Geranylgeranylacetone Protects Membranes Against Non-steroidal Anti-inflammatory Drugs

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ABBREVIATIONS: NSAID, non-steroidal anti-inflammatory drug; GGA, geranylgeranylacetone; HSP, heat shock protein; COX, cyclooxygenase; PG, prostaglandin; FBS, fetal bovine serum; PC, phosphatidylcholine.
ABSTRACT

Direct gastric mucosal cell damage mediated by non-steroidal anti-inflammatory drugs (NSAIDs) is involved in the formation of NSAID-induced gastric lesions. We recently suggested that this direct cytotoxicity of NSAIDs is due to their membrane permeabilization activity. Geranylgeranylacetone (GGA), a clinically used anti-ulcer 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 efficacious even when co-administered with NSAIDs, and was also able to restore membrane fluidity that had been compromised by NSAIDs. This mechanism appears to play an important role in the anti-ulcer activity of GGA.
Non-steroidal 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 about 15-30% of chronic users suffering from gastrointestinal ulcers and bleeding (Barrier and Hirschowitz, 1989; Fries et al., 1989; Gabriel et al., 1991; Kurata and Abbey, 1990). In the United States alone, about 16,500 people per year die as a result of these complications (Singh, 1998). Therefore, in general, anti-ulcer drugs are prescribed in combination with NSAIDs in order to prevent the NSAID-induced side effects.

Geranylgeranylacetone (GGA) was developed in Japan and has become the leading anti-ulcer 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; Pappas et al., 1987; Terano et al., 1986). Various mechanisms have been proposed for this protective effect of GGA. First, it stimulates the synthesis of mucus (Bilski et al., 1987; Rokutan et al., 2000; Terano et al.,
and increases mucosal blood flow, an important factor in maintaining the integrity of the mucosa (Kunisaki and Sugiyama, 1992). It has also recently been reported 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; Takano et al., 2002; Tomisato et al., 2001b). 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 co-administered 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 thought 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, since the increased incidence of
gastric lesions and the decrease in PG levels induced by NSAIDs do not always occur in parallel (Ligumsky et al., 1983; Ligumsky et al., 1990). We have previously demonstrated that NSAIDs induce \textit{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 \textit{in vivo} (Tomisato et al., 2004b; Tomisato et al., 2001a). Furthermore, we have recently shown 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 that a clinically used anti-ulcer 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 (FBS) was purchased from Gibco Co. (Grand Island, New York). RPMI 1640 was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Indomethacin was purchased from Wako Co. (Tokyo, Japan), while ibuprofen, diclofenac, mefenamic acid, flufenamic acid and ketoprofen came from Sigma Co. (Tokyo, Japan). Nimesulide and flurbiprofen were obtained from Cayman Chemical Co. (Ann Arbor, Michigan), and egg phosphatidylcholine (PC) from Kanto Chemicals Co. (Tokyo, Japan). GGA was kindly provided by Eisai Co. (Tokyo, Japan). Celecoxib was purchased from LKT Laboratories, Inc. (St. Paul, Minnesota). 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% FBS. Cells were exposed to NSAIDs by replacement of the entire bathing medium with fresh medium containing the NSAID
under investigation. NSAIDs were dissolved in DMSO; control experiments (without NSAIDs) were performed in the presence of the same concentration of DMSO.

**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 (Na₂HPO₄-NaH₂PO₄) (pH 6.8) containing 150 mM NaCl) to remove untrapped calcein. The final liposome precipitate was re-suspended in 5 ml 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. The release of calcein from liposomes was determined by measuring fluorescence intensity at
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 ($2.4 \times 10^6$ cells/ml). After incubation with NSAIDs for 10 min at $37^\circ$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 in the membrane permeability experiments, except for the addition of 1 ml of buffer A instead of 100 mM calcein-NaOH. Measurements were carried out using an Hitachi F-4500 fluorospectrophotometer. The degree of polarization ($P$) was calculated according to the following equation:

$$P = (I_{VV} - C_I_{VH}) / (I_{VV} + C_I_{VH})$$
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 ± standard error (S.E.M.). One-way analysis of variance (ANOVA) followed by Scheffe’s multiple comparison was used for evaluation of differences between the groups. A Student's $t$-test for unpaired results was performed to evaluate differences between two groups. Differences were considered to be significant for values of $P<0.05$. 
Results

GGA Suppresses NSAID-induced Membrane Permeabilization. We have recently reported that some NSAIDs (celecoxib, mefenamic acid, flufenamic acid, nimesulide, 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 due to 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.

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. 2B 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 non-specific.

**GGA Protects Membranes against NSAIDs even when Co-administered.**

As for the experiments described above, calcein-loaded liposomes were pre-incubated with GGA and subsequently treated with various NSAIDs in the presence of the same concentration of GGA as in the pre-incubation step. As shown in Fig. 3A, GGA suppressed the indomethacin-induced membrane permeabilization under these conditions ("pre-treated" in Fig. 3A). However, a similar result was obtained even when GGA was added simultaneously with indomethacin ("co-treated" in Fig. 3A). Treatment with GGA and either diclofenac or celecoxib (Fig. 3B 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 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. Since a similar effect was observed in calcein-loaded liposomes, which consist only of phospholipids (without membrane proteins), the membrane stabilization activity of GGA appears to be mediated by its direct interaction with phospholipids, a conclusion supported by the observation that GGA increases membrane fluidity of PC liposomes. This is the first report that a clinically used anti-ulcer 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 about 5 $\mu$M ($5 \times 10^{-6}$ M) in patients (data from interview form from the company). The maximum concentration of GGA at gastric mucosa should be higher, suggesting that concentrations of GGA used in this study are clinically significant.

Based on our previous studies (see below), we consider that this novel activity of GGA is involved in its anti-ulcer 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 \textit{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 due to 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 vs. ED$_{20}$ values for membrane permeabilization yielded an $r^2$ value of 0.94 or 0.93, respectively (Tomisato et al., 2004a and Tanaka et al., unpublished results). 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 anti-ulcer drugs.

As outlined in the Introduction, GGA has a number of pharmacological
activities that are believed to be involved in its anti-ulcer activity. These include
stimulating the synthesis of gastric mucus, increasing gastric mucosal blood flow and
inducing HSPs in gastric mucosal cells (Bilski et al., 1987; Hirakawa et al., 1996;
Kunisaki and Sugiyama, 1992; Mizushima et al., 1999; Takano et al., 2002; Terano et al.,
1986; Tomisato et al., 2000). However, these activities cannot be 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, it is possible that longer-term
indirect actions of GGA may also play a role in its anti-ulcer activity, and that the different
time-courses of these effects could confer a clinical advantage.
In the present study we have 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 previously shown that most but not all 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 can not 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 thought to be important for maintaining mucosal integrity. Lichtenberger and his co-workers 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 decrease in gastric surface hydrophobicity and gastric lesion score in rats (Darling et al., 2004; Lichtenberger et al., 1995). They also suggested that a 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 how the effect of GGA on membrane fluidity and its influences on anti-ulcer activity.
References


Foot note

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LEGENDS FOR FIGURES

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).

Fig. 2. Effect of GGA on membrane permeabilization.

Calcein-loaded liposomes were pre-incubated 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 are mean ± S.E.M. (n=3).

***P<0.001, **P<0.01, *P<0.05.

Fig. 3. Rapid protection of membranes by GGA.
Calcein-loaded liposomes were either pre-incubated with GGA for 10 min then incubated with NSAID in the presence of GGA (pre-treated), simultaneously incubated with GGA and NSAID (co-treated), or pre-incubated 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 of Fig. 1. Values 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 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 are mean ± S.E.M. (n=3). ***P<0.001; **P<0.01; *P<0.05.

**Fig. 5.** Effect of GGA on K⁺-efflux from cells in the presence of NSAIDs.

AGS cells were pre-incubated 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 of Fig. 4. Values are mean ± S.E.M. (n=3). ***$P<0.001$; **$P<0.01$. 
Table 1
Effect of GGA on membrane fluidity in the presence or absence of various NSAIDs

<table>
<thead>
<tr>
<th>NSAIDs (mM)</th>
<th>Degree of polarization (P) + GGA (M)</th>
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<tbody>
<tr>
<td></td>
<td>PC 10⁻⁵</td>
</tr>
<tr>
<td>Control</td>
<td>0.121 ± 0.007</td>
</tr>
<tr>
<td>Indomethacin 1</td>
<td>0.172 ± 0.033</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Diclofenac 2</td>
<td>0.149 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Celecoxib 0.1</td>
<td>0.133 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
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<td>1</td>
</tr>
</tbody>
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The degree of polarization (P) of PC liposomes in the presence of GGA and/or various NSAIDs was measured as described in 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 are mean ± S.E.M. (n=3). ***P<0.001; **P<0.01; *P<0.05.
Figure 1 (MOL 15784)

![Graph showing calcein release (%) vs. NSAIDs (mM) for different NSAIDs: Ketoprofen, Etodolac, Indomethacin, Ibuprofen, Nimesulide, Flurbiprofen, Mefenamic acid, Celecoxib, Diclofenac, and Flufenamic acid.](molpharm.aspetjournals.org)
Figure 2 (MOL 15784)

A. Indomethacin 6 mM

B. Diclofenac 2 mM

C. Celecoxib 0.08 mM

D. Ethanol 10%
Figure 3 (MOL 15784)

A  
Indomethacin 6 mM

B  
Diflunisal 4 mM

C  
Celecoxib 0.12 mM

Calcein release (%)
Figure 4 (MOL 15784)

A

![Bar graph showing K⁺ efflux (%)]

- Indomethacin (mM): 0.5, 1, 2, 4

B

![Bar graph showing K⁺ efflux (%)]

- Diclofenac (mM): 0.5, 1, 2, 4

C

![Bar graph showing K⁺ efflux (%)]

- Celecoxib (mM): 0.05, 0.1, 0.2, 0.4
Figure 5 (MOL 15784)