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Interaction of Indomethacin and Naproxen with Gastric Surface-active Phospholipids: A Possible Mechanism for the Gastric Toxicity of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

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ABSTRACT. The possibility that the molecular mechanism underlying the topical gastric irritancy of nonsteroidal anti-inflammatory drugs (NSAIDs) may involve alterations in the surface-active properties of gastric phospholipids was investigated. Indomethacin and naproxen were intragastrically administered to rats and the hydrophobicity of the luminal surface of the stomach wall was assessed by contact angle analysis. Both NSAIDs have the ability to attenuate the phospholipid-related hydrophobic properties of the gastric mucosa by more than 80–85% in a dose-dependent fashion. Potential molecular interactions between both NSAIDs and surface-active phospholipids were analyzed using fluorescent probes. Indomethacin has the ability to displace, in a dose-dependent manner, ANS (1-anilino-8-naphthalene sulphonate), a fluorescent anionic probe previously bound to the head group of phosphatidylcholine molecules. Estimations of the resonance fluorescence transfer between naproxen and the surface probe ANS or the hydrophobic probe, pyrene, bound to dipalmitoylphosphatidylcholine (DPPC) vesicles revealed that naproxen diffuses within the phospholipid bilayers. The dynamic of the gastric lipid material extracted from the surface scraping material (SSM) of the mucosa was altered by the NSAID as shown by the increase in the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) (at 25°, $r_{SSM} = 0.106 \pm 0.006$, $r_{SSM + indomethacin} = 0.137 \pm 0.005$, and $r_{SSM + naproxen} = 0.133 \pm 0.007$, $P < 0.001$). The thermodynamic behavior of a model bilayer containing DPPC was also perturbed by the NSAIDs tested. These results provide evidence that NSAIDs may reduce the ability of gastric surface-active phospholipids to form a hydrophobic protective layer. BIOCHEM PHARMACOL **57**;3:247–254, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. gastropathy; hydrophobicity; lipid dynamic; nonsteroidal anti-inflammatory drugs; gastric surfactant; liposomes

The relationship between the oral intake of NSAIDs‖ and the incidence of hemorrhage and ulceration in the stomach has been clearly demonstrated and correlated with life-threatening complications such as ulcer perforation [1, 2]. The rational development of safer NSAIDs will depend upon the understanding of the processes initiating and promoting gastric injury. Such mechanisms are complex and the cascade of events leading to mucosal damage has yet to be characterized.

The discovery by Vane and colleagues [3] of the inhibitory effect of aspirin on prostaglandin synthesis provided new insight into both the mechanisms of NSAIDs' therapeutic activities and side-effects. However, the sequence of events resulting from cyclooxygenase inhibition does not totally explain the overall gastric toxicity of aspirin and other NSAIDs. The pathogenesis of gastric mucosal damage induced by aspirin-like drugs can also be related to their topical irritancy. Davenport suggested, first, that the normal resistance of the gastric mucosa to back-diffusion of luminal acid can be disrupted by topical administration of lipid soluble damaging agents such as acetylsalicylic acid or aspirin [4]. Evidence of the direct superficial damaging effect of other drugs that are members of the NSAID family was subsequently provided by many investigators who showed histological, biochemical, and permeability changes

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‖ *Abbreviations:* ANS, 1-anilino-8-naphthalene sulphonate; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; GI, gastrointestinal; NSAID, nonsteroidal anti-inflammatory drug; and SSM, surface scraping material.

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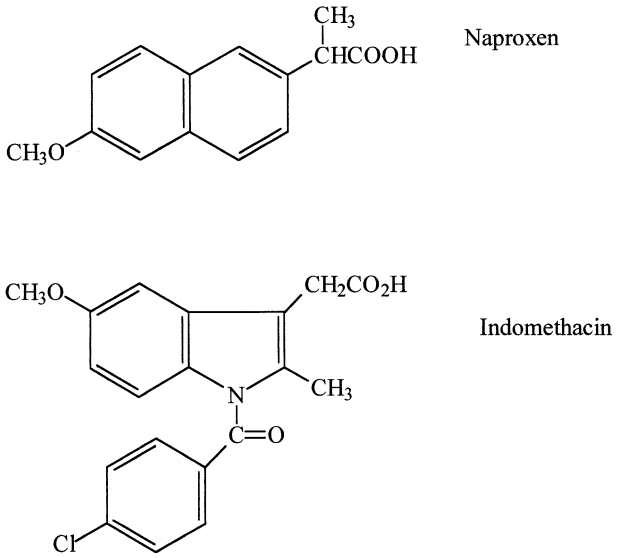


FIG. 1. Chemical structure of naproxen and indomethacin.

in the gastric mucosa [5, 6]. However, the "barrier-breaking" activity of the drugs has not been established on a molecular basis.

The acid-resistant barrier properties of the stomach are attributable to a multiplicity of factors. Among them, the extracellular lining of surfactant-like phospholipids on the surface and within the mucus gel layer represents an initial line of defense of the stomach and confers hydrophobic, non-wettable, and acid-resistant properties to the mucosa [7]. Oral administration of aspirin has the ability to rapidly transform the gastric mucosa from a hydrophobic to a more hydrophilic state [8].

In the present study, we established the ability of two nonsalicylate NSAIDs, indomethacin and naproxen (Fig. 1), to affect gastric mucosal hydrophobicity in the rat and then proceeded to study the molecular mechanism which may be involved. We used different and complementary fluorescent spectroscopic techniques to evaluate their capacity to bind to surface-active phospholipids as well as their effect on the lipid dynamic.

MATERIALS AND METHODS

Chemicals

Indomethacin, naproxen, DPPC, and 1,6-diphenyl-1,3,5-hexatriene were purchased from SIGMA. Dipalmitoylphosphatidylglycerol was purchased from Calbiochem, and 8-anilino-1-naphthalene sulfonate (ANS) and pyrene from Molecular Probes Co. The compounds were used without further purification.

Animals

Male Sprague–Dawley rats (Harlan Sprague-Dawley Inc.) weighing 150–200 g were fasted for 18–20 hr before experiments and water was provided *ad lib*. All animal protocols were reviewed and approved by our institution's Animal Welfare according to the NIH guidelines in the care and treatment of laboratory animals.

Contact Angle Analysis

Rats were intragastrically administered saline or soluble NSAID at various doses. Animals were killed at different times after administration of NSAID-test formulations by an overdose of ether and the stomachs were removed, opened along the greater curvature, blotted, and dried. The contact angle formed at the air-liquid-tissue interface by a droplet of water (5 μL) applied to the surface of the oxyntic region of the stomach was measured with a goniometer as an index of gastric mucosal hydrophobicity [9].

Preparation of Gastric Lipid Vesicles

The surface of the oxyntic region of the stomach was gently scraped with a spatula [10]. The collected SSM was extracted in chloroform–methanol (2:1) according to the Folch lipid extraction methods [11] and shaken overnight at 4°. After centrifugation, the chloroform phase was separated and dried under nitrogen. The dried film of lipids was sonicated in PBS to form liposomes (30 sec at 40 mW with a titanium probe sonicator) [10].

Preparation of DPPC Liposomes

DPPC was dissolved in chloroform. The solvent was removed by evaporation under a stream of nitrogen gas, followed by overnight drying under vacuum. Distilled water was added to the dried film of lipids. To prepare multilamellar vesicles for ANS binding and liposome precipitation experiments, a volume of buffer (PBS, pH 7.4 or citrate buffer, pH 4) was added to the dry film. The samples were then sonicated in a bath-type sonicator (Laboratory Supplies Company) for 15 min [8]. The temperature reached a value above the transition phase temperature of the phospholipids.

To prepare small unilamellar vesicles for the energy transfer and polarization fluorescence studies, the dry film of DPPC was resuspended in PBS or the NSAID solution and then sonicated at 30 mW with a Branson-type model microprobe sonicator. According to the conventional procedure, repetitive 30-sec cycles of sonication/ice cooling were performed. These conditions were previously verified to result in the generation of over 90% unilamellar sealed vesicles [12]. Samples were briefly centrifuged to remove titanium particles.

Binding of ANS

The binding of ANS to phosphatidylcholine bilayers results in an increase of its fluorescence quantum yield. The intensity of fluorescence has been reported to show a linear relationship with the concentration of bound ANS [13]. The binding of ANS to phospholipids was monitored by

measuring fluorescence intensity (excitation = 360 nm, emission = 480 nm) at 37° with a fluorescence spectrophotometer (Perkin-Elmer LS-3). Aliquots (20 μL) of ANS (1 mg/mL) were added to 3 mL of DPPC vesicles (1 mg/mL) in a 1-cm quartz cuvette until the fluorescence intensity reached a plateau, indicating that an equilibrium of the probe binding to the surface of vesicles had been reached [14]. At this point, increasing amounts of NSAID or PBS were added and the fluorescence intensity recorded. The relative binding fluorescence of ANS was calculated as a percentage of the maximum binding in absence of the drug.

This technique allowed us to investigate the effects of indomethacin on ANS binding to DPPC vesicles but was not suitable for studying the effect of naproxen, as the NSAID quenches the fluorescence of the probe ANS.

Fluorescence Resonance Energy Transfer Experiment

DPPC liposomes (1 mg/mL) were incubated with the fluorescent probes 1-OH pyrene dissolved in ethanol (final concentration 10^{-7} M) or ANS dissolved in PBS (final concentration 3.10^{-4} M). Fluorescence excitation spectra of ANS and pyrene were then recorded. The fluorescence emission spectrum of naproxen was also recorded after incubation of the drug with liposomes. The emission-excitation spectral overlap between naproxen/ANS and naproxen/pyrene suggested that they would form efficient donor–acceptor pairs for fluorescence energy transfer. The distance R_0 at which energy transfer is 50% efficient (i.e. 50% of fluorescence of the donor is quenched by the acceptor) was defined by the Förster equation:

$$R_0(\text{Å}) = 9.79.10^3(\kappa^2\, q J n^{-4})^{1/6}$$

where κ is an orientation factor, q is the fluorescence quantum yield of the donor in the absence of acceptor molecules, and n is the refractive reflex of the medium.

$$J(\text{cm}^3\,\text{M}^{-1}) = \int \varepsilon_A(\lambda) \cdot F_D(\lambda) \cdot \lambda^4\, d\lambda$$

where ε_A is the extinction coefficient of the acceptor and F_D the fluorescence emission intensity of the donor as a fraction of the total integrated intensity. J represents the normalized overlap between donor emission and acceptor excitation spectrum and was calculated from the recorded spectra. The other values were taken as n = 1.4, q = 0.8, and κ = ⅔ [15].

The fluorescence intensity (F_0) of the donor (naproxen) in the absence of acceptor and the fluorescence intensity (F) of the donor in the presence of the acceptor were recorded (emission = 360 nm; excitation = 230 nm). The efficiency of energy transfer for both sets of donor–acceptor pairs was calculated according to the relationship [16]: $E = 1 - (F/F_0)$.

Liposome Precipitation Experiment

We estimated the percentage of NSAID molecules bound to DPPC liposomes according to a technique that is based on the ability of heavy metals to bind to phospholipid vesicles, thereby increasing their density [17]. One hundred μL of DPPC liposomes-NSAID (molar ratio 1:1) were diluted with 100 mL of saline and mixed with 150 μL of a solution containing 0.1 M manganese chloride, 500 U/mL of heparin and 30 mg/mL of NaCl. The mixture was vortexed for 2 min and allowed to stand for 10 min before centrifugation (1000 *g*). Supernatant and precipitate were separated and diluted in $NaCO_3$ (2.6 mg/mL in saline).* Absorption of indomethacin (290 nm) or naproxen fluorescence (excitation = 230 nm, emission = 360 nm) were measured in both supernatant and precipitate.

Fluorescence Anisotropy Measurement

A fresh dispersion (2.75 mL) of DPH in PBS (DPH 2 μM) was incubated for 45 min at 25° with 250 μL of SSM lipid extract or a suspension of DPPC unilamellar liposomes (DPH/lipid molar ratio 1:400). The fluorescence anisotropy, r, was calculated from fluorescence measurements on an AMICO SPF 500 spectrofluorometer:

$$r = (I_{//} - I_{\perp})/(I_{//} + 2I_{\perp})$$

where $I_{//}$ and $I_{\perp}$ are the fluorescence intensities of DPH measured in directions parallel and perpendicular to the electric vector of the exciting light (emission = 460 nm, excitation = 350 nm). In all experiments, the amount of lipidic material used was chosen carefully to avoid any problem of light scattering [10].

Statistical Analysis

In most of the cases, data are expressed as means ± SEM. Statistical difference between groups was determined by Student's *t*-test and was considered significant when $P < 0.05$.

RESULTS

Effect of NSAID on the Surface hydrophobicity of the Gastric Mucosa

Intragastrically administered NSAID induced a significant decrease in the hydrophobicity of the mucosa as assessed by contact angle measurement (Figs. 2 and 3). The acute effect of the drugs on the non-wettable properties of the mucosa is dose-dependent. A maximal reduction of the contact angle of 80% was obtained after oral administration of 50 mg/kg of indomethacin (Fig. 2A) or 80 mg/kg of naproxen (Fig. 3A). The doses of NSAID used in our experiment have been shown to induce gastrointestinal damage, ranging from ultrastructural changes to macroscopic lesions in rat model systems. The time-course study of naproxen-

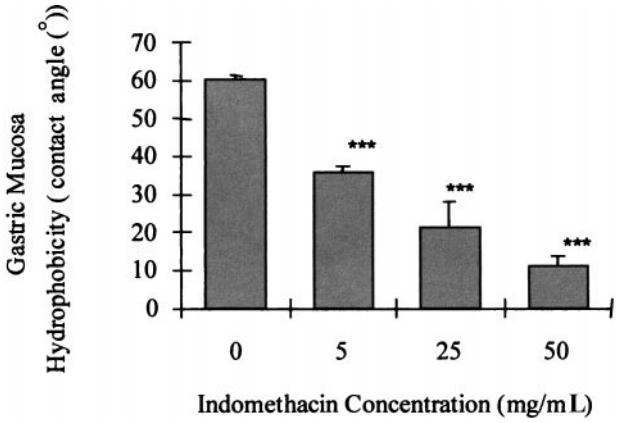
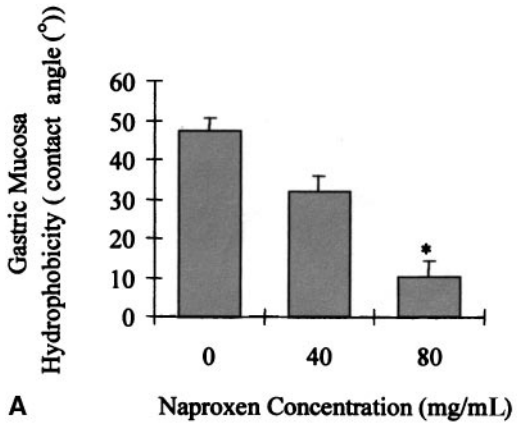


FIG. 2. Dose-response analysis of the ability of indomethacin to reduce gastric mucosal hydrophobicity. Animals were challenged intragastrically with indomethacin 2 hr before contact angle analysis. Results are expressed as means ± SEM from 5 animals. (***$P < 0.001$ vs control values).



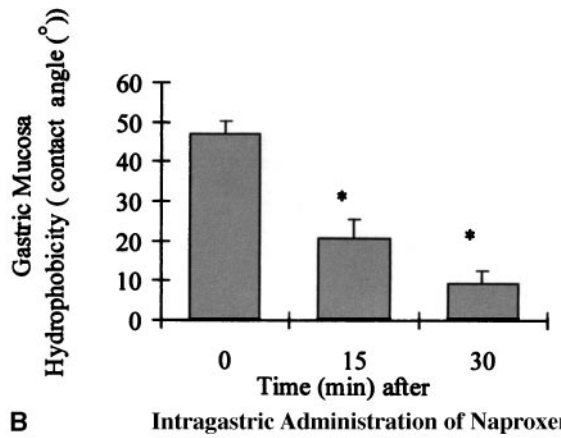
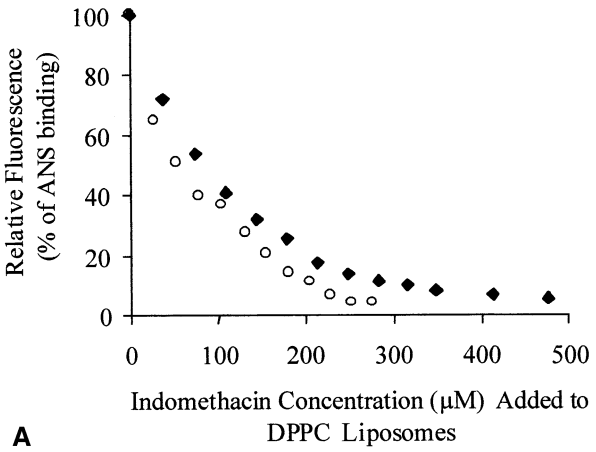


FIG. 3. Ability of naproxen to reduce gastric mucosal hydrophobicity. Results are expressed as means ± SEM from 4–9 animals. (*$P < 0.001$). (A) Dose-response study where animals intragastrically received 1 mL of naproxen 1 hr before contact angle analysis. (B) Time-dependence analysis where animals received a single dose of naproxen (80 mg/kg).



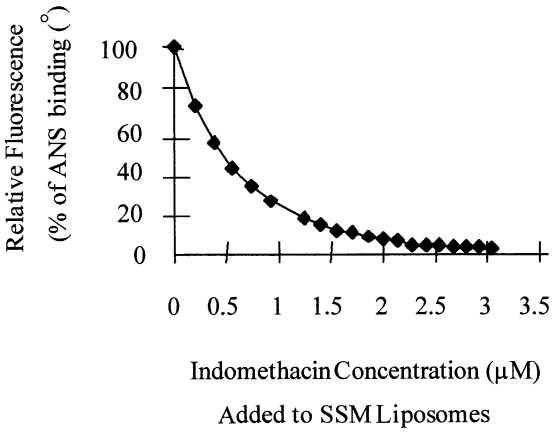


FIG. 4. Dose-dependent effect of indomethacin on the percent reduction of fluorescence of ANS bound to DPPC liposomes at pH = 7.4 (◆) or pH = 4 (○) (A) or to gastric lipid extracts from the surface scraping material (SSM) (B).

induced transformation of the stomach to a wettable state demonstrated a rapid effect of the drug (Fig. 3B).

Interaction of Indomethacin with ANS Binding to Lipid Vesicles

The lipid fraction of the mucus gel layer has been shown to predominantly contain phosphatidylcholine (30–50%) with a large concentration of the highly surface-active phospholipid DPPC [10, 18]. The following experiments were therefore performed with artificial DPPC vesicles or a lipid extract from the SSM.

The anionic probe ANS forms electrostatic binding with the polar head region of phosphatidylcholine [19]. ANS fluoresces when bound to synthetic vesicles of DPPC as well as gastric phospholipid vesicles. The probe is clearly displaced by indomethacin as shown Fig. 4A and B. Under both acidic and neutral pH conditions, increasing the concentration of indomethacin resulted in a dose-dependent decrease in the fluorescence intensity of ANS. These results reflect a decrease in the number of probe molecules actually bound to the lipid vesicles.

In order to estimate a possible quenching effect of ANS

TABLE 1. Estimations of the spectral overlap, corresponding to the Förster transfer distance and resonance energy transfer efficiency between naproxen and two different acceptor probes

Acceptor	Spectral overlap J (cm^3 M^{-1})	Distance R_0 (Å)	Transfer energy efficacy E*
ANS	$9.7\ 10^{-16}$	~70	0.87
Pyrene	$7.1\ 10^{-16}$	~70	0.46

*DPPC small unilamellar vesicles (1.35 mM) were incubated with pyrene (10^{-7} M) or ANS (3.10^{-4} M). Naproxen was added at a molar ratio naproxen:probe 1:1.

fluorescence by indomethacin, we compared the indomethacin absorption spectrum and fluorescence spectrum of ANS. No overlap was observed between the two spectra (data not shown).

Interaction of Naproxen with DPPC Liposomes

The well-established fluorescence resonance energy transfer technique [20] was used to monitor the localization of naproxen in the DPPC bilayer. We used two different probes, ANS and pyrene. ANS binds to the polar head group of DPPC. At a concentration of 10^{-7} M, pyrene is believed to be totally and deeply incorporated into the DPPC vesicles in the hydrophobic tail region. The efficiency of fluorescence energy transfer displayed in Table 1 showed that DPPC-bound ANS quenched naproxen fluorescence. Upon addition of naproxen to pyrene-labeled liposomes, significant quenching was also achieved between both molecules. The observed energy transfer is presumably a direct result of naproxen being partitioned within the DPPC bilayer.

Estimation of Bound NSAID to Liposomes

Results obtained after precipitation of the liposomes complexed with an NSAID showed that 50% of indomethacin and 45% of naproxen precipitated with the DPPC liposomes. Assuming that the gentle precipitation of intact liposomes did not displace the molecules of bound NSAID, it appeared that half of the drug molecules interact with the liposomes. It is likely that a balance exists between the molecules of NSAID bound to the liposomes and the free drug in solution.

Effect of NSAIDs on the Fluidity of SSM Lipid Extract

We used polarization fluorescence to estimate the effect of the drugs on the bulk fluidity of the gastric phospholipid vesicles. The anisotropy of fluorescence of DPH embedded in SSM lipid vesicles was measured at 25° (Table 2). The incubation of the vesicles with NSAIDs resulted in a significant increase in the anisotropy of fluorescence. Alterations of the physical properties of the SSM lipids were similar with indomethacin (0.8 mg/mL) and naproxen (1.2 mg/mL). Both of the drugs studied had a rigidifying effect on the gastric lipidic material.

Effects of NSAIDs on the Thermotropic Phase Transition Profiles of DPPC

We further explored the effects of indomethacin and naproxen on our phospholipid model system. The fluorescence anisotropy (r) of DPH is sensitive to the structural order of lipid in bilayers and is commonly used to estimate membrane fluidity as well as reveal the phase transition of a phospholipid matrix from a gel to a liquid-crystalline state [21].

Figure 5 shows the temperature profile of r for unilamellar liposomes of DPPC in the absence and presence of the NSAIDs. The thermotropic behavior of pure DPPC reveals a phase transition temperature (Tm) of 38.5°. The gel to liquid-crystal transition temperature for DPPC has been well documented in small unilamellar liposomes [22]. Our Tm value is in agreement with values reported in studies under similar conditions [22]. Addition of an equimolar concentration of indomethacin resulted in a small increase in Tm, now appearing at 39.5°. However, the main effect of indomethacin was related to the observed increase of r values across the whole range of temperature studied (25–48°). The stabilizing effect of indomethacin was also observed, to a lesser extent, with the other NSAID studied, as naproxen caused an increase in DPH anisotropy only in the gel state.

DISCUSSION

Present results show that oral administration of indomethacin and naproxen transforms the gastric mucosa from a hydrophobic to a more hydrophilic state. A similar effect was observed when we administered aspirin to rats [8]. These observations suggest that direct topical effect plays an important role in the GI toxicity of NSAIDs. This assumption is supported by studies showing that uncoated aspirin tablets cause gastric damage wherever the aspirin contacts gastric mucosa and that enteric coatings protect the stomach from the toxic effects of NSAIDs [23].

Over the past 15 years, our laboratory has provided

TABLE 2. DPH fluorescence anisotropy (r) of gastric lipid extracts in the absence or presence of indomethacin and naproxen

	DPPC	DPPC + indomethacin	DPPC + naproxen
Fluorescence anisotropy r	0.106 ± 0.006	0.137 ± 0.005*	0.133 ± 0.007*

Gastric lipid extracts were obtained from the Folch extraction of gastric surface scraping material (SSM). Each extract was dried under N_2, briefly sonicated in PBS containing DPH and incubated for 45 min at 25°. Naproxen (final concentration: 1.2 mg/mL) or indomethacin (final concentration 0.8 mg/mL) were then added to the lipid suspension. Results are expressed as the means of fluorescence anisotropy of four SSM lipid extracts ± SEM (*$P < 0.001$).

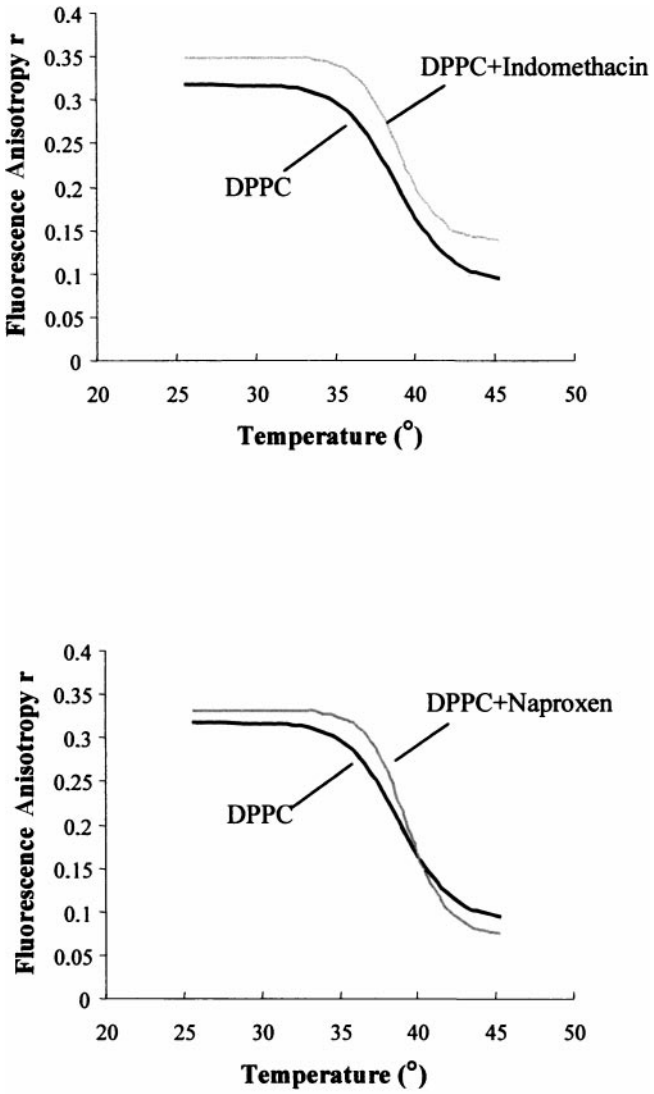


FIG. 5. Temperature dependence of the steady-state fluorescence anisotropy of DPH embedded in small unilamellar vesicles of DPPC prepared in the absence (—) and in the presence (- - -) of naproxen (A) or indomethacin (B). The NSAID:DPPC molar ratio was 1:1.

evidence for the gastroprotective role of the surface-active phospholipids, present within and on the surface of the mucus gel layer. The toxic effect of NSAIDs and their effect on mucosal hydrophobicity may be attributable to molecular interaction between NSAIDs and endogenous phospholipids. This possibility was supported by evidence that the solubility of NSAIDs in either chloroform or saline can be markedly affected by the addition of DPPC to these contrasting solvent systems [9]. The present data establish at the molecular level, in agreement with other studies [24–27], that NSAIDs markedly interact with various phospholipidic systems and change their physico-chemical properties.

Our observations suggest that indomethacin likely interacts at the surface of the lipid vesicles. We show that this NSAID displaced the probe, ANS, from the phospholipid headgroup. Competition for the surface binding site could occur between both molecules. Indomethacin, like most NSAIDs, is a weak acid with pK_a value of 4.5. Under the pH conditions of the *in vitro* experiments (pH = 7), more than 90% of the NSAID molecules are, thus, considered to be in an ionized state. Therefore, electrostatic bonding between the negatively charged carboxyl group of the NSAID and the positively charged quarternium ammonium of the phosphatidylcholine could be formed.

Change in the vesicular structures induced by indomethacin may also result in a modification of the number of ANS-binding sites. This is a possible explanation for the results observed at pH values below the pK_a, where the majority of NSAIDs are believed to be in an unionized sate.

Our study with naproxen, a more liposoluble NSAID, suggests that naproxen is localized within the bilayer, at a level closer to the polar head group than the end of the fatty acid tail. The transfer of energy is limited by the distance R_0 between donor and acceptor chromophores, and the higher quenching efficacy was obtained with the probe localized at the surface of the vesicles (ANS).

The different positions of the NSAID molecules with respect to phospholipids may account for their different effect on the physico-chemical properties of the lipid model. The great rigidifying effect of indomethacin can be compared with the earlier study of Abransom *et al.*, who similarly demonstrated an increase in viscosity of liposomes in the presence of indomethacin [28]. As indomethacin is likely to be present at the surface of the bilayer, the possible neutralization of the charge head group induced by indomethacin surface binding and the consequent elimination of the electrostatic repulsion between the phospholipid polar head may allow a better packing of the hydrocarbon chains [29].

In a bilayer system, we reported here that a rigidification of the DPPC vesicles and surface-active gastric lipids was also induced by naproxen, although it was lower than that observed with indomethacin. However, the change in the physical properties might be dependant on the lipidic system study as naproxen has also been shown to increase the mean molecular area of a monolayer of milk phospholipids [26].

Our observations emphasize that the interaction of NSAIDs with the phospholipid bilayer is a function of their partition coefficient [25]. Evidence supporting the notion that the physico-chemical state of the NSAIDs contributes to their side-effects has been reviewed by McCormack and Brune [30]. The degree of water solubility and ionization as well as the liposolubility of the NSAIDs are believed to play a role in both their absorption site [31] and GI side-effects [32].

The relationship between the fluidity and physiological function of biological lipid systems has been previously established [21]. The fundamental basis of this relationship relies on the intermolecular forces between phospholipid molecules. Lipid dynamics depend on these forces, which reciprocally control the surface tension characteristics of phospholipidic systems. Therefore, the deleterious effect of

NSAIDs on mucosal hydrophobicity may best be explained by our observations that pertain to changes in gastric phospholipid fluidity.

The mechanism responsible for NSAID-induced GI damage may differ from one region of the GI tract to another. The interaction of NSAIDs with the gastrointestinal surface-active phospholipids could be part of the cascade of events involved in the pathogenesis of NSAID-induced gastropathy as well as enteropathy. Furthermore, enterohepatic recirculation of NSAIDs such as indomethacin results in repeated exposure of the epithelium to the drug. Topical interaction of the biliary excreted NSAID with the gastrointestinal surfactant may also be implicated in the decreases in the hydrophobicity of the gastric and duodenal mucosa observed after parenteral administration [33].

In conclusion, the present study provides evidence for a new mechanism of NSAID-induced gastropathy independent of the inhibition of prostaglandin synthesis. The deleterious action of NSAIDs on the hydrophobicity of the mucosa reported here may result from their ability to perturb physical properties of gastric phospholipids. NSAIDs share the capacity to insert themselves into lipid bilayers and alter the lipid dynamic of both artificial membranes and gastric phospholipid vesicles. Changes in the optimal fluidity of the gastric phospholipids may reduce their ability to form a protective hydrophobic layer. The clinical significance of these observations becomes evident with regard to the development of a new formulation of NSAIDs, where the drug is preassociated to synthetic phospholipid before administration to limit the interactions of the NSAID with the intrinsic phospholipid lining of the stomach. These phospholipid-associated NSAID formulations have been tested in rodents and appear to prevent the acute GI toxicity of the NSAIDs while maintaining or enhancing the drugs' therapeutic activity [34].

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