Role of proteinase-activated receptors 1 and 2 in nonsteroidal anti-inflammatory drug enteropathy

Running title: PAR1 and PAR2 in NSAID-induced enteropathy

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ABSTRACT

Background: The use of nonsteroidal anti-inflammatory drugs (NSAIDs) can promote lower gastrointestinal detrimental effects. Proteinase-activated receptors 1 (PAR1) and PAR2 are involved in the pathophysiology of several digestive disorders. This study examine the contribution of PAR1 and PAR2 in NSAID-induced small intestinal injury, and to investigate the underlying mechanisms.

Methods: Male Wistar rats (40 weeks old) were treated with indomethacin (1.5 mg/kg BID) for 14 days. Subgroups of animals were treated intraperitoneally with TFFLR-NH2 (PAR1 agonist), AC55541 (PAR2 agonist), SCH79797 (PAR1 antagonist) or ENMD-1068 (PAR2 antagonist). After treatments, blood and feces were collected for the assessment of hemoglobin and calprotectin, respectively. The ileum was processed for the evaluation of myeloperoxidase (MPO), malondialdehyde (MDA), and the protein expression of occludin and activated caspase-3.

Results: Indomethacin elicited a significant intestinal damage, associated with a decrease in blood hemoglobin and an increase in tissue MPO, MDA and fecal calprotectin. In this setting, the PAR1 agonist or PAR2 antagonist counteracted these changes, with the exception of MDA, which was unaffected. By contrast, the PAR1 antagonist or PAR2 agonist did not exert any effect on all parameters. Indomethacin decreased also occludin and increased activated caspase-3 expression in ileal tissues. The PAR1 agonist or PAR2 antagonist prevented the reduced occludin expression, while the PAR2 antagonist also decreased the levels of activated caspase-3.

Conclusions: PAR2 is involved in the pathogenesis of indomethacin enteropathy, through pro-inflammatory mechanisms and an impairment of the intestinal epithelial barrier. PAR1

activation and PAR2 inhibition could represent suitable strategies for the prevention of NSAID enteropathy.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed medications for treatment of inflammatory and pain-related conditions [1-3]. However, these drugs can elicit detrimental effects at level of the digestive tract [4,5], including the occurrence of small bowel mucosal injury [6-9]. The pathophysiology of this so called NSAID enteropathy remains partly undetermined, even though a variety of factors, involved in the maintenance of intestinal mucosal integrity, have been called into play [10-12]. In particular, the uncoupling of mitochondrial oxidative phosphorylation in enterocytes, with subsequent activation of apoptosis, as well as alterations of tight junction protein expression, are among the earliest events elicited by NSAIDs [10-12]. These alterations can favor the entry of luminal antigens through the intestinal epithelium and the subsequent activation of immune/inflammatory response, leading to tissue inflammatory damage [10-12].

Among the different factors involved in the control of the intestinal epithelial barrier integrity, the proteinase-activated receptors (PARs) have been called into play as significant regulators of gut homeostasis [13-15]. PARs are G protein-coupled receptors, which include four subtypes (PAR1, PAR2, PAR3 and PAR4) expressed at the membrane surface of a large variety of cell types. In humans, PAR1, PAR2, and PAR3 are localized within the same locus on chromosome 5q13 [16-17], while PAR4 is located on a separate chromosome (19p12) [18]. Given their chromosomic location and similarities in structure, it is believed that PAR1, PAR2 and PAR3 may be derived from a common ancestral gene [16].

PARs are activated through the proteolytic cleavage of their extracellular N-terminal domain, thereby generating a new N-terminal ending that serves as a tethered ligand able to bind the second extracellular loop of the cleaved receptor. This molecular rearrangement results in a signal transduction that usually leads to the initiation of a host inflammatory response [19-20]. PAR subtypes are expressed in various regions of the intestinal tract, including the small and

large bowel. In particular, PAR1 and PAR2 are located in enterocytes and cells of the lamina propria of the small intestine. Moreover, PAR1, PAR2 and PAR4 are expressed in endothelial cells, submucosal and myenteric neurons, and immune cells, while PAR1 and PAR2 have been detected in fibroblasts, smooth muscle cells and mast cells. PAR3 has been detected in both stomach and small intestine, but its cellular localization remains still undetermined [21,22].

PARs are indeed involved in a number of inflammatory or infectious conditions of the digestive tract [23,24]. In particular, the blockade of PAR1 delayed the healing of chronic gastric ulcers induced by acetic acid in rats [25], while PAR1 agonists counteracted the development of experimental colitis in mice [14], thus suggesting a protective role mediated by PAR1 at level of the digestive tract. By contrast, data regarding the pathophysiological role of PAR2 in the gastrointestinal (GI) tract agree in supporting a pro-inflammatory action [26], and suggest an involvement of these receptors in the pathogenesis of intestinal mucosal damage, such as ischemia/reperfusion injury [27]. In addition, the activation of PAR2 increases and alters the permeability of enteric mucosa, which is of paramount importance in the maintenance of equilibrium between the external milieu (digestive contents) and the submucosal immune system [13, 22]. Data regarding the role of PAR3 and PAR4 in the development of pathological conditions in the digestive tract are quite limited. For instance, PAR4 mRNA appears to be reduced in the colonic mucosa of patients with IBS, suggesting a protective role of these receptors in the pathophysiology of such disorder [28]. By contrast, no data regarding PAR3 are currently available [21].

Of note, alterations of mucosal permeability are known to play a major role in the pathophysiology of NSAID enteropathy [29], which is regarded as a major cause of digestive bleeding [9,30,31]. However, the involvement of PARs in the pathogenesis of NSAID enteropathy is currently unknown. Accordingly, the present study has been designed to examine the role played by PAR1 and PAR2 in the detrimental intestinal effects exerted by the NSAID

indomethacin in an experimental model of enteropathy and to investigate the underlying mechanisms, by means of selective PAR1 and PAR2 ligands.

Materials and Methods

Animals

Male Wistar aged rats (40 week-old, 500-600 g body weight) (Envigo, Udine, Italy) were employed throughout the study. They were fed standard laboratory chow (Envigo, Udine, Italy) and tap water ad libitum, and were not employed for at least one week after their delivery to the laboratory. The experimental protocol was designed in order to minimize pain or discomfort to the animals. Animals were housed in solid-bottomed cages, equipped with wire-mesh bottomed inserts to prevent coprophagy, and located in temperature-controlled rooms (at 22-24°C and 50-60% humidity) under a 12-h light cycle (06:00-18:00 hours) for two weeks prior to experimental procedures. Their care and handling were in accordance with the provisions of the European Community Council Directive 210/63/EU, recognized and adopted by the Italian Government. The experiments were approved by the Ethical Committee for Animal Experimentation of the University of Pisa (Authorization n. 11483, 24/03/2015) and by the Italian Ministry of Health (Authorization n. 468/2015-PR).

Aged rats were employed since ageing is a risk factor for NSAID-induced enteropathy [32,33]. Indeed, elderly patients are known to display altered basal conditions as a consequence of aging gut and/or co-morbidities. Moreover, our model was set up to mirror the setting of clinical practice, in which very often the greatest users of NSAIDs are people in old age [12].

Experimental design

Enteropathy was induced by indomethacin, as previously described by Fornai et al. [34]. The dose of indomethacin and duration of treatment were selected in order to obtain small bowel injuries mirroring those induced by NSAIDs in humans. To pursue this goal, non-fasted rats were treated for 14 days with indomethacin, 1.5 mg/kg BID by intragastric route, suspended in

1% methylcellulose and administered in a volume of 0.3 ml/rat. Subgroups of animals were subjected to administration of selective ligands for PAR1 and PAR2, in order to examine the putative role of these receptors in the pathophysiological mechanisms underlying NSAID enteropathy. Drugs were dissolved in DMSO and administered by intraperitoneal route. Details regarding PAR ligands are reported in Table 1.

Twenty four hours after the last dose of test drugs, rats were anesthetized with sodium thiopental (50 mg/kg). Blood samples were collected by cardiac puncture from each animal for hemoglobin measurement. Animals were then sacrificed by CO_2 exposure. The whole GI tract was excised and samples of ileum were snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis of myeloperoxidase (MPO) and malondialdehyde (MDA), as well as protein expression of activated caspase-3 and occludin. Other portions of tissues were fixed in 10% formalin for subsequent evaluation of microscopic damage.

Experimental groups were arranged as follows: Group 1: animals treated with vehicle (control, n=10); Group 2: animals treated with indomethacin 1.5 mg/kg BID (IND, n=10); Group 3: animals treated with indomethacin plus TFFLR-NH2 (PAR1 agonist, 2 mg/kg *ip*) for 14 days (n=10); Group 4: animals treated with indomethacin plus AC55541 (PAR2 agonist, 10 mg/kg *ip*) for 14 days (n=10); Group 5: animals treated with indomethacin plus SCH79797 (PAR1 antagonist, 0.025 mg/kg *ip*) for 14 days (n=10); Group 6: animals treated with indomethacin plus ENMD-1068 (PAR2 antagonist, 4 mg/kg *ip*) for 14 days (n=10).

Microscopic assessment of intestinal damage

Histological evaluation of small bowel injury was carried out as previously described [34]. Upon removal, the small intestine was immediately injected with 10% formalin and left in the same fixative solution. After 30 min, it was opened along the anti-mesenteric border, cleaned

of its fecal contents and fixed again in 10% formalin for 24 h. In order to rule out any bias, intestinal tissue samples were taken in accordance to the following procedure: the full length of small intestine was measured; 1 cm of tissue proximal to the ileo-cecal valve was discarded, and two specimens of 1.5-2 cm were taken at this edge and 5 cm from the first ileum sample. Sections of ileum were embedded into paraffin blocks and cut into 3 consecutive serial 7-8 μm sections. The slices were cut at two different points of the block: two on the surface and three at a deeper level. Each slice was placed on a glass slide for staining with haematoxylin and eosin. Histological damage was assessed by two observers, blind to treatments, according to the score system adopted in our laboratory [12]. The intestinal damage was scored as reported in Table 2. Higher magnification (60X) pictures of haematoxylin & eosin-stained sections were employed to evaluate the presence and distribution of inflammatory cells infiltrating the ileal tissues.

Evaluation of tissue myeloperoxidase levels

MPO, taken as a quantitative index to estimate the degree of intestinal wall infiltration by inflammatory polymorphonuclear cells, was assessed as described by Fornai et al. [35]. Specimens of small intestinal tissues (30 mg) were homogenized on ice with a polytron homogenizer (QIAGEN, Milan, Italy) in 0.6 mL of ice-cold lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerine, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 28 μg/ml aprotinin (pH 7.4). The homogenate was centrifuged 2 times at 4°C for 15 min at 1,500 g. The supernatant was diluted 1:5 and used for determination of MPO concentration by means of an enzyme-linked immunosorbent assay (ELISA) (Hycult Biotech, Uden, Netherlands). The results were expressed as nanograms of MPO per milligram of intestinal tissue.

Evaluation of tissue malondialdehyde levels

MDA concentration in intestinal tissues was determined to obtain quantitative estimates of membrane lipid peroxidation [36]. For this purpose, intestinal tissue was excised, weighed, minced by forceps, homogenized in 2 ml of cold buffer (Tris-HCl 20 mM, pH 7.4) using a polytron homogenizer (QIAGEN, Milan, Italy), and centrifuged at 1,500 g for 10 min at 4°C. Aliquots of supernatants were then used for subsequent assay procedures. Mucosal MDA concentrations were estimated using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.). The results were expressed as nmoles of MDA per milligram of intestinal tissue.

Assessment of blood haemoglobin concentration

Haemoglobin assessment was performed on blood samples, collected as reported above, by means of Quantichrom Hemoglobin assay kit (Bioassay Systems, Hayward, CA, USA) and expressed as g/dL [37].

Assay of fecal calprotectin

Fecal calprotectin was evaluated as previously described [38]. Fecal pellets were freeze dried for 24 hours and reconstituted in 1 mL PBS, along with 50 μL 1% (wt/vol) ascorbic acid (Sigma, St Louis, MO, USA). Samples were then homogenized for 10 min (4 °C). Homogenates were diluted with 2 mL lysis buffer (0.1% sodium dodecylsulfate, 0.5% sodium deoxycholate, 0.02% sodium azide, 5 mM disodium ethylenediaminetetraacetic acid, and 1× Halt protease/phosphatase inhibitor cocktail [Thermo Fisher Scientific Inc, Waltham, MA, USA] in PBS). Homogenates were further homogenized for 30 s and centrifuged (5800×g, 10 min, 4°C), and supernatants were snap frozen in liquid nitrogen and stored at −80°C. Fecal calprotectin levels were determined using rat calprotectin enzyme-linked immunosorbent assay kit.

Homogenates were diluted 2-fold with PBS and analysed following the manufacturer's instructions.

Western blot analysis

Samples of ileum, excised as reported above, were weighed and homogenized in lysis buffer containing HEPES 10 mmol/L, NaCl 30 mmol/L, EDTA 0.2 mmol/L, phenylmethylsulfonyl fluoride 2 mmol/L, leupeptin 10 µg/ml, aprotinin 10 µg/ml, sodium fluoride 1 mmol/L, sodium orthovanadate 1 mmol/L, glycerol 2%, $MgCl_2$ 0.3 mmol/L and Triton-X 100 1%, using a polytron homogenizer (Qiagen, Milan, Italy). Homogenates were spun by centrifugation at 20,000 r/min for 15 min at 4°C, and the resulting supernatants were then separated from pellets and stored at −80°C. Protein concentration was determined in each sample by the Bradford method (Protein Assay Kit, Bio-Rad, Hercules, CA, USA).

Western blot analysis of activated caspase-3 (index of cell apoptosis) and occludin (important protein for tight junction assembly and maintenance of enteric mucosal barrier) was performed as previously described by Colucci et al. [38] and Fornai et al. [39]. Equivalent amounts of protein lysates (15 µg) were separated by electrophoresis on sodium dodecylsulfate polyacrylamide gel and transferred onto a PVDF membrane. The blots were blocked for 1 h with 5% non-fat dried milk in 0.1% Tween-20 in Tris-buffered saline (TBS-T) and then incubated overnight at room temperature with antibodies specific for activated caspase-3 (dilution 1:1000, Cell Signalling, USA) and occludin (dilution 1:500, Invitrogen, USA). After repeated washings with TBS-T, a peroxidase-conjugated secondary antibody (dilution 1:10000, Santa Cruz Biotechnology, USA) was added for 1 h at room temperature.

Immunoreactive bands were visualized by incubation with chemiluminescent reagents (Immobilon reagent, Millipore USA) and exposed to Kodak Image Station 440 for signal

detection and densitometric image analysis. To ensure equal loading and accuracy of changes in protein abundance, the protein levels were normalized to β-actin.

Statistical analysis

The results are presented as mean ± SEM or median ± IQR. Shapiro-Wilk normality test was performed to assess the data distribution. The statistical significance of differences was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test or Kruskal-Wallis followed by Dunn's multiple comparisons test, and *p* values lower than 0.05 were considered significant. All statistical procedures were performed using Graph-Pad Prism 7.0 software (GraphPad Software, Inc., San Diego, CA).

Results

Effects of PAR1 and PAR2 ligands on microscopic intestinal damage induced by indomethacin

In preliminary experiments, the administration of PAR ligands to control animals did not produce any significant microscopic alteration of small intestinal mucosa (not shown). Therefore, such groups of treatment were excluded from the experimental design.

In ileal specimens from control animals, the microscopic examination did not reveal any type of lesion (Fig. 1A). According to one-way ANOVA followed by Dunnett's test, treatment with indomethacin was associated with the appearance of type 1 (32.52±1.66%; $F_{5, 47}$=6.715; p = 0.0001) (Fig. 1B and E), 2 (18.56±0.84%; $F_{5, 43}$=13,41; p = 0.0001) (Fig. 1C and F) and 3 (4.42±0.36%; $F_{5, 40}$=3.82; p = 0.0009) (Fig. D and G) lesions, as compared with controls. In animals treated with indomethacin, the occurrence of small intestinal lesions was partly prevented by the PAR1 agonist (13.03±4.56%, p = 0.005 for type 1 lesions; 4.46±2.19%, p = 0.0001 for type 2 lesions; 1.34±0.74%, p = 0.0116 for type 3 lesions) or the PAR2 antagonist (16.32±3.51%, p = 0.0167 for type 1 lesions; 5.18±2.22%, p = 0.0001 for type 2 lesions; 1.76±0.79%, p = 0.0351 for type 3 lesions), while the PAR1 blockade or PAR2 activation did not exert any significant influence (Fig. 1E-G).

In higher magnification sections of ileal tissues from rats treated with indomethacin, the submucosa appeared enlarged with transmural inflammation, as compared with control tissues. The presence of several infiltrating inflammatory cells was detected, including neutrophils, eosinophils, macrophages and lymphocytes (Supplementary Figure 1).

Effects of PAR1 and PAR2 ligands on blood hemoglobin levels

Treatment of animals with indomethacin resulted in a significant decrease in blood hemoglobin levels compared to the control group (9.77 ± 0.35 g/dL; $F_{5, 14}$=8.778; p = 0.0006). Under these conditions, the administration of PAR1 agonist or PAR2 antagonist resulted in a significant recovery of blood hemoglobin levels (15.45 ± 0.86 g/dL, p = 0.0009, and 13.75 ± 0.89 g/dL, respectively, p = 0.0144). By contrast, the PAR1 antagonist or the PAR2 agonist did not modify the decreasing effect exerted by indomethacin (Fig.2). These values were statistically significant according to one-way ANOVA followed by Dunnett's test, compared to control group (15.27 ± 0.92 g/dL).

Effects of PAR1 and PAR2 ligands on myeloperoxidase levels in the ileum

MPO levels in the ileum from rats treated with indomethacin were significantly increased, as compared with controls (21.64±1.47 ng/mg tissue vs 9.47±1.56 ng/mg tissue, respectively) ($F_{5,21}$=15.26; p = 0.0003 by one-way ANOVA followed by Dunnett's test). The concomitant administration of indomethacin with the PAR1 agonist (6.13±1.92 ng/mg tissue, p = 0.0001) or PAR2 antagonist (9.96±1.74 ng/mg tissue, p = 0.0012), according to one-way ANOVA, resulted in a significant decrease in MPO levels (Fig. 3A). On the other hand, the PAR1 antagonist or the PAR2 agonist did not elicit any significant effect on the increment of MPO levels induced by indomethacin (Fig. 3A).

Effects of PAR1 and PAR2 ligands on malondialdehyde levels in the ileum

In the ileum form rats treated with indomethacin, one-way ANOVA analyses showed that MDA levels were significantly increased, as compared with controls (62.56±7.68 nmol/mg tissue vs 13.77±3.53 nmol/mg tissue, respectively, $F_{5,26}$=11.06; p = 0.0001). In rats treated with

indomethacin, the concomitant administration of PAR ligands did not produce any significant modification of MDA concentration in the ileum (Fig. 3B).

Effects of PAR1 and PAR2 ligands on fecal calprotectin levels

In fecal samples collected from rats treated with indomethacin, the levels of calprotectin were significantly increased, as compared with controls (2.59±0.37 ng/mg feces vs 0.83±1.09 ng/mg feces, respectively, $p = 0.0043$). Under these conditions, PAR1 activation (1.26±0.37 ng/mg feces, $p = 0.0045$) or PAR2 blockade (1.04±0.83 ng/mg feces, $p = 0.0036$) counteracted the indomethacin-induced increase in fecal calprotectin, while the PAR1 antagonist or PAR2 agonist were without effects (Fig. 4). These values were analyzed by Kruskal-Wallis followed by Dunn's test ($H=25.82$; $N_{1\text{-}6}=10$).

Effects of PAR1 agonist and PAR2 antagonist on the expression of activated caspase-3 and occludin

In animals with enteropathy induced by indomethacin, the expression of activated caspase-3 was increased compared to controls (1.26±0.14 vs 0.26±0.07, respectively) ($F_{3,10}=12.4$; $p = 0.011$ by one-way ANOVA followed by Dunnett's test). This expression pattern was reduced by the PAR2 antagonist (0.77±0.08, $p = 0.0442$), while the PAR1 agonist did not exert any significant effect (Fig. 5A). Occludin expression in the ileum from rats treated with indomethacin was significantly reduced, as compared with controls (0.34±0.01 vs 0.84±0.09, respectively) ($F_{3,13}=6.451$; $p = 0.038$ by one-way ANOVA followed by Dunnett's test). Under these conditions, the administration of PAR1 agonist (0.83±0.11, $p = 0.0179$) or PAR2 antagonist (0.84±0.10, $p = 0.0141$) prevented completely such a decrease (Fig. 5B).

Discussion

The results obtained in the present study point out an opposing role played by PAR1 and PAR2 in the development of intestinal injury induced by treatment with indomethacin. In particular, the PAR2 seems to play a constitutive role in the pathogenesis of the detrimental effects of the NSAID, as the blockade of PAR2 improved the small intestinal damage induced by indomethacin. On the other hand, PAR1 seems to mediate protective effects, which, however, become evident only upon pharmacological receptor stimulation. These findings add further support to the significant role played by PAR1 and PAR2 in the pathophysiology of different digestive disorders, and provide an original demonstration of their contribution to the determinism of NSAID enteropathy.

The majority of literature data indicates that PAR2 mediate detrimental effects at the level of digestive tract. For instance, in line with our present observations, Yoshida et al. [27] reported that the blockade of PAR2 with specific antibodies counteracted the occurrence of mucosal damage in the small intestine in a murine model of ischemia/reperfusion. Likewise, Bonnart et al. [40] showed that the occurrence of small intestinal damage induced by *Toxoplasma gondii* infection was less severe in PAR2 deficient mice, as compared with wild type animals. In addition, Lohman et al. [41] observed that treatment with a PAR2 antagonist ameliorated the intestinal injury in a model of experimental colitis induced by trinitrobenzenesulfonic acid (TNBS) in mice.

Current knowledge regarding the role played by PAR1 in digestive diseases is controversial and does not allow to draw unambiguous conclusions. Indeed, when considering the effects of PAR1 pharmacological modulation in experimental models of intestinal injury, opposite evidence have been provided. In keeping with our present findings, the notion of a protective role played by PAR1 in the digestive tract has been previously highlighted. In particular, Cenac et al. [14] observed that treatment with a PAR1 agonist counteracted the development of

intestinal damage in a mouse model of oxazolone-induced colitis. On the other hand, Saeed et al. [42] found that PAR1 deficient mice were less sensitive to the development of experimental colitis induced by *Clostridium rodentium* in mice. Of note, the observation that PAR1 can mediate either pro- or anti-inflammatory actions could depend on the different pathological conditions. Indeed, as previously hypothesized [14,42] PAR1 seems to mediate detrimental actions in Th1/Th17 cytokine-mediated colitis, while exerting protective functions in the setting of Th2 cytokine colitis.

A present there is no evidence about a role of these receptors in the pathophysiology of NSAID-induced enteropathy. However, these receptors are known to exert an influence on some pathogenic mechanisms involved in the detrimental effects of NSAIDs. Moreover, in our hands, very preliminary data suggest that indomethacin elicited changes in the expression of both PAR1 (which is decreased) and PAR2 (which is increased) in the ileum (Fornai M et al., unpublished results). These data allow to hypothesize that indomethacin (and putatively other NSAIDs) could exert, at least in part, their injuring effects through the modulation of PAR1 and PAR2 expression. However, such hypothesis needs to be verified in future investigations. In addition, although PAR3 and PAR4 are expressed in the digestive system, very scarce data are currently available about their involvement in the pathophysiology of intestinal disorders [21,22], and there is also no evidence about a possible role of these receptors in the pathophysiology of NSAID enteropathy.

The mechanisms through which PAR1 and PAR2 take part, with different roles, to the occurrence of indomethacin-induced small intestinal damage are likely to include anti-inflammatory actions, as well as responses related to the mucosal barrier integrity, such as the modulation of cell apoptosis and tight junction protein expression.

Occult bleeding, documented by a decrease in blood hemoglobin levels, is known to occur in patients receiving chronic NSAID therapy [12] and animal models of NSAID-induced

enteropathy [35,37], as a possible consequence of intestinal inflammation and tissue damage. Of note, in experimental enteropathy, both intestinal bleeding and tissue inflammation were found to decrease upon treatment with enteroprotective agents, such as rifaximin and probiotics [35,37,38]. In the clinical setting, similar findings were obtained by Bjarnason et al. [43,44], who first showed that inflammation and blood loss were significantly increased in patients with NSAID-enteropathy, these two parameters being mutually related. Interestingly, both inflammation and blood loss were significantly reduced by treatment with metronidazole [44]. In our experimental model, the PAR1 agonist and PAR2 antagonist counteracted hemoglobin decrease along with tissue injury, thus suggesting that such pharmacological interventions are able to decrease occult bleeding associated with NSAID enteropathy.

As previously observed, the model of indomethacin enteropathy is characterized by an increase in tissue MPO levels [35], an index known to reflect the inflammatory infiltration by polymorphonuclear cells [45]. In this setting, the treatment with PAR1 agonist or PAR2 antagonist resulted in a significant decrease in MPO levels in the small intestine. Therefore, it is conceivable that the beneficial effects on bowel injury, arising from such pharmacological interventions, are likely to depend, at least in part, on the modulation of the inflammatory enteric response triggered by the NSAID. Similar findings have been reported by Yoshida et al. [27], who found that the PAR2 blockade with a specific antibody counteracted the increase in MPO levels in a model of ischemia/reperfusion-induced small bowel damage. Moreover, Lohman et al. [41] showed that the PAR2 antagonism prevented the increase in colonic MPO in a mouse model of TNBS-induced colitis. Of interest, the PAR1 activation has been shown also to counteract the increment of MPO in the context of intestinal inflammation. In particular, Cenac et al. [14] observed that the increase in MPO levels in colonic tissues from mice with oxazolone-induced colitis was prevented upon administration of a PAR1 agonist.

Calprotectin is a calcium binding protein, whose fecal levels display a good correlation with neutrophil infiltration in the intestinal mucosa, thus representing a reliable marker of intestinal inflammatory response [46-48]. In previous studies, we and others demonstrated that treatment with NSAIDs resulted in a significant increase in fecal calprotectin both in animal models [38] and humans [49,50]. In keeping with these findings, in the present study the administration of indomethacin elicited an increment of fecal calprotectin levels, and, in this setting, both the PAR1 agonist and the PAR2 antagonist prevented completely such an increment, thus supporting further the hypothesis that the beneficial effects exerted by both these agents are related to the activation of enteric anti-inflammatory mechanisms.

In our experiments, treatment with indomethacin was associated with a significant decrease in ileal occludin expression. This result is consistent with the general concept that NSAID enteropathy is characterized by an impairment of the intestinal mucosal barrier [12,51]. It is also noteworthy that a decrease in intestinal occludin expression has been previously observed in models of NSAID enteropathy [38,52]. Interestingly, in our study, the activation of PAR1 or the blockade of PAR2 prevented completely such an occludin decrease, thus suggesting that, in the setting of NSAID enteropathy, the PAR2-mediated detrimental effects could depend, at least in part, on an impairment of the intestinal epithelial barrier. This conclusion is in keeping with the concept that PAR2 activation can drive a pathological increment of intestinal mucosal permeability, and thereby an increase in the susceptibility to tissue injury [13]. On the other hand, our results support the view that the pharmacological stimulation of PAR1 could restore such a deficiency.

An abnormal rate of enteric mucosal cell apoptosis is considered as one of the most important pathophysiological hallmark of several digestive disorders, including NSAID enteropathy [12,53], which could contribute significantly to the impairment of gut mucosal barrier. In line with this concept, in the present study, we observed that treatment with indomethacin was

associated with a significant increase in caspase-3 activation, regarded as one of the main markers of mucosal cell apoptosis [54]. Interestingly, the PAR2 antagonist prevented the increase in caspase-3 activation, indicating the occurrence of a PAR2-dependent pro-apoptotic signaling in the pathogenesis of NSAID enteropathy. Such an observation is in line with previous reports, which attribute pro-apoptotic actions to this receptor. In particular, the activation of PAR2 by tryptase elicited an increment of activated caspase-3 in cultured rat colonic epithelial cells, while treatment with a selective PAR2 antagonist reverted such an increase [55]. On the other hand, in our model PAR1 activation did not exert any effect on caspase-3 activation, thus suggesting a lack of influence of PAR1 on the apoptotic process.

Overall, the results obtained in the present study point out for the first time an active role of PAR2 in the pathophysiology of NSAID enteropathy, and show that the pharmacological activation of PAR1 can promote beneficial effects in this specific experimental setting. The underlying mechanisms are likely to include anti-inflammatory effects, as well as the modulation of intestinal epithelial barrier integrity. These findings could help to gain better insights into the pathogenesis of intestinal injury induced by NSAIDs, as well as to investigate novel pharmacological interventions aimed at protecting the enteric mucosa in patients undergoing NSAID therapy.

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Conflict of interest statement

The Authors declare that they have no conflict of interest to disclose

Authors Contributions

Matteo Fornai: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review and editing. Rocchina Colucci: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review and editing. Carolina Pellegrini: Data curation, Formal analysis, Methodology, Software. Laura Benvenuti: Data curation, Formal analysis, Methodology, Software. Gianfranco Natale: Conceptualization, Data curation, Resources, Writing – review and editing. Larisa Ryskalin: Conceptualization, Resources, Visualization. Corrado Blandizzi: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review and editing. Luca Antonioli: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review and editing.

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Figure legends

Figure 1 Histological analysis of damage in the ileum of rats treated with indomethacin (INDO, 1.5 mg/kg BID), alone or in combination with TFFLR-NH2 (PAR1 agonist, 2 mg/kg *ip*), AC55541 (PAR2 agonist, 10 mg/kg *ip*), SCH79797 (PAR1 antagonist, 0.025 mg/kg *ip*) or ENMD-1068 (PAR2 antagonist, 4 mg/kg *ip*), for 14 days. Representative pictures showing the microscopic appearance of ileal mucosa from control animals (A), as well as type 1 (B), type 2 (C) or type 3 (D) lesions, observed in animals treated with INDO. Effects of treatments on type 1 (E), type 2 (F) or type 3 lesions (G). Each column represents the mean ± SEM obtained from 10 animals. Statistical analysis: One-Way ANOVA followed by Dunnett's multiple comparisons test.

Figure 2 Blood hemoglobin levels in rats treated with indomethacin (INDO, 1.5 mg/kg BID), alone or in combination with TFFLR-NH2 (PAR1 agonist, 2 mg/kg *ip*), AC55541 (PAR2 agonist, 10 mg/kg *ip*), SCH79797 (PAR1 antagonist, 0.025 mg/kg *ip*) or ENMD-1068 (PAR2 antagonist, 4 mg/kg *ip*), for 14 days. Each column represents the mean ± SEM obtained from 10 animals. Statistical analysis: One-Way ANOVA followed by Dunnett's multiple comparisons test.

Figure 3 Effects of indomethacin (INDO, 1.5 mg/kg BID), alone or in combination with TFFLR-NH2 (PAR1 agonist, 2 mg/kg *ip*), AC55541 (PAR2 agonist, 10 mg/kg *ip*), SCH79797 (PAR1 antagonist, 0.025 mg/kg *ip*) or ENMD-1068 (PAR2 antagonist, 4 mg/kg *ip*), for 14 days on tissue myeloperoxidase (MPO) (A) or malondialdehyde (MDA) (B) levels. Each column represents the mean ± SEM obtained from 10 animals. Statistical analysis: One-Way ANOVA followed by Dunnett's multiple comparisons test.

Figure 4 Fecal calprotectin levels in rats treated with indomethacin (INDO, 1.5 mg/kg BID), alone or in combination with TFFLR-NH2 (PAR1 agonist, 2 mg/kg *ip*), AC55541 (PAR2 agonist, 10 mg/kg *ip*), SCH79797 (PAR1 antagonist, 0.025 mg/kg *ip*) or ENMD-1068 (PAR2 antagonist, 4 mg/kg *ip*), for 14 days. Each column represents the median ± IQR obtained from 10 animals. Statistical analysis: Kruskal-Wallis followed by Dunn's multiple comparisons test.

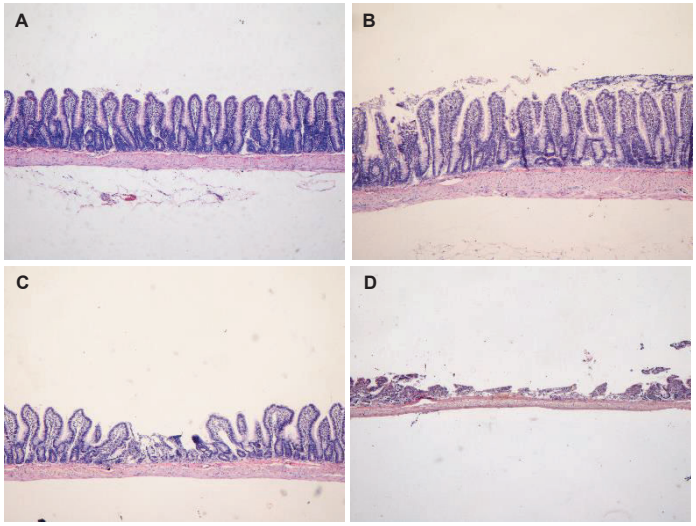
Figure 5 Western blot assay of activated caspase-3 (A) and occludin (B) expression in the ileum from animals treated with indomethacin (INDO, 1.5 mg/kg BID), alone or in combination with TFFLR-NH2 (PAR1 agonist, 2 mg/kg *ip*), AC55541 (PAR2 agonist, 10 mg/kg *ip*), SCH79797 (PAR1 antagonist, 0.025 mg/kg *ip*) or ENMD-1068 (PAR2 antagonist, 4 mg/kg *ip*), for 14 days. Each column represents the mean ± SEM obtained from 10 animals. Statistical analysis: One-Way ANOVA followed by Dunnett's multiple comparisons test.

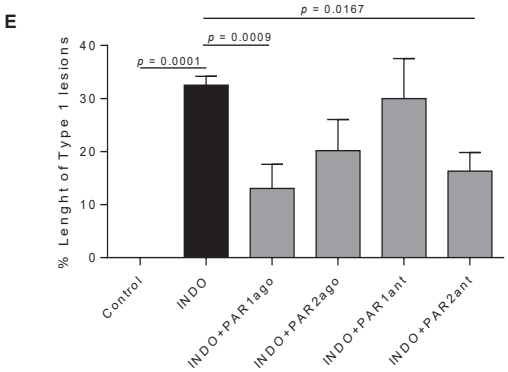
Table 1. Characteristics of PAR ligands employed in the study

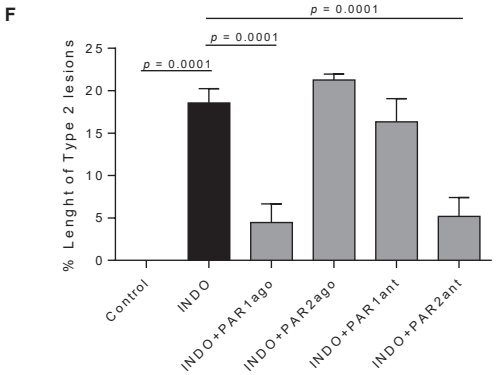
Drug	Pharmacological action	Dose *ip* (mg/kg)	Reference
TFLLR-NH2	PAR1 agonist	2	[56]
AC55541	PAR2 agonist	10	[57]
SCH79797	PAR1 antagonist	0.025	[58]
ENMD-1068	PAR2 antagonist	4	[59]

Table 2. Microscopic criteria for the evaluation of intestinal damage induced by indomethacin

Type 1 injury	• Damages confined to the tunica mucosa • De-epithelization • Significant morphologic alterations of villi
Type 2 injury	• Inflammatory infiltration in the submucosa, with thickening of the tunica muscularis or serosa • The morphologic framework of tunica mucosa is preserved
Type 3 injury	• Damage involves the full thickness of intestinal wall • The morphologic patterns of tunicae are lost • Inflammatory reaction widely extended to the tunica serosa with a significant increase in the thickness







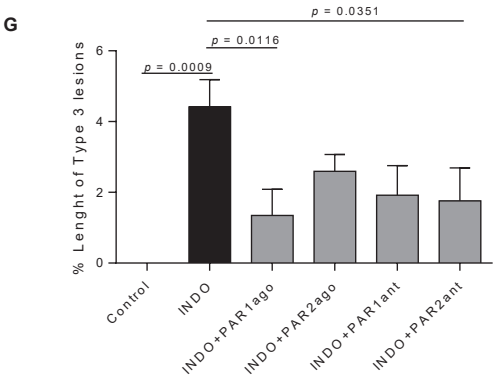


Figure 1

Figure 2

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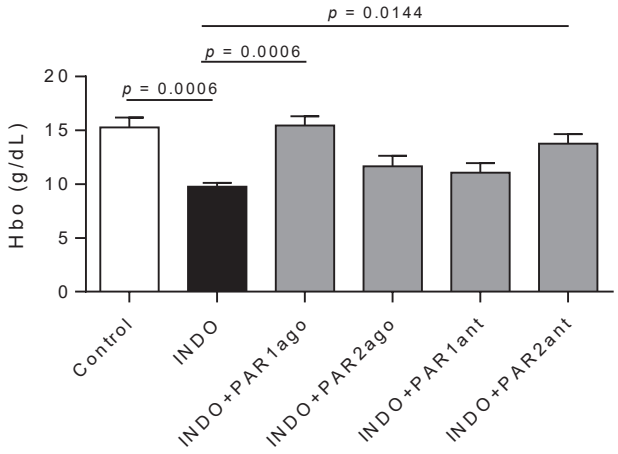

Figure 2

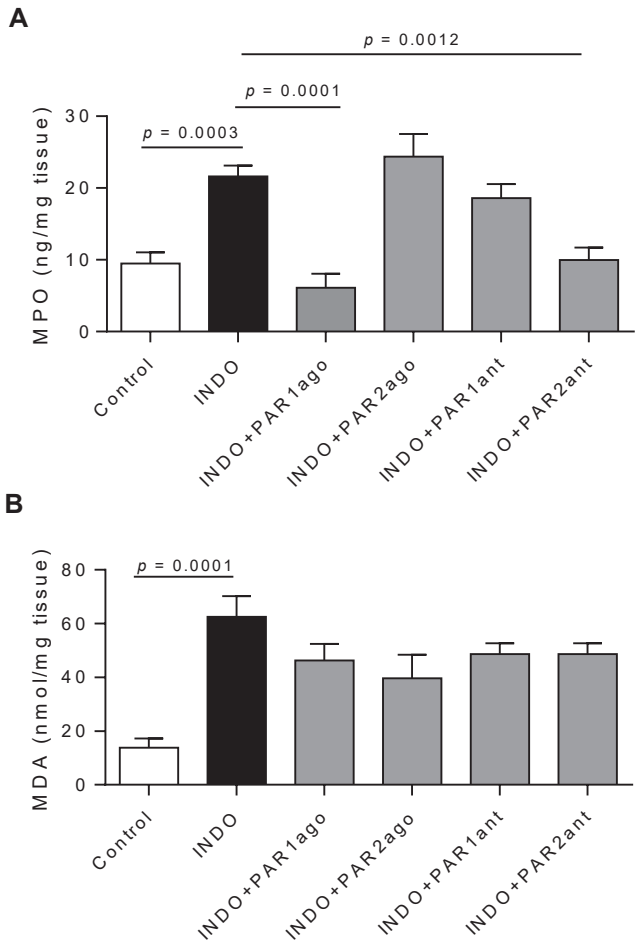

Figure 3

Figure 4

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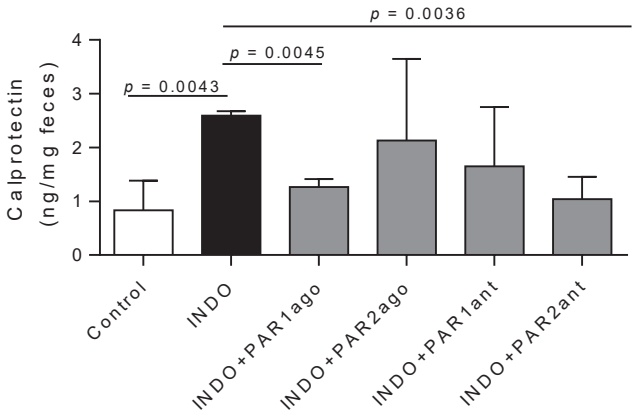

Figure 4

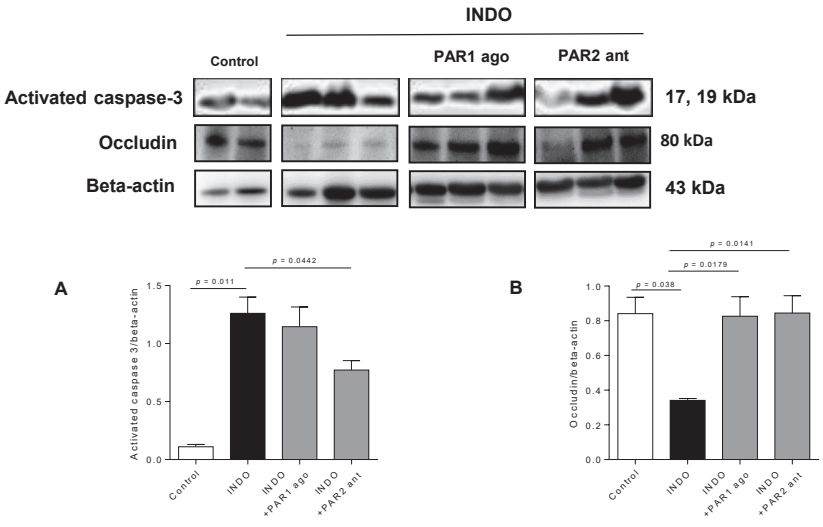

Figure 5