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 $K_{\text{at}}$-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-
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 calcine-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 mol/mol, 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 homogeneous 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 (Na₂HPO₄-NaH₂PO₄, pH 6.8) containing 150 mM NaCl) to remove untrapped calcein. The final liposome precipitate was resuspended in 5 ml of buffer A. A 6.8) containing 150 mM NaCl to remove untrapped calcein. The release of calcein from liposomes was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm).

Assay for K⁺ Efflux from Cells. K⁺ efflux from cells was monitored as described previously (Katsu et al., 1987), with some modifications. Cells were washed twice with buffer A and then suspended in fresh buffer A (2.4 × 10⁶ cells/ml). After incubation with NSAIDs for 10 min at 37°C, K⁺ efflux from the cells was measured with a K⁺ ion-selective electrode.

Fluorescence Polarization. Membrane fluidity was measured using the fluorescence polarization technique (Makise et al., 2002). Diphenylhexatriene (1%, mol/mol, of egg PC) was used as a fluorescence probe. Liposomes were prepared using a reversed-phase evaporation method similar to that used in the membrane-permeability experiments, except for the addition of 1 ml of buffer A instead of 100 mM calcine-NaOH. Measurements were carried out using a Hitachi F-4500 fluorospectrophotometer (Hitachi Software Engineering, Yokohama, Japan). The degree of polarization (P) was calculated according to the following equation: 

\[ P = \frac{I_{VV} - I_{HH}}{I_{VV} + I_{HH}} \]

where \( I_{VV} \) and \( I_{HH} \) refer, respectively, to the vertical and horizontal orientations of the excitation (first) and emission (second) polarizers. \( C_I = \frac{I_{VV}}{I_{HH}} \) is a correction factor.

Statistical Analyses. All results were expressed as the mean ± S.E.M. One-way analysis of variance 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.](image-url)
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.](https://example.com/fig2.png) 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 $5 \mu$M ($5 \times 10^{-6}$ M) in patients (data from interview form from the manufacturer). The maximum concentration of GGA at gastric mucosa should be higher,
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.](image)

### TABLE 1

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<tr>
<th>NSAIDs</th>
<th>Degree of Polarization</th>
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<td></td>
<td>M</td>
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<tr>
<td>Control</td>
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<tr>
<td>Indomethacin</td>
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<td></td>
<td>2 mM</td>
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<td>Diclofenac</td>
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<td>5 mM</td>
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<td>10 mM</td>
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<tr>
<td>Celecoxib</td>
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* 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.

References


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