

HEPATOLOGY

Importance of biliary excretion of indomethacin in gastrointestinal and hepatic injury

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Abstract

Background and Aims: A mechanism for protection of gastrointestinal (GI) and hepatic cells from damaging detergent actions of bile acids appears to involve the bile component, phosphatidylcholine (PC). Non-steroidal anti-inflammatory drugs (NSAIDs) induce intestinal injury in direct proportion to their ability to be excreted into bile, and are known to chemically associate with PC. We investigated the role of bile acids and PC in the mechanism of indomethacin-induced epithelial injury.

Methods: Rats were injected orally or intravenously with radiolabeled indomethacin and their bile was collected over time for determination of NSAID secretion. Bile from rats treated with or without indomethacin was used in studies of red blood cell (RBC) hemolysis as a measure of membrane cytotoxicity. The bile salt, sodium deoxycholate (SDC), and indomethacin were tested alone and in combination with PC on RBC and on hepatic HepG2 cells.

Results: Intravenously or orally given indomethacin was quantitatively excreted (approximately 50%) into bile over a 2-h study period. Bile from a rat treated with indomethacin or bile with exogenous indomethacin was cytotoxic to RBC, and the injury was prevented by the addition of PC. Hepatocytes exposed to SDC showed injury that could be dose-dependently prevented by PC, and reversed by indomethacin.

Conclusions: Biliary PC plays an important physiological role in protecting GI and hepatic epithelia from the cytotoxic actions of bile salts. The ability of NSAIDs excreted into the bile to associate with mixed bile salt micelles and reduce the protective action of the PC may be a critical component in the drugs' pathogenic mechanism.

Introduction

The mechanism which protects the epithelia of the gastrointestinal (GI) and hepatobiliary tracts against the detergent-like properties of bile salts has yet to be fully resolved. It has been proposed to include direct effects of luminal factors such as phospholipids and cholesterol.^{1–5} The recent description of innate defense induction by bile-induced FXR receptor activation in the ileum⁶ also offers the possibility that other protective mechanisms for the hepatic and biliary regions remain to be identified. However, it is the potential of phospholipid to counter GI injury that has been the focus of our laboratory and which was investigated in this study. Evidence from our laboratory and others has indicated that biliary phosphatidylcholine (PC) may protect intestinal epithelia,^{3,7,8} cholangiocytes,^{9,10} and erythrocytes^{1,4,5} against bile salt-induced injury. We sought to investigate a similar protective role for PC in the current study and extended our investigation into exploring its role in the mechanism of NSAIDs that are excreted into the bile and induce GI and hepatic injury.

The injurious effects of NSAIDs on the GI tract are well

documented, but the hepatic injury that can be induced by NSAIDs is not widely appreciated, even though hepatotoxicity is a class warning for NSAIDs. Several recent papers have described case-control and population studies in which NSAID use is associated with hepatic injury.^{11,12} Indeed, several NSAIDs (i.e. benoxaprofen, droxicam, bromfenac, pirofen, fenclofenac) have been removed from the European market due to hepatic adverse drug reactions. It is not known how NSAIDs cause hepatic injury, although it is well established that certain NSAIDs such as indomethacin are readily excreted into the bile and enter the enterohepatic circulation, resulting in high concentrations of NSAID in the liver and bile.^{13,14} We have shown previously that NSAIDs chemically associate with PC and this process can occur in the GI tract where the drug-induced loss of surface-protective PC results in injury to the mucosa.^{15,16} We propose that biliary PC normally protects hepatic and intestinal epithelia from bile salt-induced injury, but in the presence of NSAIDs excreted into the bile, the defensive actions of PC are compromised. This study therefore was designed to test the hypothesis that PC in bile is essential for protection from bile salt and NSAID injury, and that

interactions between these three components may alter hepatic and GI mucosal integrity.

Methods

Animals

Male Sprague–Dawley rats (200–300 g) were used in all studies. They were fasted overnight before use to ensure an empty stomach to allow for intragastric dosing. All animal protocols were approved by the Animal Welfare Committee of The University of Texas Health Science Center at Houston.

Biliary secretion of indomethacin

Rats were anesthetized with pentobarbital and were fitted with a cannula (PE 10 tubing) into the common bile duct which drained into a tube in ice. After an initial 15-min collection period, rats were dosed with 2×10^5 c.p.m. of ^{14}C -indomethacin (Perkin Elmer, Boston, MA, USA) in a volume of 0.25 mL. Dosing was by the intravenous route into a tail vein ($n = 11$), or by oral gavage with a feeding needle and syringe ($n = 8$). Bile collections continued for 2 h, with tube changes at 15–30 min intervals. The entire sample in a tube (volume < 1 mL) was mixed with 10 mL scintillation counting fluid and counted on a Beckman Liquid Scintillation Counter. Values are expressed as the cumulative percentage of the administered amount.

In separate experiments, bile was collected for 1 h from anesthetized rats dosed intragastrically with vehicle (1% sodium carbonate) or indomethacin (25 mg/kg) at least 30 min prior to initiation of collection.

Red blood cell hemolysis

Membrane cytotoxicity on RBC was analyzed as described previously.¹ Fresh human blood from volunteers was collected by venipuncture into heparinized tubes and centrifuged at 2500 *g* for 10 min. Packed RBC were washed three times with 140 mmol/L sodium chloride/15 mmol/L HEPES buffer, pH 7.4, and were then resuspended in buffer to twice their volume. For hemolysis testing, 0.25 mL RBC was mixed with 1.5 mL buffer and 0.25 mL undiluted or diluted rat bile collected from single control or indomethacin-treated rats. The RBC mixtures were incubated for 10 min at 37°C and were then centrifuged at 14 000 *g* for 1 min. The supernatant was diluted 24-fold and the percentage of hemolysis was calculated by reading absorbance of the diluted supernatant at 525 nm and comparing it to the absorbance of an uncentrifuged incubation mixture that was totally hemolyzed by diluting with water. For subsequent hemolysis testing, bile from pooled control rats was mixed with 0.35–1.4 mmol/L indomethacin and incubated

as above with RBC. Control bile was also mixed with indomethacin that had been pre-associated with PC (0.7–2.8 mmol/L).

HepG2 cell experiments

Hepatic HepG2 cells, originally derived from a human hepatoma, were obtained from the Texas Gulf Coast Digestive Diseases Center (NIH P50 DK56338). They were maintained in culture in Minimal Essential Media with 5% fetal bovine serum, 2 mmol/L glutamine, and 100 U/mL penicillin/streptomycin. Cells were grown in 6- or 12-well culture plates until confluent. Then the media was replaced with that containing concentrations of sodium deoxycholate (SDC; 0.2–0.6 mmol/L) and the plates were incubated for 5 h, after which the media were removed for analysis of the cytosolic enzyme, lactic dehydrogenase (LDH), as an index of cell injury. LDH was assayed with kits from either Sigma-Aldrich Co. (Poole, Dorset, UK) or Thermo DMA (Arlington, TX, USA), according to the manufacturer's instructions. In subsequent experiments, 0.6 mmol/L SDC was used as an injurious concentration and combinations of SDC and increasing PC (0.3–2.4 mmol/L) were tested. In a last study, the effect of increasing indomethacin (0.6–2.4 mmol/L) on SDC alone (0.6 mmol/L) or complexed with PC (2.4 mmol/L) was investigated.

Chemicals

Indomethacin and SDC were purchased from Sigma Chemical Co. (St Louis, MO, USA) and soy phosphatidylcholine (Phospholipon 90G) was obtained from Natterman Phospholipid (Cologne, Germany). Prior to use, the PC was dissolved in chloroform, dried under a stream of nitrogen gas, and resuspended by vortex and sonication in NaCl/HEPES buffer either with or without SDC, or with or without indomethacin.

Statistics

Results were analyzed by analysis of variance (ANOVA), followed by the Fisher LSD test.

Results

Biliary secretion of indomethacin

The rat does not have a gall bladder to accumulate bile, so that cannulation of the common bile duct allows immediate collection of secreted bile. The average volume of bile collected by this method after indomethacin inoculation is shown in Table 1. It can be seen that bile continued to flow following indomethacin treatment. The level of radiolabeled indomethacin detected in the bile of rats inoculated intragastrically or intravenously with

Table 1 Volume of bile collected from rats after indomethacin dosing

	Time (min)						
	30	45	60	90	120	150	180
	484 ± 50	199 ± 27	214 ± 24	362 ± 52	315 ± 47	251 ± 14	275 ± 35

Units are expressed as mean value (μL) ± standard error of the mean ($n = 6$).

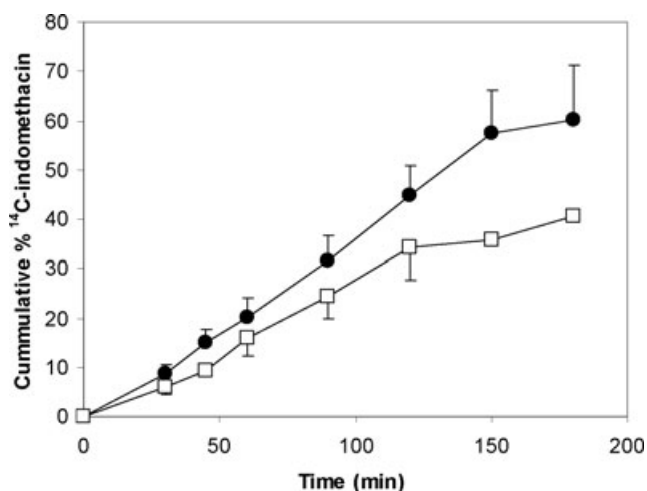


Figure 1 Biliary secretion of systemically administered indomethacin. ¹⁴C-indomethacin was dosed orally (IG; □) or intravenously (IV; ●) to rats and bile was collected at intervals for 3 h and counted for radioactivity. Values are expressed as the cumulative percent of the total administered dose. *n* = 11 (IV) and eight (IG) rats per treatment.

indomethacin was similar. Figure 1 shows that the NSAID was detectable as soon as 30 min after dosing and the level continued to rise thereafter, so that 40–50% of the total inoculum was excreted into the bile in 2 h. In subsequent studies with non-radiolabeled indomethacin, bile was collected beginning 30 min after dosing and continued for 1 h. This timing resulted in the collection of 20–30% of the administered dose in the bile.

Cytotoxicity of indomethacin in bile and its reversal by PC

The toxicity of rat bile from a control and an indomethacin-treated animal was tested on RBC for hemolytic activity as a measure of cytotoxicity. It can be seen in Fig. 2 that the control bile was not toxic to the cells. However, when bile from an indomethacin-treated rat was incubated with RBC, there was a concentration-dependent hemolysis of the cells. This finding suggested that it was the treatment with indomethacin that made the bile toxic. We estimated that the concentration of indomethacin in the undiluted bile was approximately 3.6 mmol/L (20% of a dose of 5 mg in a bile volume of 0.775 mL). Therefore, an experiment was performed in which indomethacin was added to control rat bile before incubation with RBCs. The results of this study are shown in Fig. 3 and reveal that neither control bile nor indomethacin alone (at a dose of 1.4 mmol/L), were toxic to the cells. However, the combination of bile with indomethacin produced a dose-dependent increase in RBC hemolysis that was significantly higher at 1.4 mmol/L indomethacin, confirming that the presence of NSAID and bile are both required for the drugs' cytotoxic activity to be manifest.

In order to test our hypothesis that indomethacin's chemical association to PC in bile was responsible for the toxicity, a study was performed in which RBC were incubated in buffer containing rodent bile in the presence of a concentration of indomethacin that was shown to be toxic in Fig. 2 (1.4 mmol/L) and a variable

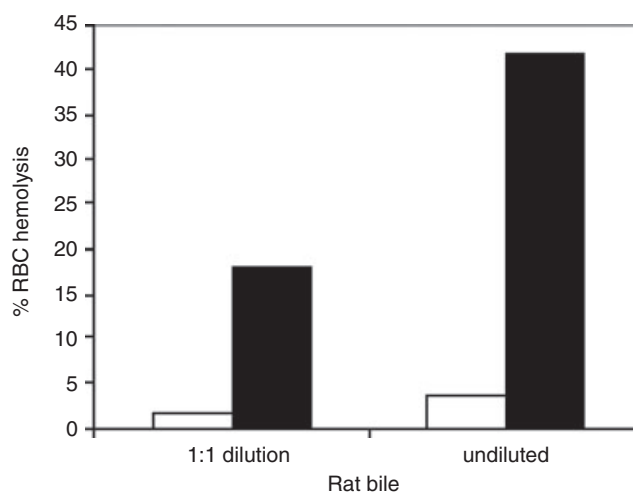


Figure 2 Cytotoxicity of bile from indomethacin-treated rat. Red blood cells (RBC) were incubated with bile from a control or indomethacin-treated rat and hemolysis was measured. Values are expressed as a percent of total hemolysis. □, Control; ■, indomethacin treated

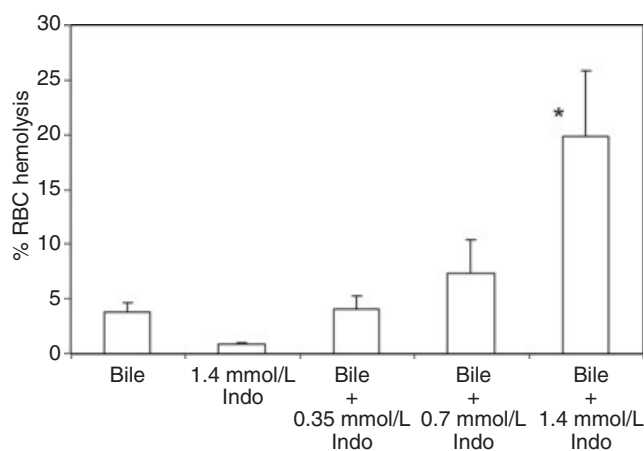


Figure 3 Cytotoxicity of indomethacin (Indo) added to normal rat bile. Red blood cells (RBC) were incubated with normal rat bile to which increasing concentrations of indomethacin were added. Hemolysis was measured and values are expressed as a percent of total hemolysis ± standard error. *n* = 6–15 per group. **P* < 0.05 vs Bile or 1.4 mmol/L Indo.

concentration of PC (0–2.8 mmol/L). The results in Fig. 4 show that the addition of PC dose-dependently reversed the indomethacin toxicity of bile. At a 2:1 molar ratio of PC to indomethacin, there was complete prevention of indomethacin-bile induced toxicity.

Cytotoxicity of indomethacin and SDC to hepatocytes and its reversal by PC

The toxic effects of a model bile containing the bile salt, SDC, were tested on a cell culture of hepatocytes, the HepG2 cell line. These cells are representative of cells that could be exposed to high

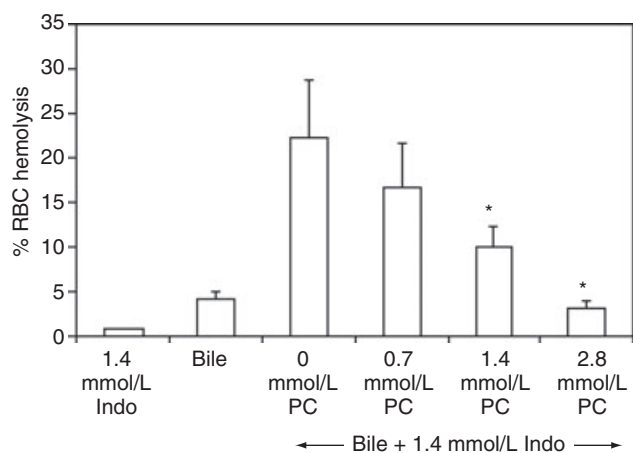


Figure 4 Phosphatidylcholine (PC) reversal of indomethacin (Indo) toxicity in rat bile. Red blood cells (RBC) were incubated with normal rat bile, bile plus added indomethacin, or bile plus indomethacin pre-associated with PC. Hemolysis was measured and values are expressed as a percent of total hemolysis \pm standard error. $n = 4-9$ per group. * $P < 0.05$ vs 0 mmol/L PC.

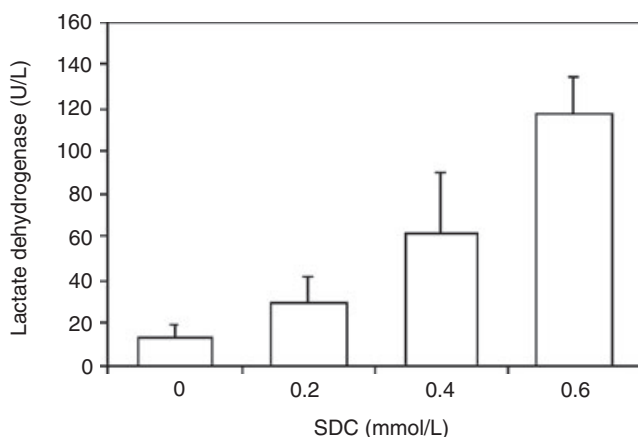


Figure 5 Toxicity of sodium deoxycholate (SDC) on hepatocytes. HepG2 cells were incubated with increasing concentrations of SDC and release of cytosolic lactate dehydrogenase (LDH) was measured. Values are expressed as the mean enzyme units \pm standard error. $n = 4$ per group. * $P < 0.05$ vs 0 SDC.

levels of bile and indomethacin in bile. It was determined that purified SDC alone had a dose-dependent damaging effect on HepG2 cells (Fig. 5), as detected by release of cytosolic LDH activity, most likely due to SDC detergent activity on cell membranes. Because consistent effects were seen with 0.6 mmol/L SDC, that concentration was used in subsequent studies. Therefore, cells were then incubated with SDC alone and SDC that had been premixed with PC in varying concentrations (0.3–2.4 mmol/L). Figure 6 reveals that SDC-induced damage to the cells was reversed by PC in a dose-dependent manner. Finally, to show that indomethacin could interfere with PC protection against bile salt injury, cells were incubated with 0.6 mmol/L SDC + 2.4 mmol/L PC (induced no injury) in the presence of increasing concentra-

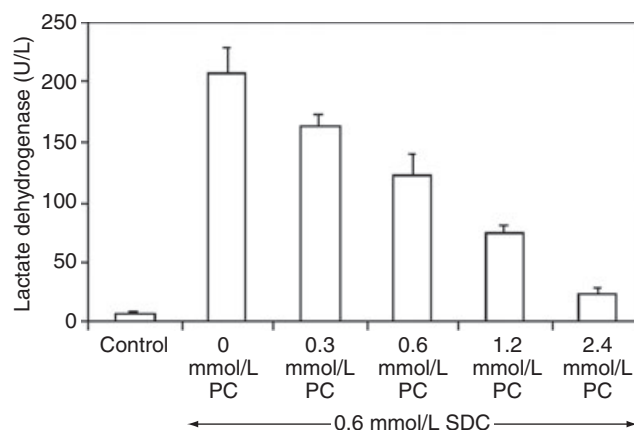


Figure 6 Phosphatidylcholine (PC) reversal of sodium deoxycholate (SDC) toxicity on hepatocytes. HepG2 cells were incubated with SDC and increasing concentrations of PC. Lactate dehydrogenase (LDH) was measured and values are expressed as the mean enzyme units \pm standard error. $n = 6$ per group. * $P < 0.05$ vs Control.

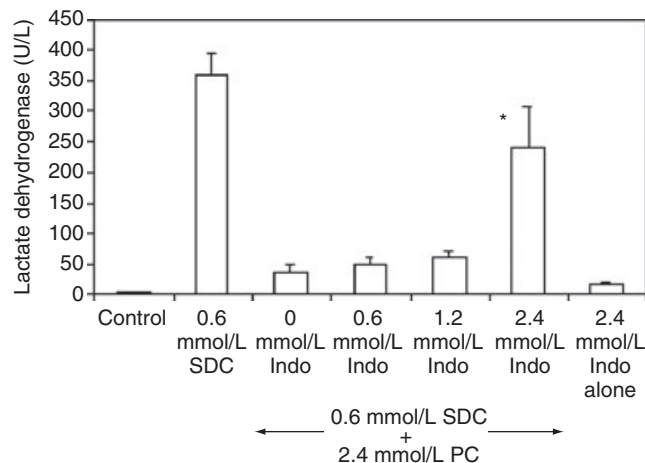


Figure 7 Indomethacin (Indo) reversal of phosphatidylcholine (PC) protection from sodium deoxycholate (SDC) toxicity to hepatocytes. HepG2 cells were incubated with SDC plus PC and increasing concentrations of indomethacin. Lactate dehydrogenase (LDH) was measured and values are expressed as the mean enzyme units \pm standard error. $n = 5-6$ per group. * $P < 0.05$ vs 0 mmol/L Indo.

tions of indomethacin (0.6–2.4 mmol/L). Figure 7 demonstrates that indomethacin dose-dependently reversed the PC protection against bile salt injury, again confirming the interactions between the three biliary components (indomethacin, PC and bile salt).

Discussion

Our laboratory and a number of others have shown that the PC in bile is crucial for protection of GI mucosal tissue from the detergent actions of bile salts.^{3,7-10} One of the means by which this PC protection is accomplished is proposed to be through promotion of mixed micelles of bile salts and PC which are much less toxic,

compared to pure bile acid micelles or monomers. Our present studies support this concept. We showed that a submicellar concentration of the bile salt SDC was toxic to hepatocytes (Fig. 5) and that this toxicity was overcome by mixing the SDC with PC before exposure of the cells (Fig. 6). This type of mixed PC-bile salt micelle has also been reported to be non-injurious to Caco-2 cells and ileal mucosa when incubated together *in vitro*.⁸ Similarly, bile from a control rat which contains a variety of bile salts (e.g. cholic, deoxycholic, chenodeoxycholic acid) and PC, presumably as mixed micelles, was not damaging to RBC membranes (Fig. 2).

Another means by which PC could protect hepatocytes is by prevention of bile salt uptake into the cell and activation of the FXR receptor which could lead to apoptosis. This has been shown to occur in cholangiocytes at low bile salt concentrations.⁹ In those studies, both egg yolk and soybean-derived PC prevented uptake of a bile salt into cells and therefore prevented FXR activation and apoptosis. It is known that HepG2 cells contain active FXR receptors,¹⁷ so that we cannot eliminate this possibility as a mechanism for PC protection. However, bile salt stimulation of FXR in HepG2 cells has not been associated with apoptosis, but rather with activation of other functions.^{17,18} In addition, RBC do not have nuclei and cannot respond in this manner to FXR activation, but they still show protection by PC. Therefore, it is most likely that PC protection of cell membranes from bile salt involves another mechanism such as a switch from monomeric to mixed micelle forms of the bile.

In addition to PC protection of epithelia from bile salts, PC also has protective effects against the topical actions of NSAIDs that are seen at the systems level. In the whole animal, the NSAID, indomethacin, produces GI bleeding, ulcerations and intestinal adhesions, depending on dosage. These injuries can be prevented by pre-association of indomethacin with PC.¹⁵ Thus, the PC-NSAIDs were developed as drugs that would be safer for the GI tract. Their safety for the hepatic and biliary tracts has not been established, so that this study is the first to report such a possibility.

We initially confirmed that indomethacin is taken up by the liver in rats and excreted into the bile (Fig. 1) in agreement with other reports.^{13,14} It did not matter whether the drug was administered orally or intravenously. Both routes resulted in a significant portion of the drug being released into the bile within a 2-h period, supporting the enterohepatic system as the primary route of excretion and recirculation. These data agree with earlier investigators who showed the importance of indomethacin excretion into bile for its injurious effects on the intestine.^{13,19} Thereafter, in our studies, bile collected from rats treated with vehicle or indomethacin was tested for its ability to disrupt membranes of RBC. It was clearly seen that only the bile from indomethacin-treated rats was toxic (Fig. 2) and not that from control rats. The primary difference, of course, is the presence of indomethacin in the bile. These results support the role of PC in bile as protective, as the natural bile, which contains both bile salt and PC, was not damaging. This result and interpretation were confirmed in the experiment in which we added indomethacin to normal bile and demonstrated that it dose-dependently increased bile's cytotoxicity to RBC (Fig. 3). Furthermore, the presence of PC in the incubation buffer prevented the injurious effect of the NSAID in a dose-dependent manner (Fig. 4). These

results are consistent with the possibility that both bile salt and indomethacin can interact with PC, but that the NSAID may have a greater affinity for PC, leaving either a higher level of bile salt monomer or a lesser level of mixed bile salt-PC micelles available. However, Petruzzelli *et al.*²⁰ examined this possibility and were unable to show an increase in bile salt concentration in the intermixed micellar-vesicular fraction which was composed of bile salt monomers and simple micelles. Rather, they speculated that indomethacin may intercalate into bile salt mixed micelles or cell membranes making the former more toxic and the latter more fluid and susceptible to disruption.²¹ Our results agree with this possibility. A recent report by Jacob *et al.* further supports the theory of toxic mixed micelles because these authors showed that indomethacin must pass through the liver and biliary tract for NSAID enteropathy to occur.²² Our results also argue against a primary role for acyl glucuronide metabolites of indomethacin²³ as the main toxic moiety, as we found that membrane toxicity was present when purified indomethacin was added to SDC model bile in the absence of any glucuronidated product (Figs 3,7). The possibility that biliary composition and bile acid pool hydrophobicity were affected by drug treatment was not assessed in this study, and remain for future investigations.

Our studies on hepatocytes were designed to verify that a synthetic bile salt, SDC, was toxic to these cells (Fig. 5), and that association of the bile salt with PC could prevent these toxic effects (Fig. 6). The logical extension of all these findings is that the addition of indomethacin to the non-injurious SDC/PC mixture would produce a toxic effect and, indeed, that is what was observed in our experiments (Fig. 7). Very similar findings have been reported on sheets of ileal mucosa, where indomethacin reversed the protective effect of PC against the bile acid taurodeoxycholate.⁸ Thus, hepatic cells along with intestinal cells, are sensitive to bile salts and require endogenous protective mechanisms.

In summary, we propose the following to explain how NSAIDs may interfere with the protective action of PC against bile salt-induced injury to the hepatic and biliary tracts. The presence of PC in bile converts simple bile salt micelles and/or monomers into mixed micelles, suppressing the bile salts from exerting their membrane disruptive actions while concentrated in the hepatic ducts and gall bladder. In the presence of indomethacin, which is avidly taken up by the liver and excreted into bile, there is a tendency for the NSAID to associate with PC and bile in the mixed micelles, which may result in the formation of more toxic micelles. These NSAID-induced macromolecular changes will result in bile becoming more toxic to cells and membranes. This scheme thus supports the concept that one way to decrease the toxicity of NSAIDs is by pre-associating the NSAID with PC. We speculate that these interactions between NSAID, bile salt and PC occur within the hepatic/biliary tracts, as well as within the small intestinal lumen, and may account for much of the previously reported NSAID-related GI injury. A recent report of hepatotoxicity associated with a coxib²⁴ underscores that no NSAID may be free of this potential side-effect.

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