INTRODUCTION

Clinical evidence is emerging that NSAID-induced gastrointestinal injury and bleeding is not limited to the gastroduodenal mucosa but also affects the lower gut, which may be manifest as erosions, ulcers, strictures, ileitis and associated pathology, such as diverticulitis/diverticular bleeding (1-8). It is also becoming apparent that this form of lower gut injury is not restricted to non-salicylate NSAIDs and may also be associated with the consumption of aspirin, notably in the enteric-coated dosage form (4, 5, 7, 9-11).

Our laboratory has recently been investigating NSAID-induced lower gut injury using a number of in vitro and in vivo model systems. In these studies we have confirmed and extended the findings of other laboratories demonstrating that the ability of non-salicylate NSAIDs to induce intestinal injury/bleeding can be prevented by bile duct ligation (12-14). Furthermore we have confirmed the findings of Brune and associates that linked NSAID-induced lower gut injury to both the secretion of specific non-salicylate NSAIDs into the bile and the balance between the concentrations of damaging bile acids and protective PC/lecithin in bile (12, 13, 15-17). This issue will be elaborated upon in a subsequent section.

Okabe and associates recently developed a novel rodent model system to study aspirin-induced intestinal injury, by administering the NSAID directly to the proximal duodenum of anesthetized rats and following histological injury and alterations in intestinal permeability, afterwards (18). We therefore used this new model to further study the role of bile acid and PC in aspirin-induced lower gut injury, a subject that is particularly relevant today, as we better appreciate that this form of GI injury is quite common notably among chronic consumers of EC-aspirin.

MATERIALS AND METHODS

Chemicals

Immediate-release (IR) generic aspirin was purchased from a local pharmacy (Walgreen Co, Lot No. P5340) and suspended in deionized distilled water until the desired concentration achieved. To prepare aspirin-PC (PL2200, provided by PLx Pharma, Houston, TX) Phosphatidylcholine (PC) enriched soy lecithin and was purchased from Lipoid Inc, (Ludwigshafen, Germany) and purified aspirin from Rhodia, Lyon, France,
respectively. Aspirin-PC conjugate (PL2200, provided by PLx Pharma Inc, Houston, TX) was prepared in accordance to previously described method (19, 20). It should be noted that we used immediate-release aspirin purchased from a retail drug store for the comparator group that received unmodified aspirin, mainly because it is packaged with dispersants that promote its rapid release and dispersability in the subjects’ GI tract. In contrast, aspirin-PC (PL2200) uses pure aspirin-HCl (provided by Rhodia), that does not contain these dispersants and other additives that may interfere with aspirin’s ability to associate with PC.

Rodent model of NSAID-induced intestinal injury/bleeding

All animal protocols described in this study were previously reviewed and approved by our institution’s Animal Welfare Committee and determined to meet or exceed guidelines of the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and NIH and conforms to the provisions of the Declaration of Helsinki on the proper care and treatment of laboratory animals. A modification of the technique previously described (18) was used to study the acute effect of aspirin to induce small intestinal mucosal injury, increase intravascular permeability and bleeding. To study NSAID-induced intestinal injury, fasted male Sprague Dawley rats (150-200 g, purchased from Harlan Labs, Indianapolis, IN) were anesthetized under isoflurane, and after making a midline incision and the pyloric junction region visualized, a 20 gauge needle was inserted into the proximal duodenum of rats where we administered either aspirin at 0 (control) 50, 100 and 200 mg/kg which was dissolved in water, or the equivalent dose of active, where the aspirin was pre-associated with PC or bile from a donor rat. In most experiments, the rats were euthanized 90 minutes later and the entire length of the small intestine was removed and flushed with ice cold saline which was subsequently analyzed for hemoglobin (Hb) concentration as an estimate of intestinal bleeding, as previously described (21-23).

Measurement of NSAID-induced increase in vascular permeability

In a second study, rats were i.v. administered Evan’s blue (EB, purchased from MP Biomedicals, Solon, OH) 10 mg/ml saline at 60 minutes after intra-duodenal administration of the aspirin test drugs and euthanasia at 90 minutes, the proximal 30 cm of the small intestine was cut open longitudinally (starting at the pyloric junction), inspected for EB staining under a dissecting microscope, photographed and extracted for EB reactivity as previously described (18, 24). EB was extracted from this tissue which extends from the proximal duodenum to proximal jejunum using the following procedure: the collected intestinal tissue was immersed in 2 ml formamide solution (Fisher Scientific) in test tubes which were in turn incubated in a water bath at temperature of 56°C overnight. After the overnight incubation, the mucosal tissue was completely dissolved in the formamide solution. The Evan’s blue extracted tissue was then vortexed and the intensity of the optical density of the color read employing a Spectrophotometer (Thermo Spectronic) at OD 610 nm against a EB standard curve from 0-31.25 µg/ml in formamide.

Effect of bile duct ligation and donor bile on aspirin-induced intestinal injury

In a separate experimental series, aspirin-induced intestinal injury was studied as described above, in anesthetized rats whose bile duct was ligated or sham operated/SO (by surgically placing loosely-tied ligatures around the exposed bile duct) 30 minutes prior to intra-duodenal administration of the aspirin either alone or in the presence of 1 ml of rat bile (collected from isoflurane-anesthetized donor animals over a 2 hr collection period) and intestinal bleeding analyzed 90 minutes later.

Histology and scoring of light microscopic injury

Tissue was collected from the region which includes distal duodenum/proximal jejunum (20-30 cm from the pyloric junction), cut into rings with the mucosa facing inward, fixed in 10% buffered formalin, embedded in paraffin and cut longitudinally into 4 µm sections for hematoxylin & eosin (H&E) staining. Then after the slides were decoded, representative sections for each treatment were photographed under low power microscopy.

Statistical analysis

Experiments were analyzed initially by one-way ANOVA, followed by post hoc testing with the Fisher LSD test. The level of significance was set at p<0.05.

RESULTS

Effect of intra-duodenal aspirin on Evan’s blue staining of the small intestine

Using Evan’s blue staining of GI tissue as a marker of an increase in vascular permeability, we investigated the effects of intra-duodenal administration of immediate-release aspirin (at a dose of 100 mg/kg) vs. the equivalent NSAID dose of aspirin-PC on the uptake of the vascular dye 30 minute prior to euthanasia. At euthanasia, the proximal 30 cm of the small intestine was subdivided longitudinally into 5 segments of 6 cm/length and viewed and photographed under a dissecting microscope. The results depicted in Fig. 1A, indicate that in comparison to saline-treated control rats which showed light Evan’s blue staining, rats intra-duodenally administered aspirin were more heavily stained with the vascular dye notably in the 2nd, 3rd and 4th 6 cm ribbon. It should be noted that the location of the ligament of Treitz (corresponds with the 3rd 6 cm ribbon) is distal to where the bile duct enters the duodenum (which would correspond to the 2nd ribbon). It also can be appreciated that rats intra-duodenally administered the equivalent dose of aspirin-PC took up less of the vascular dye, upon visual inspection, which was then quantitatively confirmed when the Evan’s blue was extracted from the dissected duodenal tissue (Fig. 1D). It also should be noted that in this analysis it was determined that intra-duodenal aspirin induced a statistically significant increase in the tissue uptake of Evan’s blue concentration of the proximal (30 cm) of the small intestine over both control levels and the tissue levels of the dye of rats administered aspirin-PC, with the latter group not being different from saline-treated control values.

Effect of intra-duodenal aspirin on the light microscopic structure of the intestinal mucosa

When H&E stained sections of the intestinal mucosa were observed under a light microscopy, it became evident that intra-duodenal aspirin at a dose of 100 mg/kg induced significant surface injury to the villus structure with considerable cell sloughing into the lumen (see Fig. 2B) in comparison to saline-treated control rats (Fig. 2A) or rats intraduodenally administered aspirin-PC (Fig. 2C), where the villus/crypt architecture was well preserved.
Effect of intra-duodenal aspirin on intestinal bleeding

Based upon the above findings, we next performed a dose-response analysis investigating the effects of intra-duodenal aspirin upon intestinal bleeding by measuring the hemoglobin concentration of a perfusate of the gut lumen, using a method previously described to measure the effect of non-salicylate NSAIDs on lower gut bleeding (22, 23, 25). In this study we compared intra-duodenally administered saline with aspirin concentrations ranging from 50-200 mg/kg vs. the equivalent NSAID doses of aspirin-PC. The results of this experiment, which are depicted in Fig. 3, clearly demonstrated that intra-duodenally administered aspirin induced a dose-dependent increase in intestinal bleeding over control values, which were remarkably and significantly attenuated in rats intra-duodenally administered aspirin-PC. It should be noted that PC-conjugated aspirin even at the highest dose tested (200 mg aspirin/kg) induced only a modest apparent increase in intestinal bleeding that was not statistically significantly different from saline-treated control values.

**Effect of intra-duodenal aspirin on intestinal bleeding in rats with bile duct ligation and its dependence on the presence of rat bile**

In this experimental series we studied the bile dependence of aspirin induced intestinal injury, by investigating the effect of bile duct ligation on intestinal bleeding induced by the intra-duodenal administration of aspirin. As depicted in Fig. 4, in contrast to the pronounced increase in intestinal bleeding in sham-operated (SO)
rats that received intra-duodenal aspirin, bile duct ligated (BDL) rats were resistant to aspirin’s injurious action. Fig. 4 also demonstrates that the apparent protective action of BDL to aspirin-induced intestinal bleeding could be overcome, in part, if bile collected from a donor rat was mixed with the immediate-release aspirin prior to intraduodenal administration to BDL rats, though the administration of bile in the absence of aspirin did not have an injurious action on it’s own.

**DISCUSSION**

The chronic consumption of aspirin has been associated over the years with gastroduodenal ulcers and bleeding, some of which may be life-threatening (11, 26-30). Recently, there has been a growing body of evidence, based upon both prospective wireless, capsule endoscopic studies, and restrospective/epidemiological analyses that chronic aspirin consumption may also be associated with injury/bleeding to the lower gut and occur in >50% aspirin users (4, 5, 8, 10). Clinical studies evaluating the benefit of enteric coated (EC) aspirin have been conflicting and therefore it is presently uncertain whether delayed aspirin-release provides symptomatic relief or prevents tissue injury as determined by endoscopic observation or histological examination of mucosal biopsies (6, 9, 31).

Thus, it appears that aspirin, particularly EC-aspirin that currently dominates the market, may act in a similar manner to non-salicylate NSAIDs to induce intestinal injury, which may result in perforation or life-threatening GI bleeds. In support of this, aspirin consumption has been linked to distal gut pathology, notably the development of ileitis and/or diverticulitis that may be perforating in nature (1-8). In support of these clinical findings, it has recently been reported that oral administration in rats with experimentally-induced colitis exacerbates TNBS-induced colonic mucosal inflammation and delays the healing of this lesion to the lower gut (32). The work presented here, confirms and extends earlier work of Okabe and associates (18) and demonstrates that intra-duodenal administration of aspirin induces mucosal injury to the small intestine of rats as determined both by macroscopic and microscopic methods, an increase in vascular permeability as indicated with Evan’s blue staining of the proximal gut, and a dose-dependent increase in intestinal bleeding.

Ever since Brodie’s demonstration that indomethacin-induced intestinal injury can be blocked by bile duct ligation (33), we have grown an appreciation of the importance of bile in the pathogenic mechanism of intestinal injury caused by non-salicylate NSAID. This bile dependence may in part be attributable to the fact that many of these non-salicylate NSAIDs are secreted into the bile and enter the enterohepatic circulation.
In fact Brune’s lab demonstrated that the efficiency of biliary excretion of an NSAID was directly associated with its toxicity to induce intestinal injury (15, 16). In these studies, the investigators emphasized that this pathway did not apply to immediate release (IR) aspirin and/or salicylate as their biliary excretion is very low in comparison to non-salicylate NSAIDs, such as indomethacin that induces severe injury/perforation to the lower gut. Although these findings may explain why GI injury due to the consumption of IR-aspirin is primarily confined to the gastroduodenal mucosa, it would not apply to EC-aspirin, where the aspirin is released in the lumen of the small intestine.

It is also of particular importance to note that in addition to serving as a pathway for NSAIDs to gain access to the intestinal lumen, even after systemic administration, bile may either aggravate or protect against NSAID-induced injury depending on its composition. Our lab has demonstrated that the surface injurious action of bile acids (both unconjugated and conjugated) on membranes, and notably intestinal epithelial cells can be exacerbated in the presence of NSAIDs (12-14, 34). This has been demonstrated in a number of model systems including synthetic phospholipid membranes, erythrocyte membranes, established GI culture lines (AGS and IEC-6 cells) and rodent model systems. Similarly in this study we demonstrated that intra-duodenally administered aspirin failed to induce intestinal bleeding in the absence of bile (experimentally induced with BDL) and this protection could be overcome in part by the administration of bile from a donor rat. The molecular mechanism of this bile acid-dependence of NSAID-induced membrane injury has yet to be elucidated and we have proposed that it may be attributable to the formation of toxic mixed micelles composed of NSAIDs and bile acids due to the common amphipathic properties of these two classes of molecules (12-14, 34).

Lastly, it is of note that we have demonstrated that the bile-acid induced increase in the toxic potency of NSAIDs to the enterocyte membrane can be reversed in a dose-dependent manner by PC, which requires equimolar or higher concentrations of PC (12, 14). As lecithin or PC is normal constituent of bile, though at rather low concentrations that would have little or no protective action (representing 20-30% of the total bile acid concentration), an alternative approach our lab has taken is to pre-associate the PC and NSAID prior to administration. Both preclinical studies and a recently published clinical endoscopic study have demonstrated that aspirin-PC (PL2200) induces significantly fewer gastroduodenal ulcers and erosions (~70% reduction) than the equivalent dose of PL2200 may empty into the small bowel, we compared the intestinal toxicity of intra-duodenally administered aspirin-PC vs. aspirin in this study and in confirmation with our previous observations with PC-NSAIDs demonstrated little or no mucosal injury and/or intestinal bleeding even at the highest doses tested.

It should be noted, that this study focused on the cyclooxygenase (COX) independent surface injurious actions of aspirin on the intestinal mucosa, as would occur at the site of NSAID absorption. Indeed aspirin induced COX-inhibition, and the depletion of “cytoprotective” prostaglandins likely plays an important role in ulcer formation and healing based upon considerable evidence that prostaglandins promote the barrier properties of the tissue and it’s ability to respond to tissue injury, which include stimulation of mucus/bicarbonate secretion and the maintenance of mesenteric blood flow (36-38). However a number of studies, indicate that aspirin can induce acute surface injury to the tissue by COX-independent mechanisms. This is based upon the findings of a number of studies including from our laboratory which indicate that aspirin-induced systemic inhibition of COX can be dis-associated from tissue injury and ulcer development (34). For example Ligumsky, demonstrated that parenteral administration of aspirin led to >95% inhibition of COX, as indicated by depletion of mucosal PGE2, but did not induce ulcer development, whereas gastric injury was induced by the intragastric administration of the NSAID (39). Furthermore our lab reported that intragastric aspirin induces gastric ulceration/bleeding in COX-1 and COX-2 knockout mice (40), and that rats administered aspirin-PC in combination with celecoxib were protected from aspirin-induced injury/bleeding, although mucosal PGE2 concentration was inhibited >85% (21). It is thereby likely that both surface injury and COX-inhibition are required for the development of chronic mucosal small bowel injury and bleeding.

In summary, we have demonstrated in a rodent model system that intra-duodenally administered aspirin induces mucosal injury, an increase in vascular permeability and intraluminal bleeding of the proximal small bowel that appears to be dependent on the presence of luminal bile acids. This aspirin-induced intestinal injury/bleeding can be abrogated by the administration of a PC-conjugate of the NSAID (PL2200). The findings support the case that the presence of aspirin in the intestinal lumen as likely occurs with EC-aspirin does induce mucosal ulceration/bleeding of the lower gut, most likely due to a surface injurious action that is aggravated by bile acids and reversed by PC.

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REFERENCES


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