In vitro evidence that phosphatidylcholine protects against indomethacin/bile acid-induced injury to cells

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Dial EJ, Dawson PA, Lichtenberger LM. In vitro evidence that phosphatidylcholine protects against indomethacin/bile acid-induced injury to cells. Am J Physiol Gastrointest Liver Physiol 308: G217–G222, 2015. First published December 4, 2014; doi:10.1152/ajpgi.00322.2014.—Indomethacin is a powerful analgesic nonsteroidal anti-inflammatory drug (NSAID), but is limited in use by its primary side effect to cause gastrointestinal bleeding and serious injury. One factor important for exacerbating NSAID injury is the presence of bile acids, which may interact with indomethacin to form toxic mixed micelles in the gut. The development of a safer gastrointestinal formulation of indomethacin that is chemically complexed with phosphatidylcholine (PC-indomethacin) may offer an improved therapeutic agent, particularly in the presence of bile acid, but its potential protective mechanism is incompletely understood. Intestinal epithelial cells (IEC-6) were tested for injury with indomethacin (alone and plus various bile acids) compared with PC-indomethacin (alone and plus bile acids). To explore a role for bile acid uptake into cells as a requirement for NSAID injury, studies were performed using Madin-Darby canine kidney cells transfected with the apical sodium-dependent bile acid transporter (ASBT). Indomethacin, but not PC-indomethacin, was directly and dose-dependently injurious to IEC-6 cells. Similarly, the combination of any bile acid plus indomethacin, but not PC-indomethacin, induced cell injury. The expression of ASBT had a modest effect on the acute cytotoxicity of indomethacin in the presence of some conjugated bile acids. Complexing PC with indomethacin protected against the acute intestinal epithelial injury caused by indomethacin regardless of the presence of bile acids. The presence of luminal bile acid, but not its carrier-mediated uptake into the enterocyte, is required for acute indomethacin-induced cell injury. It is likely that initial cell damage induced by indomethacin occurs at or near the cell membrane, an effect exacerbated by bile acids and attenuated by PC.

THE NONSTEROIDAL ANTI-INFLAMMATORY drug (NSAID) indomethacin is a potent and effective anti-inflammatory and analgesic drug that is used therapeutically to induce closure of the patent ductus arteriosus in low-birth-weight neonates, and in adults for the treatment of gout and other painful inflammatory disorders (6, 19). However, the usefulness of indomethacin is limited by an associated high incidence of gastrointestinal bleeding (18). A newer formulation of indomethacin that may spare the gastrointestinal tract (9) is under development and is based on an understanding of gastrointestinal physiology (2, 8, 22).

The basis for this new formulation is that the NSAID can be noncovalently associated with the natural surfactant phosphatidylcholine (PC) (11) to reduce the associated gastrointestinal toxicity. In preclinical trials, PC-indomethacin has equivalent efficacy to inhibit pain and inflammation as traditional indomethacin, but with significantly less gastrointestinal bleeding and intestinal adhesions (9). This gastrointestinal protection is thought to occur by several related PC-associated mechanisms (12). These include preservation of the hydrophobic characteristics of PC-containing mucus in the gastrointestinal tract that limits back-diffusion of gastric acid in the upper gastrointestinal tract or translocation of noxious luminal contents into or across the gastrointestinal epithelia in the lower gut. The preassociation of indomethacin with PC in the drug formulation prevents indomethacin from associating with and destabilizing mucus PC and the resultant surface disruption. Although indomethacin is secreted into bile primarily as the glucuronide conjugate, a modification that may protect the enterocytes of the upper small intestine, it should be noted that a significant amount of the drug is converted to free indomethacin in the lower gut (where injury does occur) by bacterial glucuronidases (1, 3, 4, 17). Thus we contend that a significant amount of unconjugated indomethacin may be available in the lower gut to associate with bile acids and form potentially toxic mixed micelles. Previously, we reported that cultured gastric (AGS) and intestinal (IEC-6) cells were injured by short-term incubation with indomethacin in the presence of bile acid (deoxycholate), and the concept that this greater cytotoxicity is due to the formation of toxic mixed micelles (23) is supported by physicochemical analysis [nuclear magnetic resonance (NMR)] and computational [molecular dynamics (MD)] modeling (3, 11, 16). This combination may resemble the in vivo situation, where bile acids and indomethacin are both secreted into bile via enterohepatic cycling.

It is not known whether the addition of PC would protect cells from acute bile acid/NSAID injury in the absence of a hydrophobic (mucus) layer. Therefore, in the current study, we examined the cellular toxicity of traditional indomethacin vs. PC-associated indomethacin in combination with bile acids. We also investigated the mechanisms for NSAID/bile acid injury using cells where the expression of the human apical sodium-dependent bile acid transporter (ASBT) was experimentally manipulated. These in vitro studies, therefore, were designed to provide insight into whether NSAIDs and bile acids induce injury via their capacity to act extracellularly to disrupt the surface and membrane barriers of the luminal mucosa (extracellular action hypothesis) or whether their ability to induce injury is dependent on an intracellular interaction of the NSAID and bile acid (intracellular action hypothesis). NSAIDs preferentially induce injury in the distal small intestine (2, 8, 22). The molecular basis for this localization is not known and could reflect the higher levels of β-glucuronidase-expressing bacteria in the distal gut. As suggested above, this
would increase generation of free indomethacin to interact with bile acids to form toxic mixed micelles and induce mucosal injury/bleeding. Alternatively, in support of the intracellular action hypothesis, the ileum is the only region of the small intestine that actively takes up conjugated bile acids (via the ASBT). Hence, while NSAIDs could be passively taken up by enterocytes along the length of the small intestine based upon their hydrophobic/membrane permeability, the ileum may be preferentially susceptible to NSAID-induced injury because this tissue would also have appreciable intracellular levels of both NSAIDs and bile acids, notably conjugated species that are actively transported via ASBT. At the cellular level, the apical brush-border membrane is cholesterol- and sphingomyelin-rich and would theoretically be more resistant to the detergent effects of NSAID/bile acids compared with the cholesterol-poor membranes of intracellular organelles or the inner leaflet of the plasma membrane. In this way, NSAID/bile acid complexes that form intracellularly may be especially cytotoxic.

METHODS

Materials. Indomethacin and bile acids were purchased from Sigma Aldrich (St. Louis, MO). PC in the form of Phospholipon 90G (95% PC) or Lipoid S100 (100% PC) was obtained from Lipoid (Ludwigshafen, Germany). PC-indomethacin was prepared by combining indomethacin and 90G or S100 at a 1:2 weight ratio, as previously described (9). This ratio results in a drug complex that is approximately equimolar for both components. [14C]indomethacin was purchased from American Radiolabeled Chemicals (St. Louis, MO). [3H]taurocholic acid was purchased from PerkinElmer (Waltham, MA).

Cell culture. IEC-6 cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum, penicillin, streptomycin, and insulin. Cells were seeded onto 24-well plates at 0.1 × 10^6 cells/well. After overnight growth, media was removed and replaced with DMEM containing test agents for 3 h. Media was then removed, centrifuged, and used to assay for the cytosolic enzyme lactate dehydrogenase (LDH; Clontech Laboratories, Mountain View, CA) as a measure of cell membrane disturbance. Cells remaining on the plate were incubated with media containing 5 mg/ml of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) for 2 h to measure cell viability and number, as previously reported (23).

To examine the role of ASBT-mediated bile acid uptake in indomethacin-induced cell injury, an epithelial cell model was selected that lacks endogenous ASBT expression and could be manipulated to express this transporter. Thus, Madin-Darby canine kidney (MDCK) cells were chosen and were stably transfected with the human ASBT cDNA as described previously (5, 21). Cells were initially incubated overnight in media containing 1 mM butyrate to induce expression of transfected ASBT. Cell injury (LDH) and cell number/viability (MTT) were examined after incubation with test agents, as described above for IEC-6 cells.

Uptake of indomethacin or taurocholate into MDCK cell lines was measured as described previously (21). Briefly, cells were rinsed three times with Hank’s buffered salt solution containing either sodium chloride or choline chloride (to monitor sodium dependence), and then were incubated in the same solution containing 10 μM [3H]taurocholate [500 counts·min⁻¹·(cpm)·pmol⁻¹] or 20 μM [14C]indomethacin (300 cpm/pmoul) for 30 min at 37°C. Thereafter, cells were rinsed three times with ice-cold buffer to remove excess radiolabel and dissolved in 0.5 N sodium hydroxide, and aliquots were taken to determine cell-associated radioactivity and protein (Bio-Rad Protein Reagent, Hercules, CA). Uptake was expressed as picomoles of taurocholate or indomethacin per milligram protein per 30 min.

Statistics. Differences between treatment groups were evaluated by the Student’s t-test and/or ANOVA, followed by the Fisher’s least significant difference test. A value of P < 0.05 was considered significant.

Fig. 1. Dose-response of indomethacin/phosphatidylcholine (PC)-indomethacin on intestinal epithelial-6 cells (IEC-6). A: effect of indomethacin alone (Indo) or indomethacin complexed to PC (PC-Indo) on cell permeability. Cells were incubated in medium containing increasing concentrations of Indo or PC-Indo for 3 h. The level of the cytoplasmic enzyme lactate dehydrogenase (LDH; Clontech Laboratories, Mountain View, CA) as a measure of cell membrane disturbance. Cellular cytotoxicity was determined to be 0.7% of the maximal LDH release and is not shown due to a significant difference test. 

B: effect of Indo or PC-Indo on cell viability and number was measured by the level of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) for 2 h to measure cell viability and number, as previously reported (23).
RESULTS

Effects of indomethacin vs. PC-indomethacin on cell integrity and viability. IEC-6 cells were incubated for 3 h with varying concentrations of indomethacin or PC-indomethacin, and then LDH and MTT were assessed. It was found that indomethacin dose-dependently damaged cells as indicated by the release of LDH and reduction of MTT (Fig. 1, A and B). Maximal toxicity with indomethacin was seen at 3.5 mM. In contrast, PC-indomethacin was not injurious at concentrations up to 3.5 mM under these acute in vitro conditions. A moderate (<50% effective dose) concentration of 2.5 mM indomethacin was chosen for subsequent studies to identify potential interactions with other potential damaging agents such as bile acids.

Effects of bile acids and PC on NSAID injury. In preliminary experiments, cells were incubated with varying concentrations of primary and secondary bile acids or their tauro and glycine conjugates to determine the highest dose that did not produce significant injury. These concentrations were then used in subsequent studies as noted. The effect of combinations of 2.5 mM indomethacin or PC-indomethacin and cholic (Fig. 2), deoxycholic (Fig. 3), and chenodeoxycholic (Fig. 4) acid, as well as their tauro (T)- and glycoconjugates, can be seen. It is clear that the combination of any of the major primary/secondary bile acids with 2.5 mM indomethacin can be damaging as assessed by LDH and MTT. The potential to induce cell injury was in the order of deoxycholic (DCA) > chenodeoxycholic (CDCA) > cholic (CA), which is probably related to their chemical hydrophobicity index. It was for this reason that we evaluated the cytotoxicity of DCA, CDCA, and CA at 0.3, 0.6, and 4 mM, respectively. Also, the bile acid N-acyl amidates were generally less injurious than their unconjugated forms at the same concentration. However, the conjugates were still capable of cell damage at higher concentrations and could induce marked injury in combination with indomethacin. In comparison, PC-indomethacin was noninjurious to cells when combined with any of the bile acids or their conjugates at the concentrations tested.

Potential role for bile acid uptake with indomethacin injury. Cells expressing the human ASBT were used to examine the
importance of bile acid uptake for indomethacin-associated injury. To verify the active uptake of bile acid into cells transfected with ASBT, the experimental cell lines were stimulated with butyrate and then tested for [3H]taurocholate uptake. Figure 5 shows that parental MDCK cells did not actively take up taurocholate, whereas the ASBT-transfected cells showed significant sodium-dependent uptake of the conjugated bile acid (demonstrating a $10^2$-fold increase vs. parental MDCK). Under these same conditions, the cell lines (ASBT) were exposed to the taurine-conjugated or -unconjugated forms of the major bile acids alone (at concentrations determined to not produce injury on their own) and in combination with either indomethacin or PC-indomethacin. As shown in Fig. 6, A and B, there was significant bile acid/indomethacin-induced cell injury in the MDCK cells in the absence and presence of the ASBT. After accounting for differences in LDH release induced by indomethacin alone (control), the cell injury induced by indomethacin plus bile acids was generally comparable between the parental MDCK cells and MDCK plus ASBT cell lines (Fig. 6C). For the conjugated bile acids, modest differences were found for TDCA and TCDCA but not TCA. No differences were found for the unconjugated bile acid treatments between MDCK and MDCK plus ASBT cells. These results suggest that active uptake of bile acid into the cultured cells generally is not required for cell injury associated with acute exposure to bile acid/indomethacin but that injury may be enhanced in the presence of active uptake. It is noteworthy that PC-indomethacin showed little or no injury with either cell type (±ASBT expression) in the presence of bile acid.

To determine whether the uptake of indomethacin may be affected by bile acid, the MDCK cell lines were tested for [14C]indomethacin uptake. Figure 7 shows that there was modest indomethacin uptake into the cells, which was independent of the presence of bile acid (taurocholate) in the media or expression of the ASBT. Under these conditions, ~2.2% of the indomethacin was taken up by the cells during the 30-min incubation period.

### DISCUSSION

**Indomethacin injury and protection with PC.** As seen in Fig. 1, indomethacin is directly injurious to intestinal cells (IEC-6) in a dose-dependent manner. At 3.5 mM, indomethacin alone is clearly injurious, whereas PC-indomethacin at the same NSAID concentration is not. Complexing indomethacin with approximately equimolar amounts of PC appears to protect against indomethacin-induced acute cell injury. It should be noted that the in vitro concentrations of indomethacin used in subsequent studies (2.5 mM) are likely higher than that achieved intraluminally in humans along the length of the small intestine but may simulate local regions of high concentrations (e.g., entry of common bile duct into the proximal gut or the site of deglucuronidation in the lower gut). As such, it was used to examine potential mechanisms of injury under acute cell culture conditions. This protection against acute injury conferred by the addition of PC, which is the focus of the current study, may be different from that seen with longer periods of exposure. We previously reported that indomethacin-PC was also less injurious than indomethacin alone after a 24-h incubation, (13); however, the longer time period allowed...
Indomethacin/bile acid injury and protection with PC. Figures 2–4 show that PC-indomethacin is not injurious in the presence of any of the tested unconjugated and conjugated bile acids of varying hydrophobicity. This remarkable protection helps to explain our in vivo findings where PC-indomethacin was much less damaging to the gastrointestinal tract of rats compared with indomethacin alone (9). Interestingly, complexing the PC and indomethacin before addition to the cells may not be required for this in vitro protection, as we have previously shown that adding bile acid, indomethacin, and PC separately to cell media also protects against LDH release, if the concentration of PC is sufficiently high (i.e., 2-fold greater than indomethacin) (7). We have speculated that bile acid and indomethacin can form a toxic mixed micelle in the gut lumen, which is rendered less toxic in the presence of PC. This concept is supported by powerful NMR/MD techniques, which demonstrated that these two classes of amphipathic molecules (bile acids and NSAIDs) have a natural affinity to associate into mixed micelles that are further transformed in the presence of PC (3, 11, 16). This preassociation of PC and indomethacin may be even more important under in vivo conditions in the biliary tract system and/or intestinal lumen where indomethacin would have access to bile acid, both free and conjugated forms. In previous in vivo studies, we showed that the administration of an NSAID and PC separately was not protective, but rather, the complex of NSAID with PC was essential for protection of the gastrointestinal tract (10).

Indomethacin/bile acid site of injury on cells. We performed studies using MDCK cells expressing the human ASBT to delineate the importance of uptake of bile acid and potential activation of intracellular pathways for indomethacin-induced cell injury. It was shown that the acute NSAID/bile acid injury was induced in the absence of active uptake, and presence of the transporter only modestly increased injury for select

Fig. 6. Effect of CA, TCA, DCA, TDCA, CDCA, and TCDCA acids and indomethacin/PC-indomethacin on LDH release from: MDCK cells (A) and MDCK + ASBT cells (B). *P < 0.05 vs. control/bile acid and PC-indomethacin. C: increase in LDH release following incubation with indomethacin and bile acid in MDCK vs. MDCK-ASBT cells. The values for each cell line were corrected for the LDH release in the presence of indomethacin alone. *P < 0.05 vs. MDCK cells.

Fig. 7. Effect of sodium or choline buffers, or presence of TCA, on indomethacin uptake into MDCK and MDCK + ASBT cells.
jugated bile acids. Of the four bile acids tested that require the transporter for cell uptake (CA, TCA, TDCA, and TCDCA), only TDCA and TCDCA showed greater injury in the transfected cells. Therefore, uptake of bile acid into cells does not appear to be an absolute requirement for cell injury under the acute conditions of this study. Rather, this finding points to a cytotoxic action of indomethacin and bile acid on or at the exterior of the cell, such as an effect on the cell membrane, as we have postulated previously (23), where membrane perturbation could be relevant. Thus, we speculate that an early event in NSAID-induced enteropathy occurs on the cell membrane by NSAID/bile acid mixed micelles and results in membrane changes such as pore formation, increases in fluidity, and/or membrane lysis, all of which result in an attenuation in the barrier properties of the lower gut. This explanation is also consistent with the possibility that a longer incubation time may allow for the additional activation of intracellular mechanisms that may be of importance under in vivo conditions.

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DISCLOSURES
LML is a stockholder and member of the Scientific Advisory Board of PLx Pharma LLC (Houston TX), a university-based startup company that is developing PC-NSAIDs for a variety of therapeutic uses. EJD possesses stock options in PLx Pharma LLC. PAD has no disclosures.

AUTHOR CONTRIBUTIONS
L.M.L., E.J.D., and P.A.D. conception and design of research; L.M.L., E.J.D., and P.A.D. interpreted results of experiments; L.M.L., E.J.D., and P.A.D. analyzed data; E.J.D. performed experiments; E.J.D. and P.A.D. drafted final version of manuscript; E.J.D. and P.A.D. drafted manuscript.

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