Amelioration of TNBS-induced colon inflammation in rats by phospholipase A₂ inhibitor

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Am J Physiol Gastrointest Liver Physiol 285: G586–G592, 2003. First published April 30, 2003; 10.1152/ajpgi.00463.2002.—The pathophysiology of inflammatory bowel disease (IBD) involves the production of diverse lipid mediators, namely eicosanoids, lysophospholipids, and platelet-activating factor, in which phospholipase A₂ (PLA₂) is the key enzyme. Accordingly, it has been postulated that control of lipid mediator production by inhibition of PLA₂ would be useful for the treatment of IBD. This hypothesis was tested in the present study by examining the therapeutic effect of a novel extracellular PLA₂ inhibitor (ExPLI), composed of carboxymethylcellulose-linked phosphatidylethanolamine (CMPE), on trinitrobenzenesulfonic acid-induced colitis. Intrapertoneal administration of CMPE suppressed the colitis as measured by mortality rate, intestinal permeability, plasma PLA₂ activity, intestinal myeloperoxidase activity, and histological morphometry. Current therapeutic approaches for inflammatory conditions focus on the selective control of a lipid mediator(s) (e.g., prostaglandins or leukotrienes). The present study supports the concept that inclusive control of lipid mediator production by PLA₂ inhibition is a plausible approach to the treatment of colitis and introduces the ExPLIs as a prototype of a novel NSAID for the treatment of intestinal inflammation.

INFLAMMATORY BOWEL DISEASE (IBD) is a chronic disorder of the intestine, relating mainly to ulcerative colitis and Crohn’s disease (43), associated with abdominal pain, diarrhea, fever, weight loss, anemia, arthritic pain, and impairment of liver function. Although the etiology of IBD is not known, its pathophysiology is postulated to have two stages: an insult triggering initial tissue damage, followed by an amplification stage, which propagates the tissue destruction and the duration of the disease. It is assumed that in the amplification stage, inflammatory cells migrate to the injured site and produce diverse proinflammatory mediators, mainly eicosanoids [prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs)] and cytokines (34, 40, 43).

Because different eicosanoids are potent mediators of inflammatory processes, the inhibition of their production has long been proposed and tested for therapeutic purposes. The eicosanoids include two main families of arachidonic acid (AA) metabolites derived through the cyclooxygenase pathways (COX-1 and COX-2), producing PGs and TXs, or the lipoxygenase (LOX) pathways, producing LTs and hydroxyeicosatetraenoics (HETEs). In addition, eicosanoid production in inflammatory processes is linked to cytokine production, although the cause-and-effect relationship and interaction between these two groups of inflammatory mediators is not clear (1, 5, 7, 40, 43). Accordingly, the control of eicosanoid production has been a major target in the treatment of inflammatory processes. The prevalent approach has been aimed at selective inhibition of the production of a specific central eicosanoid or a related enzymatic pathway (e.g., COX-2 inhibition). However, these compounds play a complex role in the pathophysiology of inflammatory conditions: each of these pathways may produce agonists and antagonists of the same biological process. Moreover, similar effects may be exerted by products of COX-1, COX-2, and LOX (25, 39). Thus it often happens that selective blocking of one pathway diverts the AA pool to the alternative pathway(s) (17, 25), leading to exacerbation of the pathological state (3, 17, 26). One such situation is IBD, in which products of both the cyclooxygenase pathway (PGE₂, PGF₂α, PGD₂, thromboxane B₂) and the lipoxygenase pathway (leukotriene B₄, 5-HETE, 12-HETE) have been reported to be involved in the acceleration of the inflammatory process (13, 40, 43). In addition, COX-1 and COX-2 play a complex role in gastrointestinal inflammation, and disparate views have been expressed as to their part in its pathophysiology (13, 40, 48).

On these grounds, the “dual-pathway inhibition” has been proposed, aiming at suppressing the activation of eicosanoid production by controlling the release of eicosanoids...
their precursor, AA (17, 19, 26), i.e., inhibiting phospholipase A₂ (PLA₂) activity.

PLA₂ hydrolyzes membrane phospholipids to produce free fatty acids and lysophospholipids and thus initiates the production of the different families of inflammatory lipid mediators: the AA-derived eicosanoids, different lysophospholipids, and platelet-activating factor (PAF) (20, 33). Diverse lipid mediators, specifically derived by the COX and lipoxygenase pathways, as well as PAF, have been found to be strong mediators of intestinal inflammation (40). In addition, PLA₂, which is secreted excessively into body fluids in inflamed tissues, synergizes with reactive oxygen species to induce cell lysis in host tissues (12, 15), and, ultimately, the actual lysis of the cell membrane lipids is done directly by the extracellular PLA₂ (12, 39). In addition, PLA₂ activity is linked to cytokine production by inflammatory cells, either directly or via its effect on eicosanoid production, although the interrelation between them is not yet clear (1, 5, 31). Activation of PLA₂, particularly the secretory enzyme (sPLA₂), has been implicated in different types of intestinal inflammation in humans (7, 18, 29, 30, 41) and in animal models (14, 21). Altogether, PLA₂ has long been considered a pivotal enzyme in inflammatory and allergic processes (21, 33), and inhibition of its activity might exert multiple effects in amelioration of related diseases (21, 26, 36).

Accordingly, steroids, which are often used for treating IBD, are assumed to suppress PLA₂ activity through lipocortin synthesis (27). In addition, it has been shown that sulfasalazine, used to treat IBD, inhibits sPLA₂ release (32). Inhibitors of PLA₂, such as mepacrine (41), quinacrine, and p-bromophenacylbromide (PBPP) (14, 21, 29), have been shown to suppress intestinal inflammation in animal models (14, 21) or in biopsies of patients’ intestine (29, 42).

Because of the central role of PLA₂ in inflammatory processes, inhibitors of its activity have been sought for treatment of related diseases (8, 26, 38, 39). However, since inhibitors that penetrate the cell (e.g., mepacrine, quinacrine, PBPP) might impair the cell metabolism and viability, it has long been suggested that the appropriate treatment requires an inhibitor of PLA₂ activity at the cell membrane that is not internalized by the cell (8). Extracellular PLA₂ inhibitors (ExPLIs) that fulfill these requirements have been designed and synthesized in the lab of S. Yedgar by binding PLA₂-inhibiting molecules to polymeric carriers in a way that enables the inhibiting moiety to control the cell membrane PLA₂ activity, but its internalization is prevented by the polymeric carrier (11, 47). The ExPLIs have been found effective in protecting the membrane from different types of exogenous PLA₂, including porcine pancreatic and human recombinant synovial fluid PLA₂ (11), protecting cells from activation of endogenous PLA₂ and concomitant cell damage induced by proinflammatory agents (16, 45), inhibiting LPS-induced activation of endogenous PLA₂ in macrophages (2), suppressing IFN-γ-induced activation of endothelial cells (44), suppressing LPS-induced chemokine production by endothelial cells (5), as well as ameliorating bleomycin-induced lung injury in hamsters (10) and endotoxin-induced sepsis in rats (4).

On these grounds, the present study was undertaken to test the prospect of the multiple pathway control of lipid mediator production in the treatment of IBD by testing ExPLIs’ effect on experimental intestinal inflammation. For this purpose, we have examined the effect of the ExPLI composed of N-derivatized phosphatidylethanolamine linked to carboxymethylcellulose (CMPE) (11) on colitis induced in rats by trinitrobenzenesulfonic acid (TNBS). Human IBD is unique, and animal models do not fully simulate its pathophysiology and clinical course. Despite these drawbacks, this model is widely accepted for the study of colon inflammation and its treatment. In the present study, the treatment with ExPLIs was found considerably effective in amelioration of TNBS-induced colitis, as measured by mortality, intestinal permeability and histology, and biochemical markers.

**MATERIALS AND METHODS**

TNBS-induced colitis. Following a conventional method (21, 22, 35), TNBS (Sigma, St. Louis, MO) was administered rectally (25 mg in 1 ml of 50% ethanol) to Hebrew University Sabra rats (200–250 g) after 24 h of fasting, and the course of the disease was monitored for 2 days, according to our preliminary experiments and previous reports (25). On the basis of previous experience, in the ExPLI-treated group, the rats were treated intraperitoneally with 10 mg/100 g body wt CMPE (hereafter CMPE-treated) in 1 ml saline (to obtain ~10 μM in body fluid) at 18 and 0.5 h before as well as 3, 18, and 36 h after TNBS administration to maintain a significant level of CMPE during the experiment course. The untreated rats (hereafter TNBS rats) received 1 ml of the vehicle (saline) intraperitoneally at the same time points.

**Histological morphometry of intestinal damage.** The rats that survived the disease 48 h after induction were terminated under ether anesthesia, and 10-cm segments of the distal colon were evaluated for macroscopic histological damage and then dissected longitudinally into two parts. One part was immediately fixed in 10% formalin and embedded in paraffin following dehydration with ethanol; the other part was used for determination of myeloperoxidase (MPO) activity.

Macroscopic scoring of intestinal damage, from 0 (no damage) to 5 (maximal damage) was assessed by a naked-eye examination of areas of mucosal discoloration, erosion, ulceration, bowel wall thickening, and percentage of damaged area.

For histological morphometry, serial sections 5-μm thick were cut perpendicularly to the intestinal wall and stained with hematoxylin and eosin. The ulcerative area was determined on histological slides by using light microscopic image processes by a CUE-3 image analysis system (Galal, Migdal Haemek, Israel). As previously described (6), the CUE-3 system comprises high-resolution color charge-coupled device cameras (M-852; Sony) and a monitor (Trinitron; Sony). Image analysis was carried out in a predefined image window (with a surface of 0.3 mm² and by color thresholding representing the “actual objects” in a given window). For each rat, histology slides were taken from five loci along the intestine dissection (the same loci were taken from all dissections).
The measurements were quantified from 20–30 microscopic fields in 5 histology slides for each rat.

**Intestinal permeability.** Intestinal permeability was evaluated by determining the level of inulin-fluorescein (InFl, Sigma) in the plasma following its rectal administration (25). In a preceding study (25), we found that, although InFl does not permeate the intestine of normal rats, it is readily adsorbed in the colitic rats. That study showed that, in rats with TNBS-induced colitis, the intestinal permeability, as measured by InFl in plasma, reaches its peak at 12 h after administration of TNBS. Accordingly, in the present study, InFl (4 mg in 0.2 ml saline) was given rectally to both TNBS and CMPE-treated groups 12 h after administration of TNBS, and a blood sample (0.5 ml) was taken 2 h later from the tail vein of the ether-anesthetized rats (25). For determination of blood level of InFl, the plasma was separated by centrifugation and the fluorescence intensity was determined at 488 nm (excitation) and 517 nm (emission) as previously described (25).

**PLA₂ activity secreted into blood serum.** Blood samples (0.3 ml) were drawn from the rats by cardiac puncture under anesthesia at several time points. Before this experiment, we tested the possible effect of this procedure on plasma PLA₂ level by performing the same repeated cardiac puncture on normal (untreated) rats and found that this procedure did not affect the plasma PLA₂ activity. To avoid frequent anesthesia, in the present experiment, blood samples were drawn from each rat only three times. Accordingly, for each treatment (TNBS and TNBS + CMPE), we used two sets of five rats: one set of rats was subjected to blood sampling at 1, 6, and 12 h and the other set at 2, 6, and 24 h after TNBS administration (see Fig. 3). A slight difference was observed between the two sets at 6 h, and to obtain a continuous curve for all time points, the data for the two sets were normalized (by adjustment of the 6-h values of the 2 sets).

The plasma of the drawn blood samples was separated by centrifugation, and its PLA₂ activity was determined by the hydrolysis of phospholipid membranes (liposomes), as previously described (11). In brief, for preparation of lipid vesicles, 1-oleoyl-lyso phosphatidylcholine and dipalmitoylphosphatidylcholine (DPPC) containing 2-14C-DPPC (specific radioactivity = 1 nCi/nmol) dissolved in chloroform were mixed at a molar ratio of 1:1, respectively, dried under nitrogen, and suspended in Ca²⁺-free Tris buffer (100 mM Tris buffer [pH 8.0]) by cyclomixer to obtain total lipid concentration of 1.0 mM. For determination of serum PLA₂ activity, 25 μl of serum were mixed with 50 μl lipid vesicles and supplemented with Tris buffer containing 1 mM Ca²⁺ and 2% fatty acid-free bovine serum albumin to a final volume of 200 μl and were incubated at 37°C for the desired time. The reaction was terminated by the addition of organic solvent (Dole) to form two phases (37). The organic phase was collected, and its radioactivity was determined in a liquid scintillation counter. The activity in blood before induction of disease was taken as background and subtracted from the values obtained after disease induction. It has previously been shown, using thin-layer chromatography, that with the use of this method, the organic phase contains all of the free fatty acid released but the amount of substrate is insignificant (23, 46). This was confirmed by us using thin-layer chromatography before employing this method in the present study. As noted above, the amount of CMPE given to the treated rats was aimed at obtaining a concentration of ~10 μM in body fluid. Accordingly, to rule out the possibility that the change in plasma PLA₂ activity in CMPE-treated rats is due to its presence in plasma, the same concentration of CMPE was added to blood samples drawn from the TNBS rats (untreated with CMPE) before the PLA₂ activity was assayed.

The substrate used here (DPPC) is not hydrolyzed by cytosolic PLA₂ (cPLA₂), which is specific to AA-carrying phospholipid (2), and its hydrolysis thus represents the activity of sPLA₂ secreted to blood plasma.

**MPO activity.** Colon mucosa was homogenized in PBS (pH 7.4), and MPO activity was determined spectroscopically by o-dianisidine/H₂O₂ reaction (9, 14). Because of the high mortality rate among the TNBS (untreated) rats, MPO determinations were limited to the rats that survived the course of the experiment. This introduced a methodological problem, because it is likely that the rats that died had the most severe damage but these could not be included in the comparison between treated and untreated animals. This drawback was taken into consideration when analyzing the data of MPO activity and InFl permeation, as described in RESULTS.

All of the procedures applied to the rats were approved by the Hebrew University Institutional Animal Care and Use Committee (IACUC; permit OPRR-A01–5011).

**Statistical analysis.** The results are expressed as means ± SE. For Figs. 1 and 2, the difference between the TNBS and CMPE-treated groups was examined for significance by the Mann-Whitney U-test. For Fig. 3, the difference was examined for significance by the Mann-Whitney X-square test for combined probabilities (which compare the two entire time curves).

To avoid interference of the assays with each other (e.g., InFl with PLA₂ determination, cardiac puncture with MPO), each assay was performed with a separate set of rats. Due to our institution’s regulations concerning use of experimental animals, the possibility of repeating experiments was limited, and the number of rats used in the above assays was determined primarily by the survival of the TNBS rats through the respective experiment (12 h for InFl permeation, 24 h for plasma PLA₂, 48 h for MPO and survival).

### RESULTS

**Effect of CMPE on mortality rate.** Table 1, depicting the number of rats that survived the disease over the number of rats in each group, shows that treatment with the PLA₂ inhibitor CMPE was markedly effective in reducing the mortality rate among the rats with TNBS-induced colitis.

**Intestinal permeation.** As noted in MATERIALS AND METHODS, the assessment of the effect of CMPE on the

| Table 1. Effect of CMPE on mortality rate in rats with induced colitis |
|------------------|------------------|------------------|------------------|
| No. of Dead Rats/No. of Rats in Group | PBS | CMPE |
| 4/8 | 1/8 | 50 | 12.5 |
| 4/10 | 0/10 | 40 | 0 |
| 7/10 | 3/10 | 70 | 30 |
| 5/8 | 1/8 | 62 | 12.5 |
| 7/10 | 4/10 | 70 | 80 |
| Total | 27/46 | 9/46 | mean ± SE 58.4 ± 5.9 | 190 ± 7.1 |

Colitis was induced in rats by rectal administration of trinitrobenzenesulfonic acid. Rats were treated intraperitoneally with either PBS or carboxymethylcellulose-linked phosphatidylethanolamine (CMPE) as described in MATERIALS AND METHODS, and the mortality rate in the 2 groups was monitored for 48 h.
parameters of intestinal damage was obviously limited to the surviving animals, in which the related manifestations were less severe than in the rats that did not survive the experiment. However, in a separate study (25), we found that pathological intestinal permeation of InFl precedes the other markers of TNBS-induced colitis and reached its peak at ∼12 h after induction of the disease (25). In TNBS-induced colitis, animals started dying as early as 24 h after induction of the disease. Therefore, to obtain data from all of the animals in the experiments, intestinal permeation was examined 12 h after administration of TNBS, when most of the untreated rats were still alive. This was done by rectal administration of InFl and determination of its plasma levels after 3 h as previously described (25). As shown in Fig. 1, treatment with CMPE maintained the intestinal permeation of InFl at a much lower level than in the untreated rats, suggesting that this treatment attenuates the development of the disease at an early stage.

Colon tissue damage. Naked-eye examination of colon of rats with TNBS-induced colitis revealed damage mainly in the mucosa, with no infiltration to the serosa, and this was practically ameliorated in the CMPE-treated rats. The corresponding macroscoring (by the criteria described in MATERIALS AND METHODS) were 3.93 ± 0.43 and 1.19 ± 0.29 (n = 5; P < 0.001) for TNBS rats and CMPE-treated rats, respectively.

Figure 2A, depicting representative micrographs of colon tissue of rats with TNBS-induced colitis either
left untreated or treated with CMPE and compared with normal rats, clearly demonstrates that the treatment with CMPE markedly reduced the TNBS-induced ulceration and maintained it practically at the level of normal rats. This is confirmed by the quantitative morphometry of histological damage, shown in Fig. 2B, depicting the colon ulcerative area for the untreated and CMPE-treated colitic rats.

**Plasma PLA2 activity.** To investigate the association of the disease with the level of plasma PLA2 activity, we monitored the time course of its activity in plasma of the CMPE-treated and untreated rats subsequent to induction of colitis by TNBS. Figure 3 shows that the level of plasma PLA2 activity is enhanced during the course of the disease and appears at an early stage after induction of the disease, considerably before the expression of intestinal permeation. Figure 3 further shows that treatment with the PLA2 inhibitor suppressed the elevation in plasma sPLA2 activity through the entire time course and brought it to the basal level within 24 h (and possibly earlier) after induction of disease.

**MPO activity in intestinal tissue.** MPO activity in inflamed tissues, which is a measure of inflammatory cell infiltration into the inflamed tissue (9) and is commonly taken as a measure of intestinal inflammation (14, 21), has been linked to PLA2 activity and shown to be suppressed by PLA2 inhibitors (14, 28). In accord with this, in the present study the treatment with CMPE considerably reduced the MPO activity in the colon of colitic rats. The respective MPO activity in the untreated and the CMPE-treated groups was 19.1 ± 2.6 and 7.9 ± 1.1 U/mg tissue (means ± SE; n = 5; P < 0.01).

**DISCUSSION**

The results of the present study demonstrate that treatment with the PLA2 inhibitor CMPE impressively attenuated the development of colon injury induced in rats. This was assessed by clinical (mortality rate, intestinal permeation), histological, and biochemical parameters (PLA2, MPO). As noted in the introduction, PLA2 activity has been reported to be involved in pathophysiology of intestinal inflammation. Previous studies have reported elevated expression of Group II sPLA2 (29) and its gene (18) in the mucosa of inflamed human intestine, and PLA2 isoenzyme is abundant in rats and other mammals as well. Inflammatory conditions are typically associated with elevated sPLA2 activity, secreted to body fluids by activated inflammatory cells and inflamed tissues. This is further supported in the present study, showing elevated plasma activity of PLA2 as determined by the hydrolysis of sPLA2-specific substrate. It is likely that the plasma PLA2 activity observed in the present study is secreted to body fluids from the injured colon, but since the same isoenzyme may be secreted from inflammatory cells on their activation, they can also contribute to the elevated plasma level of sPLA2 as measured in the present study. The ExPLIs have been shown to protect cells from activation and secretion of cellular sPLA2 induced by inflammatory stimuli, such as endotoxins (2, 4). Furthermore, they block subsequent cell-signaling processes, including NF-κB stimulation (4). Because this transcription factor is involved in the activation of the intracellular cPLA2, it is likely that the ExPLIs, although cell impermeable, affect a wider range of inflammatory processes beyond sPLA2.

As discussed in the introduction, PLA2 is responsible for the production of a host of inflammatory mediators and is involved in the development of inflammation directly and indirectly, mainly via eicosanoid production. In addition, inhibition of PLA2 and subsequent production of lysophospholipids suppress the production of PAF, which is a potent inflammatory agent and has been shown to be involved in IBD pathophysiology (40). The present study, showing that experimental injury of rat colon is ameliorated by ExPLI, strongly supports the therapeutic strategy of inclusive control of the production of inflammatory lipid mediators by inhibition of PLA2 activation.

Nonsteroidal anti-inflammatory drugs (NSAIDs), which are COX inhibitors, are commonly used in clinical practice but are often associated with gastrointestinal complications, such as ulceration, bleeding, and perforation, which may be life-threatening. Treatment with corticosteroid is also accompanied by numerous adverse effects. The type of PLA2 inhibitors used here introduces a novel approach to the treatment of IBD and presents a potential alternative prototype of NSAID for the treatment of IBD, with a protecting strategy against damage induced by the currently used NSAID and steroid treatments. As noted in the introduction, the ExPLIs have been found effective in amelioration of other inflammatory conditions, including...
bleomycin-induced lung injury in hamsters (4) and endotoxin-induced sepsis in rats (10). Together with the present study, these suggest that this kind of PLA2 inhibitor may be considered a plausible candidate for therapy of intestinal injury and other inflammatory diseases.

DISCLOSURES

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REFERENCES


G591AMELIORATION OF COLITIS BY PHOSPHOLIPASE A2 INHIBITOR


