experimental paradigm, gastric lesions developed in a manner that depended on both an intravenously administered low dose of indomethacin (inhibition of COX activity at the gastric mucosa without direct gastric mucosal cell damage) and an orally administered cytotoxic COX-2–selective NSAID, such as celecoxib (direct gastric mucosal cell damage without inhibition of COX) (Tomisato et al., 2004b). We subsequently suggested that the direct gastric mucosal cell damage is caused by the membrane permeabilization activity of NSAIDs; the ED_{50} values of the 10 NSAIDs for gastric mucosal cell death (concentrations of NSAID required for 50% inhibition of cell viability by necrosis or apoptosis) correlated well with the ED_{20} values for membrane permeabilization (concentration of NSAID required for 20% release of calcein); plotting ED_{50} values for necrosis or apoptosis versus ED_{20} values for membrane permeabilization yielded an r^2 value of 0.94 or 0.93, respectively (Tomisato et al., 2004a; Tanaka et al., 2005). We therefore consider that the membrane stabilization activity of GGA causes suppression of NSAID-induced direct gastric mucosal cell damage, conferring protection against the development of ulcers. This raises the possibility that the membrane stabilization assay can be used as a rapid screening technique for potential new antiulcer drugs.

As outlined in the Introduction, GGA has a number of pharmacological activities that are believed to be involved in its antiulcer activity. These include stimulating the synthesis of gastric mucus, increasing gastric mucosal blood flow, and inducing HSPs in gastric mucosal cells (Terano et al., 1986; Bilski et al., 1987; Kunisaki and Sugiyama, 1992; Hirakawa et al., 1996; Mizushima et al., 1999; Tomisato et al., 2000; Takano et al., 2002). However, these activities cannot be

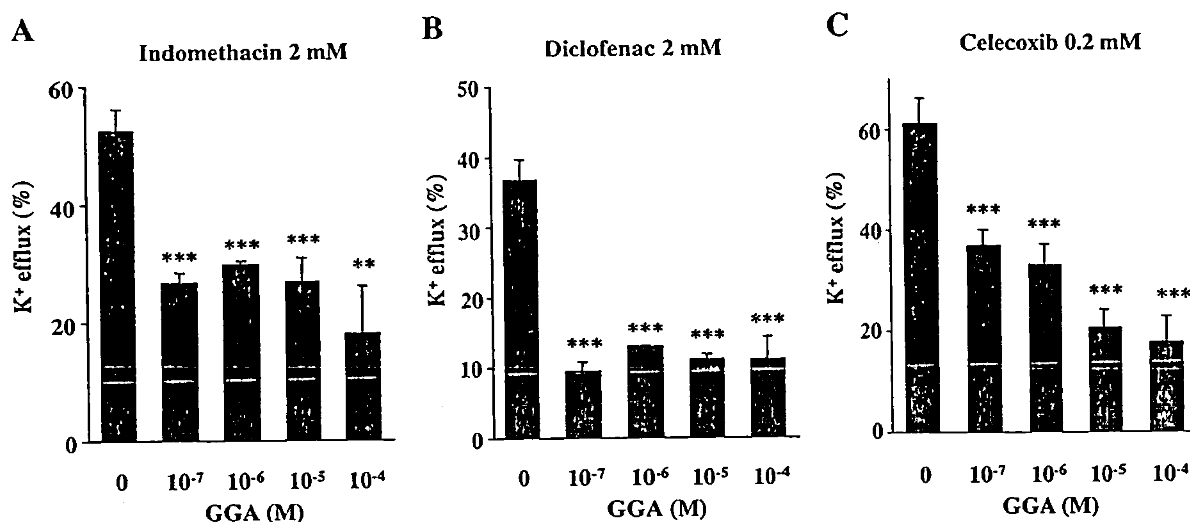

Fig. 5. Effect of GGA on K^+ efflux from cells in the presence of NSAIDs. AGS cells were preincubated with varying concentrations of GGA and then treated with NSAID in the presence of the same concentrations of GGA. The level of K^+ efflux was measured and expressed as described in the legend to Fig. 4. Values shown are mean ± S.E.M. (n = 3). **, $P < 0.01$; ***, $P < 0.001$.

TABLE 1

Effect of GGA on membrane fluidity in the presence or absence of various NSAIDs

The degree of polarization of PC liposomes in the presence of GGA and/or various NSAIDs was measured as described under *Materials and Methods*. The final lipid concentration was adjusted to 30 μM. Fluorescence polarization was measured by excitation at 360 nm and emission at 430 nm using a Hitachi F-4500 fluorospectrophotometer equipped with polarizers and thermoregulated cells. Values shown are mean ± S.E.M. (n = 3).

NSAIDs	Degree of Polarization		
	PC	+ GGA 10^{-5}	+ GGA 10^{-4}
		M	
Control	0.121 ± 0.007	0.097 ± 0.005**	0.071 ± 0.003***
Indomethacin			
1 mM	0.172 ± 0.033	0.165 ± 0.037	0.170 ± 0.020
2 mM	0.217 ± 0.017	0.177 ± 0.021	0.165 ± 0.024*
Diclofenac			
2 mM	0.149 ± 0.012	0.153 ± 0.008	0.114 ± 0.007*
5 mM	0.151 ± 0.012	0.152 ± 0.010	0.154 ± 0.010
10 mM	0.159 ± 0.015	0.167 ± 0.010	0.160 ± 0.014
Celecoxib			
0.1 mM	0.133 ± 0.006	0.113 ± 0.004**	0.080 ± 0.007***
0.5 mM	0.210 ± 0.006	0.175 ± 0.005***	0.148 ± 0.010***
1 mM	0.210 ± 0.009	0.187 ± 0.005*	0.159 ± 0.006***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

measured experimentally without an initial incubation period (for example, induction of HSPs by GGA requires at least 1 h incubation both in vitro and in vivo) (Hirakawa et al., 1996). In contrast, in clinical situations, GGA can suppress gastric lesions even when administered simultaneously with NSAIDs, suggesting a more direct protective mechanism, such as the membrane stabilization proposed here. Nonetheless, longer-term indirect actions of GGA may also play a role in its antiulcer activity and that the different time courses of these effects could confer a clinical advantage.

In the present study, we also demonstrated that GGA restores membrane fluidity that has been compromised by NSAIDs. At present, it is not certain that this activity of GGA underpins its membrane-stabilizing ability. Nor is the relationship between decreased membrane fluidity and NSAID-induced membrane permeabilization clear, given that we have shown previously that most but not all of the NSAIDs tested (mefenamic acid, flufenamic acid, celecoxib, and nimesulide, but not flurbiprofen) decrease membrane fluidity, and that cholesterol, which ameliorates the NSAID-induced decrease in membrane fluidity, renders liposomes resistant to some but not all NSAIDs (Tomisato et al., 2004a). Restoration of membrane fluidity by GGA also differed between NSAIDs (Table 1), suggesting that this effect cannot fully explain the membrane stabilization activity of GGA. However, if holes develop in membranes, such holes become more stable (in other words, the membrane becomes more permeable) when membrane fluidity decreases. It is also possible that a GGA-mediated increase in membrane fluidity is involved in the maintenance of surface hydrophobicity at the gastric mucosa, which is believed to be important for maintaining mucosal integrity. Lichtenberger and his coworkers have proposed that NSAIDs disrupt the hydrophobic barrier properties of the gastric mucosal surface, rendering it susceptible to attack by luminal acid. They showed that NSAIDs cause a marked decrease in surface hydrophobicity and observed a close relationship between a decrease in gastric surface hydrophobicity and gastric lesion score in rats (Darling et al., 2004; Lichtenberger et al., 1995). They also suggested that an NSAID-induced decrease in membrane fluidity is related to the decrease in surface hydrophobicity mediated by NSAIDs (Giraud et al., 1999). However, further studies are necessary to elucidate the effect of GGA on membrane fluidity and its influences on antiulcer activity.

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ORIGINAL ARTICLE

Celecoxib upregulates endoplasmic reticulum chaperones that inhibit celecoxib-induced apoptosis in human gastric cells

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Nonsteroidal anti-inflammatory drugs (NSAIDs) induce apoptosis in cancer cells and this effect is involved in their antitumor activity. We recently demonstrated that NSAIDs upregulate GRP78, an endoplasmic reticulum (ER) chaperone, in gastric mucosal cells in primary culture. In the present study, induction of ER chaperones by NSAIDs and the effect of those chaperones on NSAID-induced apoptosis were examined in human gastric carcinoma cells. Celecoxib, an NSAID, upregulated ER chaperones (GRP78 and its cochaperones ERdj3 and ERdj4) but also C/EBP homologous transcription factor (CHOP), a transcription factor involved in apoptosis. Celecoxib also upregulated GRP78 in xenograft tumors, accompanying with the suppression of tumor growth in nude mice. Celecoxib caused phosphorylation of eukaryotic translation initiation factor 2 kinase (PERK) and eukaryotic initiation factor-2α (eIF2α) and production of activating transcription factor (ATF)4 mRNA. Suppression of ATF4 expression by small interfering RNA (siRNA) partially inhibited the celecoxib-dependent upregulation of GRP78. Celecoxib increased the intracellular Ca^{2+} concentration, while 1,2-bis(2-aminophenoxy) ethane-*N,N,N′N′*-tetraacetic acid, an intracellular Ca^{2+} chelator, inhibited the upregulation of GRP78 and ATF4. These results suggest that the Ca^{2+}-dependent activation of the PERK-eIF2α-ATF4 pathway is involved in the upregulation of ER chaperones by celecoxib. Overexpression of GRP78 partially suppressed the apoptosis and induction of CHOP in the presence of celecoxib and this suppression was stimulated by coexpression of either ERdj3 or ERdj4. On the other hand, suppression of GRP78 expression by siRNA drastically stimulated cellular apoptosis and production of CHOP in the presence of celecoxib. These results show that upregulation of ER chaperones by celecoxib protects cancer cells from celecoxib-induced apoptosis, thus may decrease the potential antitumor activity of celecoxib.
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Keywords: celecoxib; endoplasmic reticulum chaperones; apoptosis; calcium

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used therapeutic agents in the treatment of pain, inflammation and fever (Smalley *et al.*, 1995). In addition, recent epidemiological studies clearly show that prolonged NSAID use is associated with a reduced risk of cancer, while preclinical and clinical studies have shown that some NSAIDs are effective in the treatment and prevention of cancer. This effect is particularly well documented in relation to colon and rectal cancer, with recent studies showing that NSAID use reduces the risk of stomach cancer (Farrow *et al.*, 1998; Husain *et al.*, 2002; Sorensen *et al.*, 2003; Hu *et al.*, 2004). Of the various mechanisms proposed to explain the antitumor action of NSAIDs, such as cell growth suppression, inhibition of angiogenesis and inhibition of metastasis, NSAID-induced apoptosis in cancer cells is thought to play an important role (Gupta and Dubois, 2001; Kismet *et al.*, 2004).

The anti-inflammatory action of NSAIDs is mediated through the NSAID-induced inhibition of cyclooxygenase (COX). COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong propensity for inducing inflammation. PGs, such as PGE_2, inhibit cellular apoptosis and the overexpression of COX-2 (a subtype of COX) has been reported to play a role in the development of various types of tumors (Eberhart *et al.*, 1994; Piazza *et al.*, 1995; Ristimaki *et al.*, 1997; Hoshino *et al.*, 2003). Based on these reports, NSAID-induced apoptosis was believed to be based solely on its inhibitory effects on COX activity. However, several lines of evidence suggest that NSAID-induced apoptosis also involves COX-independent mechanisms. A derivative of the NSAID sulindac (sulindac sulfone), which has no COX-inhibitory activity, induced apoptosis in tumor cells and some NSAIDs have been shown to induce apoptosis in COX-null fibroblasts and in tumor cells in which COX expression was absent (Hanif *et al.*, 1996; Elder *et al.*, 1997; Zhang *et al.*, 1999). Therefore, it is important that the COX-independent mechanisms governing NSAID-induced apoptosis be identified.

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NSAID-induced apoptosis in normal gastric mucosal cells seems to be involved in the production of gastric lesions by NSAIDs. We recently suggested that, in addition to COX inhibition by NSAIDs, the direct cytotoxicity of NSAIDs (induction of necrosis and apoptosis) contributes to the production of NSAID-induced gastric lesions (Tomisato *et al*., 2001, 2004b). We examined the mechanism of NSAID-induced apoptosis in guinea pig gastric mucosal cells in primary culture and found that NSAIDs induce apoptosis by acting as endoplasmic reticulum (ER) stressors. Various NSAIDs induce C/EBP homologous transcription factor (CHOP), which is known to be important for the induction of apoptosis by ER stressors. Further to this, we showed, using CHOP-deficient mice or a dominant-negative form of CHOP, that this CHOP induction is essential for NSAID-induced apoptosis (Tsutsumi *et al*., 2004).

In addition to inducing apoptosis, ER stressors cause upregulation of ER chaperones, which protect the ER against ER stressor activity by refolding unfolded proteins in the ER (Lee, 2001). In fact, we reported that various NSAIDs induced the expression of glucose-regulated protein (GRP)-78, a representative ER chaperone, in gastric mucosal cells in primary culture (Tsutsumi *et al*., 2004). However, it is not known if NSAIDs upregulate other ER chaperones such as ERdj3 and Erdj4, which act as cochaperones for GRP78 and activate the ATPase and refolding activity of GRP78 (Yu *et al*., 2000; Shen *et al*., 2002b). Furthermore, it is also not known if NSAIDs induce ER chaperones in other types of cells, such as tumor cells. It was reported that overexpression of GRP78 makes cells resistant to apoptosis induced by anticancer drugs (topoisomerase inhibitors) and ER stressors (tunicamycin and Ca^{2+} ionophores) (Morris *et al*., 1997; Reddy *et al*., 2003). Therefore, it is possible that the induction of GRP78 by NSAIDs contributes to the protection of cells from NSAID-induced apoptosis. In the present study, we have examined perturbations to ER chaperones by NSAIDs and the effect of such chaperones on NSAID-induced apoptosis in human gastric carcinoma cells. Several NSAIDs upregulated not only GRP78 but also ERdj3 and ERdj4. We suggest that this upregulation is mediated by an increase in intracellular Ca^{2+} concentration. Furthermore, the contribution of ER chaperones to the protection of cells from celecoxib (a NSAID)-induced apoptosis was supported by experiments using overexpression plasmid and small interfering RNA (siRNA) for GRP78.

Results

NSAIDs upregulate ER chaperones

In a previous report, we showed that NSAIDs (such as celecoxib, indomethacin and diclofenac) upregulated GRP78 expression in guinea pig gastric mucosal cells in primary culture (Tsutsumi *et al*., 2004). Here, we used immunoblotting techniques to examine the increase in GRP78 production in AGS cells caused by a number of different NSAIDs. As shown in Figure 1a, all NSAIDs tested clearly increased cellular levels of GRP78. The concentrations of celecoxib, indomethacin and diclofenac required for these increases in AGS cells were similar to those previously reported to have caused similar effects in guinea pig gastric mucosal cells (Tsutsumi *et al*., 2004). COX exists as two subtypes, COX-1 and COX-2, for which celecoxib and nimesulide are COX-2 selective in their action. The results shown in Figure 1a suggest that NSAIDs increased cellular GRP78, irrespective of their COX-2 specificity. Furthermore, although celecoxib and nimesulide have similar IC_{50} values for COX-inhibition (Riendeau *et al*., 1997; Ben-Chetrit *et al*., 2005), higher concentrations of nimesulide than celecoxib were required for similar increases in GRP78 production, suggesting that NSAIDs increase GRP78 independently of COX inhibition. Of the NSAIDs tested (see results in Figure 1a), we selected celecoxib for use in most of subsequent experiments because it increased GRP78 at the lowest concentration

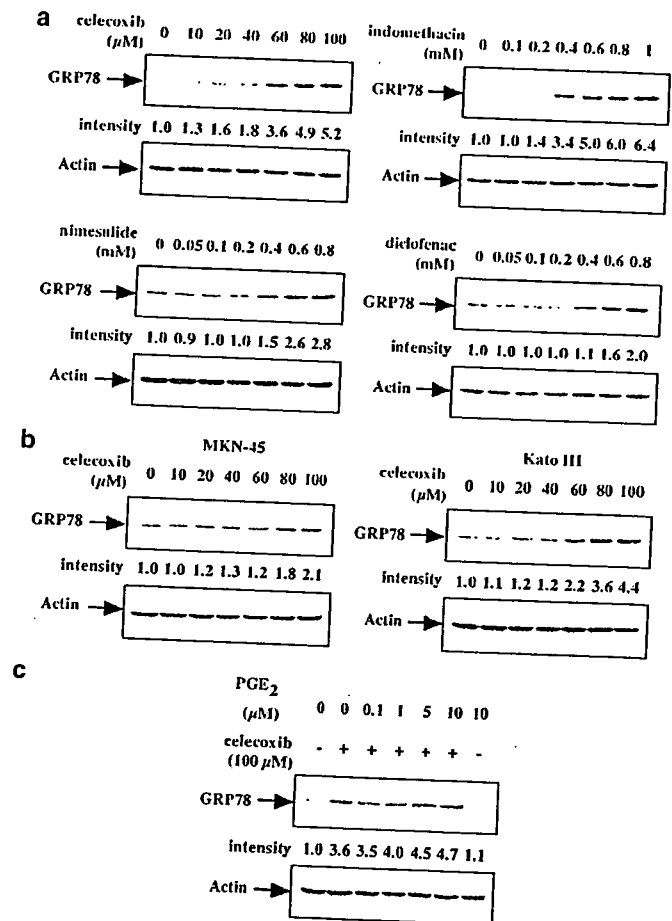

Figure 1 Upregulation of GRP78 by NSAIDs. AGS (**a** and **c**) or MKN-45 and Kato III (**b**) cells were incubated with indicated concentrations of stated NSAIDs for 12 h (celecoxib) or 24 h (NSAIDs other than celecoxib). Cells were pretreated with indicated concentrations of PGE_2 for 2 h before the celecoxib treatment (**c**). Whole cell extracts (5 μg protein) were analysed by immunoblotting with an antibody against GRP78 or actin. Band intensity of GRP78 was determined by densitometric scanning, gel-loading levels compensated against the band intensity of actin, and expressed relative to the control sample (i.e. without NSAIDs).

and its effectiveness in cancer therapy has been well established (Koki and Masferrer, 2002).

We also examined the upregulation of GRP78 by celecoxib in other cell types. The MKN-45 and Kato III cell lines are derived from gastric cancer cells (Okada et al., 2000). As shown in Figure 1b, celecoxib increased GRP78 mRNA in both of these cell lines at concentrations similar to those used for the AGS cells. It has been reported that both COX-1 and COX-2 mRNA are expressed in AGS and MKN-45 cells, whereas COX-1 but not COX-2 mRNA expression is detectable in KATO-III cells (Kawai et al., 1998; Fan et al., 2001; Lim et al., 2001). We confirmed these phenotypes by RT–PCR, that is, COX-1 mRNA expression was confirmed in each of the cell lines tested, whereas COX-2 mRNA was detected only in AGS and MKN-45 cells (data not shown). Thus, the results in Figure 1 show that the COX-2-selective NSAID, celecoxib, upregulated GRP78 mRNA not only in COX-2-expressing cells but also in cells lacking COX-2 expression, again suggesting that GRP78 upregulation can be induced by NSAIDs independently of COX inhibition. For further confirmation of this independence, we examined the effect of PGE_2 on the GRP78 upregulation by celecoxib. As shown in Figure 1c, PGE_2 did not affect the expression of GRP78 in both presence and absence of celecoxib, suggesting that the GRP78 upregulation by celecoxib cannot be explained by decrease in PGE_2 by COX inhibition.

GRP78 belongs to the HSP70 family of proteins for which cochaperones are also known (Lee, 2001). For example, HSP40 binds to HSP70 and stimulates its ATPase and refolding activities (Landry, 2003). Various cochaperones have been suggested for GRP78, among which ERdj3 and ERdj4 have been shown to bind to GRP78 and activate its ATPase activity (Yu et al., 2000; Shen et al., 2002b). We found, using real-time RT–PCR analysis, that not only *GRP78* but also *ERdj3* and *ERdj4* mRNAs were upregulated by celecoxib (see Figure 2). The concentrations of celecoxib required for the increase of both *ERdj3* and *ERdj4* mRNAs were similar to that required for the increase of *GRP78* mRNA (Figure 2a). Moreover, the curve describing the time course for the upregulation by celecoxib of *GRP78* mRNA was indistinguishable from those for *ERdj3* and *ERdj4* mRNAs (Figure 2b), showing that celecoxib simultaneously upregulates *GRP78*, *ERdj3* and *ERdj4*.

We also examined the effect of treatment with celecoxib on GRP78 expression in xenograft tumors in nude mice. Tumors were developed in nude mice by inoculation (s.c.) of MKN-45 cells and were treated with celecoxib by its oral administration. Xenograft tumor growth was clearly inhibited by the oral administration of celecoxib (Figure 3a), being consistent with results in a previous report (Williams et al., 2000; Leahy et al., 2002; Zweifel et al., 2002; Kulp et al., 2004). As shown in Figure 3b and c, the amount of GRP78 in tumors was increased by this celecoxib treatment. Results showed that celecoxib upregulates GRP78 also in tumors *in vivo*, accompanying with the suppression of tumor growth by this drug.

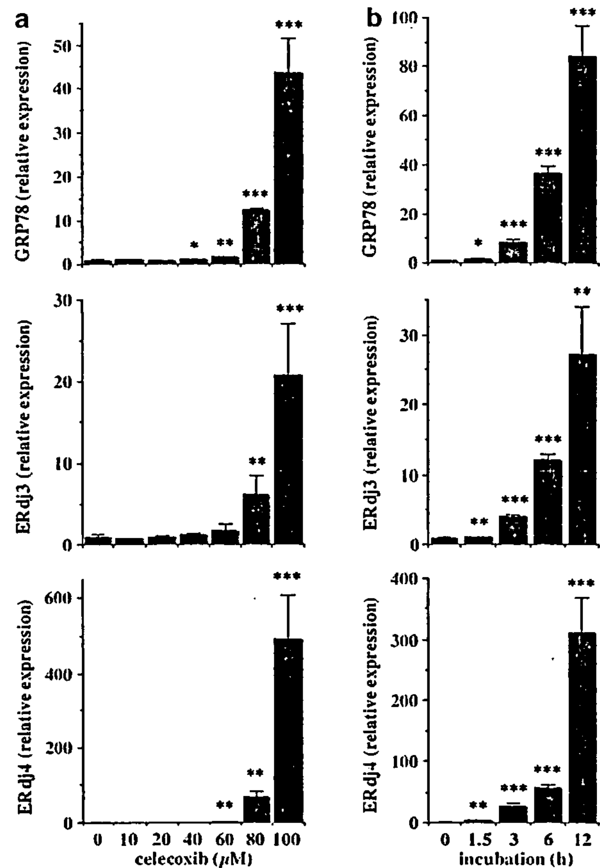

Figure 2 Upregulation of ER chaperone genes by celecoxib. AGS cells were incubated with indicated concentrations (a) or 100 μM (b) of celecoxib for 12 h (a) or the time periods indicated (b) and total RNA extracted. Samples were subjected to real-time RT–PCR by use of a specific primer for each gene. Values were normalized to actin gene expression and expressed relative to the control sample (i.e. without celecoxib). Values given are mean ± s.d. ($n = 3$). ***$P<0.001$; **$P<0.01$; *$P<0.05$.

Mechanism for upregulation of ER chaperones by celecoxib

Eukaryotic translation initiation factor 2 kinase (PERK) is an ER transmembrane protein that plays an important role in ER chaperone induction by ER stressors. Previous studies revealed that ER stressors activate PERK by its phosphorylation, the PERK then activates eukaryotic initiation factor-2α (eIF2α) by its phosphorylation, the eIF2α induces activating transcription factor (ATF)4 expression, and finally, ATF4 binds to the promoter of the GRP78 gene, resulting in the increased production of GRP78 (Harding et al., 2000; Luo et al., 2003). We used DNA microarray techniques to search for genes whose expression is stimulated by NSAIDs in AGS cells (Mima et al., 2005). *ATF4* was identified as one such gene, suggesting that its upregulation is involved in the induction of ER chaperones by NSAIDs. As shown in Figure 4a, both PERK and eIF2α were phosphorylated in the presence of celecoxib. The PERK phosphorylation was transient; it decreased after 3 h and we have no

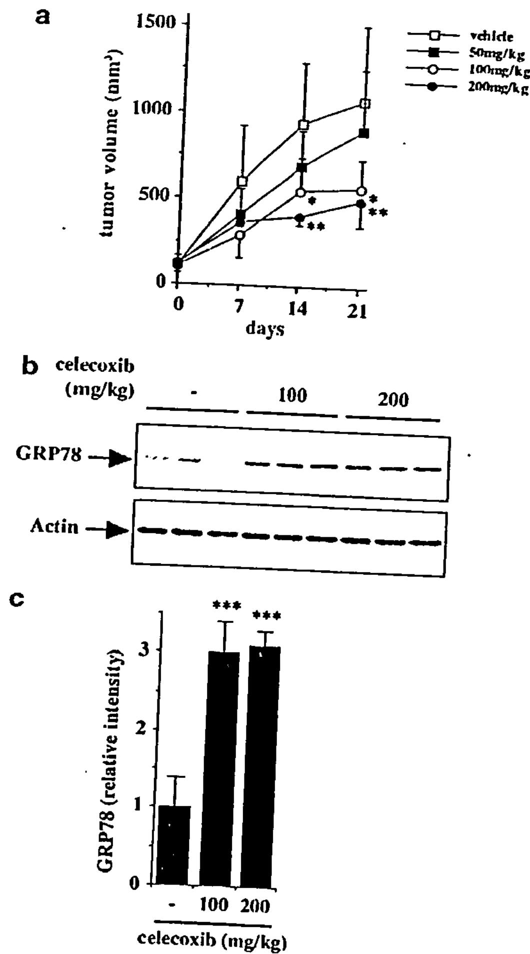

Figure 3 Effect of celecoxib on growth of xenograft tumor and expression of GRP78 in nude mice. Each nude mouse was inoculated s.c. with MKN-45 cells and tumors were developed until size of tumors reached a mean volume of 116±34 mm³. Then indicated dose of celecoxib was administered single daily orally for the duration of the study. Tumors were measured weekly and their volumes calculated (a). After 4 days from the start of celecoxib administration, cell lyzates prepared from tumors were analysed by immunoblotting with an antibody against GRP78 or actin (b). Band intensity of GRP78 was determined by densitometric scanning, compensated against the band intensity of actin, and expressed relative to the control sample (i.e. without celecoxib) (c). Values given are mean±s.d. ($n=6$ for (a) and $n=3$ for (b and c)). ***$P<0.001$; **$P<0.01$; *$P<0.05$.

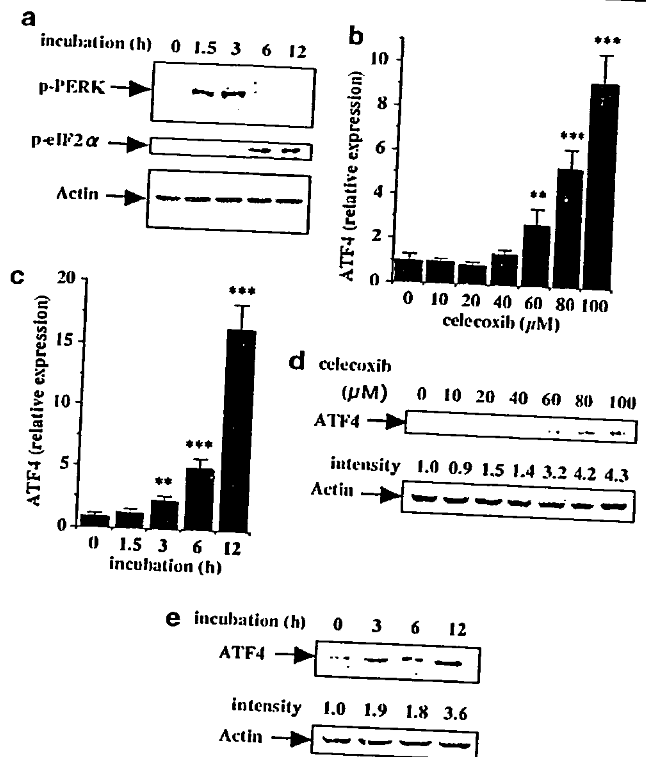

Figure 4 Activation of PERK, eIF2α and ATF4 by celecoxib. AGS cells were incubated with 100 μM (a, c and e) or indicated concentrations (b and d) of celecoxib for the time periods indicated (a, c and e) or 12 h (b and d). For (a, d and e), whole-cell extracts (5 μg protein for actin, 10 μg protein for ATF4 and 20 μg protein for PERK and eIF2α) were analysed by immunoblotting with an antibody against phosphorylated PERK (p-PERK), phosphorylated eIF2α (p-eIF2α), ATF4 or actin. For (b and c), total RNA was extracted and subjected to real-time RT–PCR by use of a specific primer for ATF4. Values were analysed and expressed as previously described in the legend of Figure 2. Values shown are mean±s.d. ($n=3$). ***$P<0.001$; **$P<0.01$.

clear explanation for this phenomenon. Furthermore, upregulation of *ATF4* mRNA and ATF4 protein by celecoxib was confirmed by real-time RT–PCR analysis and immunoblotting analysis, respectively (Figure 4b–e). Both time–course and dose–response curves for upregulation of *ATF4* mRNA were similar to those observed for the increase of *GRP78* mRNA by celecoxib (Figures 2 and 4). Interestingly, phosphorylation of PERK was detected within 1.5 h of the addition of celecoxib, maximal eIF2α was reached 6 h after addition and peak *ATF4* mRNA and protein was observed 12 h after addition (Figure 4), suggesting that the sequential activation of PERK, eIF2α and ATF4 is involved in the upregulation of GRP78. To test this possibility, we examined the effect of siRNA for ATF4 on the celecoxib-dependent upregulation of GRP78. Transfection of siRNA for ATF4 clearly inhibited the expression of *ATF4* mRNA, both in the presence and absence of celecoxib (Figure 5a). As shown in Figure 5b, transfection of siRNA for ATF4 partially suppressed the increase of *GRP78* mRNA production caused by celecoxib, suggesting that ATF4 is involved in this celecoxib-dependent GRP78 upregulation. In order to estimate the specificity of this siRNA, we examined its effect on the expression of mRNA of other CREB protein family member (ATF2 and ATF3). As shown in Figure 5c, transfection of siRNA for ATF4 did not affect the celecoxib-dependent induction of *ATF2* mRNA so clearly as that of *ATF4*, suggesting that this siRNA specifically inhibited the expression of *ATF4* mRNA. On the other hand, transfection of this siRNA inhibited the induction of *ATF3* mRNA by celecoxib (Figure 5d). This may be due to the dependence of ATF3 expression on ATF4; the upregulation of ATF3 by thapsigargin was significantly suppressed in ATF4 knockout cells (Jiang *et al.*, 2004).

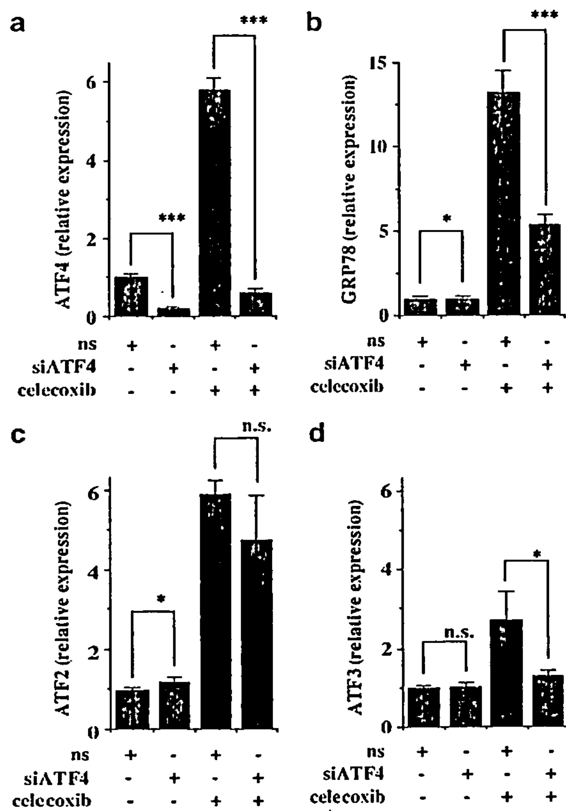

Figure 5 Effect of siRNA for ATF4 on the celecoxib-dependent upregulation of GRP78. AGS cells transfected with siRNA for ATF4 (siATF4) or nonsilencing (ns) siRNA were incubated with or without 100 μM celecoxib for 12 h. Total RNA was extracted and subjected to real-time RT-PCR by use of a specific primer for ATF4 (a), GRP78 (b), ATF2 (c) and ATF3 (d). Values were analysed and expressed as previously described in the legend of Figure 2. Values shown are mean$\pm$s.d. ($n=3$). ***$P<0.001$; *$P<0.05$. n.s., not significant.

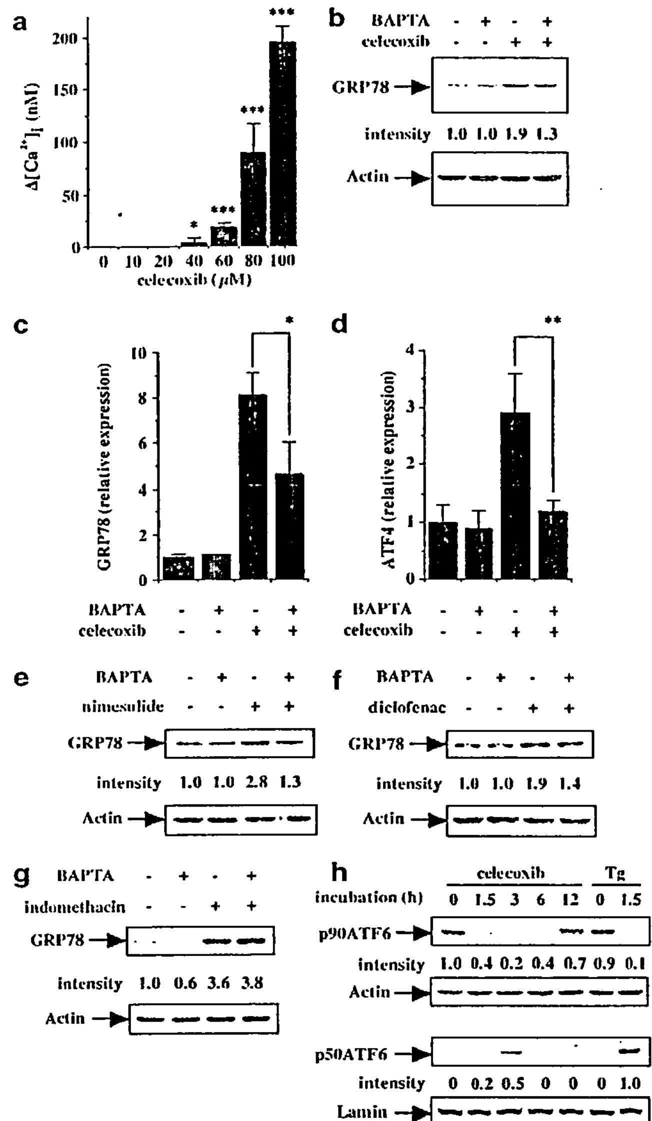

Figure 6 Changes in intracellular Ca^{2+} concentration in the NSAID-dependent upregulation of GRP78. The intracellular Ca^{2+} concentration was monitored by a fluo-3/AM assay system. Indicated concentrations of celecoxib were added to fluo-3/AM-loaded cells and the time–course of fluo-3 fluorescence change monitored. The maximum value for the increase in the intracellular Ca^{2+} level ($\Delta[Ca^{2+}]_i$) is shown (a). AGS cells were preincubated with or without 2 μM BAPTA-AM for 1 h and further incubated with or without 80 μM celecoxib (b–d), 800 μM nimesulide (e), 800 μM diclofenac (f) or 400 μM indomethacin (g) in the presence or absence of 2 μM BAPTA-AM for 6 h (celecoxib) or 12 h (other NSAIDs). The levels of GRP78 protein (b, e–g), GRP78mRNA (c) and ATF4 mRNA (d) were estimated by immunoblotting or real-time RT–PCR experiments as described in the legends of Figures 1 and 2. AGS cells were incubated with 100 μM celecoxib or 2 μM thapsigargin for indicated periods (h). Whole cell extracts (25 μg protein for ATF6 and 10 μg protein for actin) (upper panel in (h)) or nuclear extracts (20 μg protein for p50 ATF6 and 5 μg protein for lamin B) (lower panel in (h)) were analysed by immunoblotting with an antibody against ATF6, actin or lamin B as described in the legends of Figure 1. As for p50 ATF6 band, intensity of each band was expressed relative to the positive control sample (i.e. cells treated with thapsigargin for 1.5 h). Values shown are mean$\pm$s.d. ($n=3$). ***$P<0.001$; **$P<0.01$; *$P<0.05$.

Some NSAIDs have been reported to increase intracellular Ca^{2+} concentrations (Johnson *et al.*, 2002; Tomisato *et al.*, 2004a). We recently found that all of the NSAIDs tested can cause membrane permeabilization, resulting in an increase in intracellular Ca^{2+} levels. This activity correlates well with the NSAID-induced apoptosis (Tomisato *et al.*, 2004a). On this basis, we have tested whether the increase in intracellular Ca^{2+} by celecoxib is responsible for the induction of ER chaperones. First, we confirmed the presence of an increase in intracellular Ca^{2+} concentration in the presence of celecoxib under the same conditions as those used for the upregulation of GRP78 in AGS cells. As shown in Figure 6a, celecoxib increased intracellular Ca^{2+} concentration in a dose-dependent manner, similar to that observed for the increase in *GRP78* mRNA (Figure 2a). Furthermore, 1,2-bis(2-aminophenoxy)-ethane-*N,N,N′N′*-tetraacetic acid (BAPTA-AM), an intracellular Ca^{2+} chelator, inhibited the celecoxib-dependent upregulation of GRP78, *GRP78* mRNA and *ATF4* mRNA (Figure 6b–d), but had no effect when celecoxib was not present. At the concentrations used, BAPTA-AM did not affect the cell viability (data

not shown). These results strongly suggest that upregulation of GRP78 and ATF4 by celecoxib is mediated, at least in part, through an increase in intracellular Ca^{2+} concentration.

We also examined the effect of BAPTA-AM on the upregulation of GRP78 induced by other NSAIDs. As is the case of celecoxib, BAPTA-AM inhibited the upregulation of GRP78 by nimesulide or diclofenac (Figure 6e and f). On the other hand, BAPTA-AM did not affect the upregulation of GRP78 by indomethacin (Figure 6g).

ATF6 is another type of ER transmembrane protein that also plays an important role in ER chaperone induction by ER stressors. We previously suggested that ATF6 is activated in the presence of NSAIDs in gastric mucosal cells in primary culture (Tsutsumi *et al*., 2004), and it was recently reported that ATF6 is activated by nitric oxide through an increase in the intracellular Ca^{2+} level (Xu *et al*., 2004). Therefore, we examined the effect of celecoxib on the activation of ATF6. In the presence of ER stressors, such as thapsigargin, p90 ATF6 (the inactive form of ATF6 for ER stress response) is cleaved into p50 ATF6, which translocates to the nucleus where it specifically activates transcription of genes related to ER stress response (Yoshida *et al*., 2000). As shown in Figure 6h, as well as thapsigargin, treatment of cells with celecoxib caused appearance of p50 ATF6 and disappearance of p90 ATF6, suggesting that celecoxib activated ATF6. This activation was transient; both appearance of p50 ATF6 and disappearance of p90 ATF6 was apparent 3 h but not observed 12 h after the addition of celecoxib.

Effect of ER chaperones on celecoxib-induced apoptosis
It is well known that celecoxib induces apoptosis in various types of tumor cells (Koki and Masferrer, 2002). As shown in Figure 7a and b, celecoxib induced apoptosis in AGS cells in both a dose- and time-dependent manner. Real-time RT–PCR analysis showed that celecoxib induced *CHOP* mRNA production, with the dose–response and time–course curves for this response (Figure 7c and d) being similar to those seen for the induction of apoptosis (Figure 7a and b). This finding suggests that the induction of CHOP expression is responsible for the celecoxib-induced apoptosis, as reported previously (Tsutsumi *et al*., 2004).

The dose–response and time–course curves for the upregulation of GRP78 by celecoxib (Figure 2) were also similar to those for the induction of apoptosis (Figure 7a and b), showing that GRP78 upregulation and apoptosis occur simultaneously. Previous reports showed that overexpression of GRP78 in cells suppresses apoptosis induced by topoisomerase inhibitors and ER stressors (Morris *et al*., 1997; Reddy *et al*., 2003). Therefore, it is possible that celecoxib-induced GRP78 protects cells from celecoxib-induced apoptosis. In order to test this possibility, we examined the effect of overexpression of GRP78 on celecoxib-induced apoptosis. Transfection of pcDNA3.1 containing the *GRP78* gene caused both an increase in the level of GRP78 in

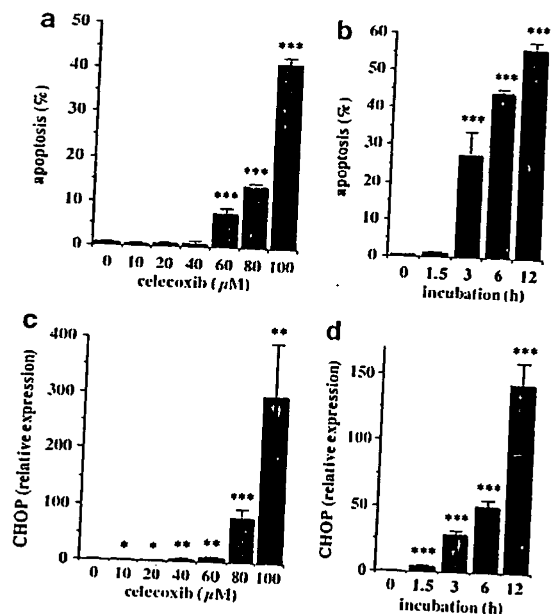

Figure 7 Induction of apoptosis by celecoxib. AGS cells were incubated with the indicated concentrations (a and c) or 100 μM (b and d) of celecoxib for 12 h (a and c) or indicated periods (b and d). For (a and b), apoptotic cell numbers were determined by FACS (a and b). For (c and d) total RNA was extracted and subjected to real-time RT–PCR by use of a specific primer for CHOP. Values were analysed and expressed as previously described in the legend of (Figure 2c and d). Values shown are mean±s.d. ($n=3$). $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

cells and partial suppression of celecoxib-induced apoptosis in a manner that depended on the dose of transfected DNA (Figure 8a and b). Real-time RT–PCR analysis revealed that the transfection increased *GRP78* mRNA both in the presence and absence of celecoxib (Figure 8c). Furthermore, the transfection partially suppressed the celecoxib-dependent induction of *CHOP* mRNA (Figure 8d). We confirmed that overexpression of GRP78 did not affect the spontaneous apoptosis (apoptosis in the absence of celecoxib) (Figure 9d). These results suggest that the celecoxib-induced increase in GRP78 expression protects cells from celecoxib-induced apoptosis by repressing the expression of *CHOP* mRNA. Overexpression of GRP78 did not diminish the celecoxib-dependent *GRP78* upregulation (Figure 8c), which is inconsistent with previous results showing that overexpression of GRP78 diminished tunicamycin-dependent GRP78 production (Morris *et al*., 1997). This discrepancy may be explained by differences in stressors or in the extent of overexpression (the extent of overexpression of GRP78 in the paper by Morris *et al*. was much higher than that found here).

It was recently reported that overexpression of ERdj4 in cells inhibits apoptosis induced by tunicamycin (Kurisu *et al*., 2003). We here examined the effect of overexpression of ERdj4, ERdj3, or their coexpression with GRP78 on celecoxib-induced apoptosis. As shown in Figure 9a, transfection of plasmid resulting in overexpression of *ERdj3* partially suppressed the cele-

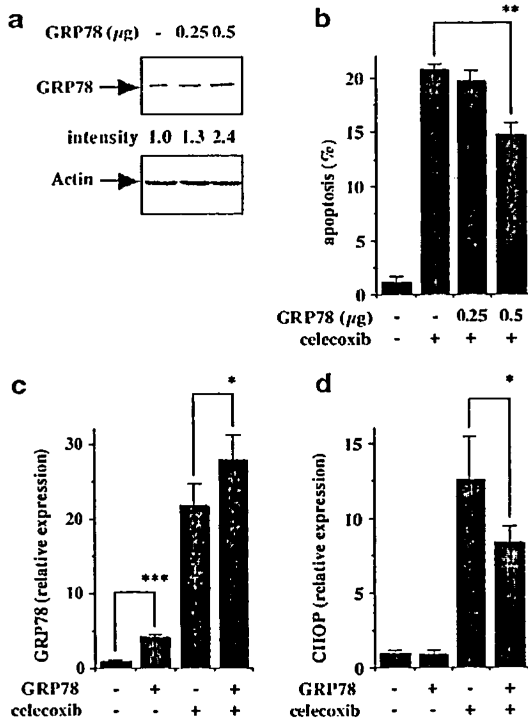

Figure 8 Effect of overexpression of GRP78 on celecoxib-induced apoptosis. AGS cells were transfected with the indicated amount (a and b) or 0.5 μg (c and d) of plasmid for the overexpression of GRP78 and pcDNA3.1 vector (total DNA amounts were fixed at 4 μg). After 48 h, cells were incubated with or without 100 μM celecoxib for 6 h (b–d). The levels of GRP78 protein (a), GRP78mRNA (c) and CHOP mRNA (d) were estimated by immunoblotting or real-time RT–PCR experiments as previously described in the legends of Figures 1 and 2. Apoptotic cell numbers were determined by FACS as described in the legend of Figure 7(b). Values shown are mean±s.d. ($n = 3$). ***$P<0.001$; **$P<0.01$; *$P<0.05$.

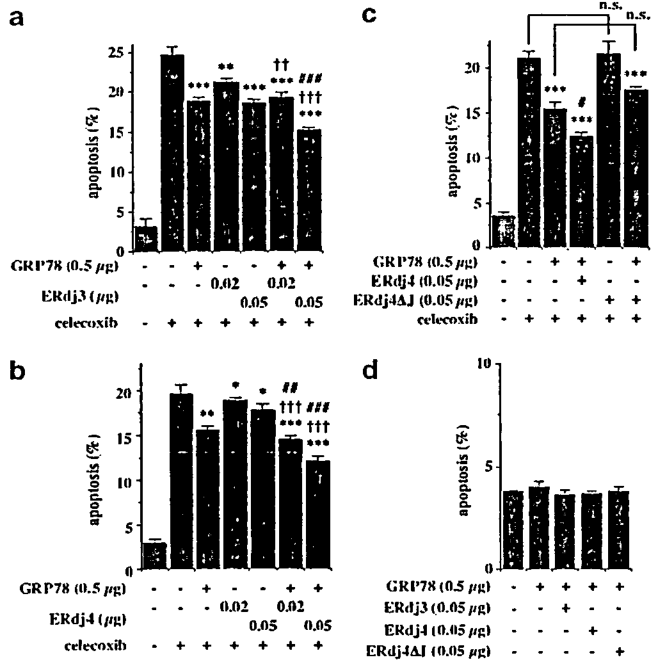

Figure 9 Stimulation of the antiapoptotic effect of GRP78 against celecoxib by coexpression of ERdj3 or ERdj4. AGS cells were transfected with the indicated amounts of each expression plasmid and pcDNA3.1 vector (total DNA amounts were fixed at 4 μg). After 48 h, AGS cells were incubated with or without 100 μM celecoxib for 6 h. Apoptotic cell numbers were determined by FACS as described in the legend of Figure 7. Values shown are mean±s.d. ($n = 3$). ***, ††† and ###$P<0.001$; **, †† and ##$P<0.01$; * and #<0.05. * (versus celecoxib-treated cells only), # (versus celecoxib-treated and GRP78 overexpressing cells), † (versus celecoxib-treated and ERdj3 (or ERdj4) overexpressing cells). n.s, not significant (a–d).

coxib-induced apoptosis in a manner that was dependent on the amount of transfected DNA. Furthermore, the cotransfection of plasmids for the overexpression of both GRP78 and ERdj3 caused a more clear-cut suppression of celecoxib-induced apoptosis than did transfection of each plasmid alone (Figure 9a). Similar results were obtained for ERdj4 (Figure 9b). We confirmed that overexpression of both GRP78 and ERdj3 or ERdj4 did not affect the spontaneous apoptosis (apoptosis in the absence of celecoxib) (Figure 9d). These results suggest that the ERdj4 and ERdj3 cochaperones stimulate the antiapoptotic effect of GRP78 against the actions of celecoxib.

The J domain of HSP40 family proteins is responsible for their interaction with HSP70 family proteins (Landry, 2003). It was shown that J domain-deleted ERdj4 (ERdj4ΔJ) could not interact with GRP78 and activate the ATPase activity of GRP78 (Shen *et al.*, 2002b). As shown in Figure 9c, in contrast to the results obtained with wild-type ERdj4, transfection of plasmid for the overexpression of ERdj4ΔJ caused neither the suppression of celecoxib-induced apoptosis nor stimulation of an antiapoptotic effect of GRP78 against celecoxib. These findings suggest that the antiapoptotic effects of ERdj4 are achieved via its interaction with GRP78.

The siRNA technique was used to further confirm that celecoxib-induced GRP78 protects cells from celecoxib-induced apoptosis. Transfection of siRNA for GRP78 decreased the expression of GRP78 protein (Figure 10a) and *GRP78* mRNA (Figure 10b), both in the presence and absence of celecoxib, and also stimulated celecoxib-induced apoptosis and *CHOP* mRNA expression (Figure 10e and f). In order to estimate the specificity of siRNA for GRP78, we examined its effect on the expression of mRNA of other ER chaperons (*ERdj3* and *ERdj4*). As shown in Figure 10c and d, transfection of siRNA for GRP78 significantly increased the *ERdj3* or *ERdj4* mRNA in both presence and absence of celecoxib, suggesting that this siRNA specifically inhibit the expression of GRP78. The stimulation of the *ERdj3* or *ERdj4* mRNA expression by this siRNA may be due to that GRP78 negatively regulated the ER stress response; GRP78 binds to PERK and protein-kinase and site-specific endoribonuclease (IRE1) and inhibits their activity for inducing ER stress response (Bertolotti *et al.*, 2000). These results strongly suggest that celecoxib-induced GRP78 protects

cells from apoptosis in the presence of celecoxib. Furthermore, the stimulation of apoptosis and CHOP mRNA expression by transfection of siRNA for GRP78 in the absence of celecoxib suggests that constitutively expressed GRP78 under nonstress conditions contributes to cell survival.

We also tried to examine the effect of siRNA for GRP78 on apoptosis induced by chemotherapy drug without ability to induce ER stress response. As shown in Figure 10h, staurosporine, a chemotherapy drug, did not upregulate GRP78 at concentrations that are enough to induce apoptosis (Figure 10g). As shown in Figure 10g, transfection of siRNA for GRP78 slightly stimulated apoptosis induced by staurosporine. This slight stimulation can be explained by its effect on the spontaneous apoptosis (apoptosis in the absence of drugs). In other words, the extent of the stimulation was not so drastic as is the case of celecoxib (Figure 10e). Suppression of GRP78 through siRNA for GRP78 sensitized human breast cancer cells to etoposide-mediated cell death (Dong et al., 2005). These results suggested that the stimulatory effect of siRNA for GRP78 on apoptosis is apparent for apoptosis induced by chemotherapy drugs that induce ER stress response.

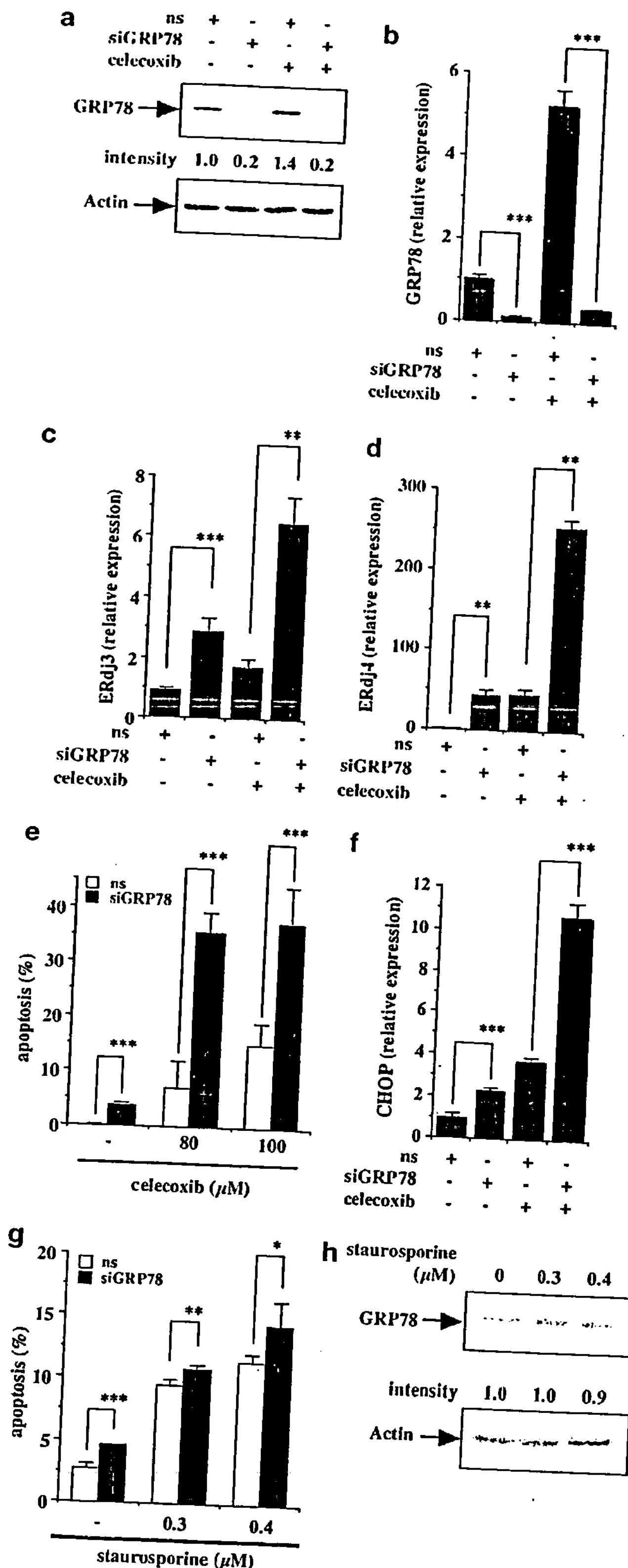

Figure 10 Effect of GRP78 siRNA on celecoxib-induced apoptosis. AGS cells were transfected with 5 μg of siRNA for GRP78 (siGRP78) or nonsilencing (ns) siRNA. After 48 h, cells were incubated with or without 80 μM celecoxib (a–d and f), indicated concentrations of celecoxib (e) or indicated concentrations of staurosporine (g and h) for 6 h. The levels of GRP78 protein (a and h), GRP78 mRNA (b), ERdj3 mRNA (c), ERdj4 mRNA (d) and CHOP mRNA (f) were estimated by immunoblotting or real-time RT–PCR experiments as described in the legends of Figures 1 and 2. Apoptotic cell numbers were determined by FACS as described in the legend of (Figure 7e and g). Values shown are mean±s.d. ($n=3$). ***$P<0.001$; **$P<0.01$; *$P<0.05$.

Discussion

In this study, we have shown that several NSAIDs upregulate ER chaperones not only in various types of cultured human gastric cancer cells but also in xenograft tumors in nude mice. We reported previously that a number of different NSAIDs upregulate GRP78 in guinea pig gastric mucosal cells in primary culture (Tsutsumi et al., 2004). Furthermore, we recently found that NSAIDs upregulate ER chaperones in HEK293 cells (human embryonic kidney cells) (Hoshino T et al., unpublished results). Therefore, it seems that NSAIDs upregulate ER chaperones as a part of their general mechanism of action. Concentrations of NSAIDs required for induction of GRP78 in vitro (60–100 μM) are relatively higher than clinical available concentrations of celecoxib (1–10 μM) (Lopez-Parra et al., 2005; Patel et al., 2005). It was reported that oral administration of celecoxib (100–200 mg/kg/day, similar conditions to our experiments in Figure 3) caused serum concentrations of 8.6–11.3 μM (Kulp et al., 2004). Therefore, under in vivo conditions, lower concentrations of celecoxib may be able to induce GRP78 than under in vitro conditions. This induction is likely to be mediated by COX-independent mechanisms, given that NSAIDs

increased GRP78 production irrespective of their COX-2 specificity and that COX-2-selective NSAIDs (celecoxib) upregulated GRP78 in cells that did not express COX-2. Furthermore, although indomethacin at a concentration of less than 1 μM inhibited both COX-1 and COX-2 (Kawai *et al.*, 1998), the upregulation of GRP78 required higher concentrations. The IC_{50} value of celecoxib for COX-2 inhibition is about 40 nM (Schroeder *et al.*, 2004; Ben-Chetrit *et al.*, 2005), which is much lower than the concentration required for the upregulation of GRP78 (about 60 μM).

The results in this study suggest that the upregulation of ER chaperones by celecoxib involves the PERK-eIF2α-ATF4 pathway. Other recent papers also suggest the importance of this pathway in the upregulation of ER chaperones by other ER stressors (Luo *et al.*, 2003; Roybal *et al.*, 2004). However, siRNA for ATF4 did not completely inhibit the celecoxib-dependent GRP78 upregulation, suggesting that pathways other than the PERK–eIF2α–ATF4 pathway are involved in this mechanism. In addition to PERK, two other ER transmembrane proteins (ATF6 and IRE1) are involved in the ER stress response. Activated ATF6 translocates to the nucleus where it specifically binds to the promoter of ER chaperone genes, while IRE1 splices X box-binding protein XBP-1 mRNA, to convert it into a potent activator for the transcription of ER chaperone genes.(Yoshida *et al.*, 2000; Kaufman, 2002; Ron, 2002) We suggest that both ATF6 and IRE1 are activated in the presence of NSAIDs in gastric mucosal cells in primary culture (Tsutsumi *et al.*, 2004), and we here showed that ATF6 is activated by celecoxib even in AGS cells.

It is well known that an increase in the intracellular Ca^{2+} concentration induces ER chaperone; Ca^{2+} ionophores, for example, induce ER chaperone (Drummond *et al.*, 1987; Wooden *et al.*, 1991). We suggested that an increase in intracellular Ca^{2+} concentration is involved in the celecoxib-dependent upregulation of ER chaperones based on the following observations: celecoxib increased the intracellular Ca^{2+} concentration and expression of ER chaperones simultaneously; and the intracellular Ca^{2+} chelator, BAPTA-AM, inhibited the celecoxib-dependent upregulation of ER chaperones. BAPTA-AM also inhibited the GRP78 upregulation caused by nimesulide or diclofenac but not by indomethacin. We recently showed that all of NSAIDs tested (including nimesulide and diclofenac) increased the intracellular Ca^{2+} level (Tomisato *et al.*, 2004a; Tanaka *et al.*, 2005). Since indomethacin absorbed fluo-3 fluorescence (530 nm), we could not measure the intracellular Ca^{2+} level in the presence of indomethacin by the assay system using fluo-3 (Tanaka *et al.*, 2005). Therefore, it seems that not only celecoxib but also most of NSAIDs induce GRP78 through increasing the intracellular Ca^{2+} level and that indomethacin induces GRP78 through different unknown mechanism. As for the mechanism for the increase in the intracellular Ca^{2+} level, both inhibition of sarcoplasmic/ER Ca^{2+} ATPase (sarco endoplasmic reticulum Ca^{2+} ATPase; an ER-located Ca^{2+} pump that is responsible for accumulation of Ca^{2+} in the ER) and stimulation of the influx of extracellular Ca^{2+} have been proposed (Johnson *et al.*, 2002; Wang *et al.*, 2004). We recently found that all of the NSAIDs tested permeabilize cellular membranes and this capacity of NSAIDs is closely related to their activity for increasing intracellular Ca^{2+} concentration, suggesting that NSAIDs stimulate the influx of extracellular Ca^{2+} by permeabilizing cytoplasmic membranes (Tomisato *et al.*, 2004a; Tanaka *et al.*, 2005). Since the suppression of celecoxib-dependent GRP78 upregulation by BAPTA-AM was partial, a further mechanism other than an increase in the intracellular Ca^{2+} level may be involved in the upregulation of GRP78. Accumulation of unfolded proteins in the ER by inhibition of the proteasome system may be involved in this upregulation; it was suggested that NSAIDs inhibit the proteasome system and that specific inhibitors of the proteasome system, such as MG132, induce the ER stress response (Bush *et al.*, 1997; Huang *et al.*, 2002).

Overproduction of GRP78 in cells was reported to make them resistant to apoptosis induced by topoisomerase inhibitors, tunicamycin or Ca^{2+} ionophores (Morris *et al.*, 1997; Reddy *et al.*, 2003). We showed here that this phenomenon can be applied to celecoxib-induced apoptosis and that the antiapoptotic effect of GRP78 is stimulated by coexpression of its cochaperones, ERdj3 and ERdj4. This effect of ERdj4 depends on its J domain, as was the case for stimulation of the antiapoptotic effect of HSP70 by HSP40 (Gotoh *et al.*, 2001). Since the J domain of ERdj4 is essential for its interaction with GRP78 and its activation of GRP78 ATPase activity, the stimulation by ERdj4 (and also ERdj3 presumably) of the antiapoptotic activity of GRP78 should be mediated by this physical and functional interaction between them.

We previously used CHOP-deficient mice or a dominant-negative form of CHOP to show that CHOP, a transcription factor with apoptosis-inducing activity, is essential for NSAID-induced apoptosis (Tsutsumi *et al.*, 2004). Since induction of CHOP by celecoxib is partially suppressed by the overproduction of GRP78, it is reasonable to speculate that this suppression is involved in the antiapoptotic effect of GRP78. It was suggested that GRP78 binds to PERK (and also IRE1 and ATF6) and inactivates PERK's ER stress response inducing capabilities under nonstress conditions; when ER stressors accumulate unfolded proteins in the ER, PERK (and also IRE1 and ATF6) is activated to induce the ER stress response by releasing GRP78 from PERK, as a result of the binding of unfolded proteins to GRP78 (Bertolotti *et al.*, 2000; Shen *et al.*, 2002a). Induction of GRP78 by proteasome inhibitors has been reported (Hong *et al.*, 2004). It was reported that overexpression of GRP78 inhibited the phosphorylation of PERK in the presence of an ER stressor (thapsigargin) (Bertolotti *et al.*, 2000). Therefore, we consider that by this mechanism, overproduced GRP78 suppressed the celecoxib-dependent CHOP induction and resulting apoptosis. It was also suggested that GRP78 inhibits topoisomerase inhibitor-induced apoptosis by inhibiting

caspase-7 (Reddy *et al.*, 2003), which may also be involved in the antiapoptotic effect of GRP78 against NSAIDs.

Resistance to chemotherapy is one of the major obstacles of cancer therapy. Owing to poor vascularization, solid tumors usually exist under conditions of glucose starvation and hypoxia, which causes induction of the ER stress response. In fact, overproduction of ER chaperones was reported in various types of tumors and artificial overproduction of ER chaperones stimulated tumor progression (Jamora *et al.*, 1996; Koomagi *et al.*, 1999; Fernandez *et al.*, 2000; Song *et al.*, 2001; Huo *et al.*, 2004). Therefore, the finding in this study that overproduction of GRP78 makes cancer cells resistant to celecoxib is very important in considering the use of celecoxib in chemotherapy; it seems that not only constitutively overproduced ER chaperones in tumors but also ER chaperones induced by NSAIDs can make them resistant to chemotherapy by NSAIDs. In fact, we showed that orally administered celecoxib caused both inhibition of xenograft tumor growth and induction of GRP78 simultaneously in nude mice. We consider that GRP78 upregulation by celecoxib decreases the activity of this drug for the suppression of tumor growth and thus propose that an inhibitor of ER chaperones may be of clinical benefit by making tumor cells more responsive to NSAIDs.

Materials and methods

Chemicals, plasmids and animals

RPMI 1640 medium was obtained from Nissui Pharmaceutical Co. Fetal bovine serum (FBS) was obtained from Gibco Co. Pluronic F127, fluo-3/AM and BAPTA-AM were from Dojindo Co. Thapsigargin, staurosporine, diclofenac, RNaseA and propidium iodide (PI) were obtained from Sigma Co. PGE_2 and indomethacin were obtained from Wako Co. Celecoxib was from LKT Laboratories Inc. Nimesulide was from Cayman Chemical Co. Antibodies against GRP78, ATF6, lamin, ATF4 and actin were purchased from Santa Cruz Biotechnology Inc., and those against phosphorylated PERK and phosphorylated eIF2α were from Cell Signaling Technology Inc. The RNeasy kit, siRNAs and RNAiFect transfection reagent were from Qiagen. A first-strand cDNA synthesis kit was purchased from Amersham. Lipofectamine (TM2000) and pcDNA3.1 plasmid were obtained from Invitrogen. SYBR GREEN PCR Master Mix was from ABI. A plasmid of pEGFP-N1 was obtained from Clontech. Plasmids of pcDNA3.1/GRP78 and pCR3.1/ERdj3 were gifts from Drs Austin R and Haslam D, respectively. Plasmids of pcDNA3.1/ERdj4 and pcDNA3.1/ERdj4ΔJ were a gift from Dr K Imaizumi. Female ICR nude mice (5 weeks of age) were obtained from the Kyudoh Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health and were approved by the Animal Care Committee of Kumamoto University.

Cell culture and overexpression of ER chaperones

Cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO_2 at 37°C. NSAIDs were dissolved in DMSO and control experiments (without NSAIDs) were performed in the presence of the same concentrations of DMSO. Cells were exposed to NSAIDs by changing the medium. Unless otherwise noted, cells (0.8×10^4 cells per well in 24-well plates, 4×10^4 cells per well in six-well plates, 6×10^5 cells in 100-mm plates) were cultured for 24 h and used in experiments. For transient expression of each gene, cells were seeded 24 h before transfection in six-well plates at a density of 5×10^5/well. The transfection with pcDNA3.1 containing each gene was carried out using Lipofectamine (TM2000) according to the manufacturer's instructions. Cells were used for experiments after a 48 h recovery period. Transfection efficiency was determined in parallel plates by transfection of the pEGFP-N1 control vector. Transfection efficiency was more than 80% in all experiments.

Analysis of apoptosis by fluorescence-activated cell sorting (FACS)

Apoptosis was monitored by FACS analysis as described previously (Alves da Costa *et al.*, 2002). Briefly, cells were cultured in 100-mm plates and collected by centrifugation. Pellets were fixed with 70% ethanol for 4 h at −20°C and centrifuged again. Pellets were resuspended in phosphate-citrate buffer (0.2 M Na_2HPO_4 and 4 mM citric acid) and incubated for 20 min at RT. After centrifugation, the pellets were resuspended in DNA staining solution (50 mg/ml PI and 10 μg/ml RNaseA) and incubated for 20 min at RT. Samples were scanned with a FACSCalibur (Becton Dickinson) cell sorter under conditions to measure only specific PI-mediated fluorescence. The signal threshold was determined based on the fluorescence of nontransfected cells. Apoptotic cells appeared as a hypodiploid peak due to nuclear fragmentation and loss of DNA.

Real-time RT–PCR analysis

Total RNA was extracted from cells using an RNeasy kit according to the manufacturer's protocols. Samples (10 μg RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was applied to real-time RT–PCR (ABI PRISM 7700) using SYBR GREEN PCR Master Mix and analysed with ABI PRISM 7700 Sequence Detection Software according to the manufacturer's instructions. Real-time cycle conditions were 2 min at 50°C, followed by 10 min at 90°C and then for 45 cycles at 95°C for 30 s and 63°C for 60 s. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, the actin gene was used as an internal standard.

Primers were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers are listed as follows: gene name: forward primer, reverse primer. ATF4: 5′-tcaaacctcatgggttctcc-3′, 5′-gtgtcatccaacgtggtcag-3′; AT F2: 5-ctccagctcacacaactcca-3′, 5′-gtttcagctgtgccacttca-3′; ATF3: 5′-atgatgcttcaacacccaggc-3′, 5′-ttagctctgcaatgttccttcc-3′; CHOP: 5′-tgcctttctcttcggacact-3′, 5′-tgtgacctctgctggttctg-3′; GRP78: 5′-tag cgtatggtgctgctgtc-3′, 5′-tttgtcaggggtctttcacc-3′; ERdj3: 5′-cggt tccgaatcaaagttgt-3′, 5′-cttccatagcttcgctcctg-3′; ERdj4: 5′-aaaataag agcccggatgct-3′, 5′-cgcttcttggatccagtgtt-3′; actin: 5′-ggacttcgagcaa-gagatgg-3′, 5′-agcactgtgttggcgtacag-3′.

Immunoblotting analysis

Whole-cell extracts were prepared as described previously (Tsutsumi *et al.*, 2002). The protein concentration of samples

was determined by the Bradford method. Samples were applied to 8% (for PERK and GRP78) or 10% (for eIF2α and actin) polyacrylamide SDS gels, subjected to electrophoresis, and proteins then immunoblotted with respective antibodies.

Xenograft tumor growth
Effect of celecoxib on xenograft tumor growth was examined as described previously (Kulp *et al.*, 2004). Each nude mouse was inoculated s.c. in the right hind footpad with 2×10^6 cells of MKN-45 suspended in 0.1 ml of serum-free medium. When tumors reached a mean volume of $116 \pm 34\,mm^3$, mice started to receive single daily oral administration of celecoxib (100 or 200 mg/kg (body weight)) in 1% methylcellulose and this administration was continued for the duration of the study. Control mice received vehicle (1% methylcellulose only). Tumors were measured weekly using calipers and their volumes calculated using the following standard formula: $width^2 \times length \times 0.5$. Body weights were measured daily. For examination of GRP78 expression in tumors, tumor xenografts (dissected into <1-mm pieces) were solubilized with RIPA buffer (50 mM Tris-HCl (pH7.2), 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.05% SDS) and subjected to immunoblotting analysis.

Measurement of intracellular Ca^{2+} levels
The intracellular Ca^{2+} levels were monitored according to manufacture's protocols (Kao *et al.*, 1989). Cells were washed with assay buffer (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). Cells were then incubated with 4 μM fluo-3/AM in the assay buffer also 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 in assay buffer also containing 2 mM probenecid. Fluo-3 fluorescence of cells in a water-jacketed cuvette (1.6×10^6 cells/cuvette) was measured with a HITACHI F-4500 spectrofluorophotometer by recording excitation signals at 490 nm and the emission signal at 530 nm at 1-second 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 fluorescent dye–Ca^{2+} complex.

SiRNA targeting of genes
We used siRNA of 5′-ggagcgcauugauacuagadTdT-3′ and 5′-ucuaguaucaaugcgcuccdTdT-3′ or 5′-gccuaggucucuuagaugadTdT-3′ and 5′-ucaucuaagagaccuaggcdTdT-3′ as annealed oligonucleotides for repressing GRP78 or ATF4 expression, respectively. AGS cells were transfected with siRNA using RNAiFect transfection reagent according to the manufacturer's instructions. Nonsilencing siRNA (5′-uucuccgaacgugucacgudTdT-3′ and 5′-acgugacacguucggagaadTdT-3′) was used as a negative control.

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

Abbreviations

ATF, activating transcription factor; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′N′*-tetraacetic acid; CHOP, C/EBP homologous transcription factor; COX, cyclooxygenase; eIF2α, eukaryotic initiation factor-2α; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; IRE1, protein-kinase and site-specific endoribonuclease; NSAIDs, non-steroidal anti-inflammatory drugs; PERK, eukaryotic translation initiation factor 2 kinase; PG, prostaglandin; PI, propidium iodide; SERCA, sarco endoplasmic reticulum Ca^{2+} ATPase; siRNA, small interfering RNA; XBP-1, X box binding protein.

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