ROLE OF PROTEASES AND PROTEASE-ACTIVATED RECEPTOR 2 IN THE REGULATION OF PHYSIOLOGICAL COLONIC BARRIER FUNCTION AND IN THE PATHOGENESIS OF VISCERAL HYPERSENSITIVITY

Ph.D. Thesis

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2008

LIST OF FULL PAPERS THE THESIS BASED UPON

I. **Róka R**, Rosztóczy A, Leveque M, Izbéki F, Nagy F, Molnár T, Lonovics J, Garcia-Villar R, Fioramonti J, Bueno L, Wittmann T. A széklet szerinproteáz-aktivitása: új patofiziológiai faktor a diarrhoea-predomináns irritábilis bél szindrómában. *Magy Belorv Arch* 2006; **61**: 267-273.

II. **Róka R**, Demaude J, Cenac N, Ferrier L, Salvador-Cartier C, Garcia-Villar R, Fioramonti J, Bueno L. Colonic luminal proteases activate colonocyte proteinase-activated receptor-2 and regulate paracellular permeability in mice. *Neurogastroenterol Motil.* 2007; **19**: 57-65. ***IF: 3.338***

III. **Róka R**, Rosztóczy A, Leveque M, Izbéki F, Nagy F, Molnár T, Lonovics J, Garcia-Villar R, Fioramonti J, Wittmann T, Bueno L. Fecal serine-protease activity: a pathophysiological factor in diarrhea-predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol.* 2007; **5**: 550-5.

IV. **Róka R,** Ait-Belgnaoui A**,** Salvador-Cartier C**,** Garcia-Villar R**,** Fioramonti J**,** Eutamène H**,** Bueno L. Dexamethasone prevents visceral hyperalgesia but not colonic permeability increase induced by luminal protease-activated receptor-2 agonist in rats. *Gut* 2007; **56**: 1072-8. ***IF: 9,002***

LIST OF PUBLICATIONS RELATED TO THE THESIS

I. Cenac N, **Róka R**, Demaude J, Salvador-Cartier C, Garcia-Villar R, Fioramonti J, Bueno L. Colonic luminal proteases regulate colonocyte PAR2 expression and paracellular permeability in mice. *Gastroenterology* 2005; **128**: A498 ***IF: 12.457***

II. **Róka R**, Cenac N, L. Ferrier, Salvador-Cartier C, Garcia-Villar R, Fioramonti J, Bueno L. Colonic luminal proteases activate colonocyte PAR2 and regulate paracellular permeability in mice. *Neurogastroenterol Motil.* 2005; **17 (Suppl 2)**: 9A ***IF: 3.338***

III. **Róka R**, Demaude J, Cenac N, Salvador-Cartier C, Garcia-Villar R, Fioramonti J, Bueno L. Colonic luminal proteases regulate colonocyte PAR2 and paracellular permeability in mice. *Gut* 2005; **54 (Suppl VII)**: A252

IF: 9,002

IV. **Róka R**, Rosztóczy A, Leveque M, Nagy F, Lonovics J, Garcia-Villar R, Fioramonti J, Wittmann T, Bueno L. Fecal serine-protease activity: a pathophysiological marker and possible therapeutical target in diarrhoea-predominant irritable bowel syndrome.

Gastroenterology 2006; **130 (4, suppl 2**): A67

IF: 12.457

V. **Róka R**, Ait-Belgnaoui A, Salvador-Cartier C, Waget A, Fioramonti J, Bueno L. Dexamethasone prevents visceral hyperalgesia induced by proteinase-activated receptor-2 agonist through modulation of colonic mast cell activation in rats. *Gastroenterology* 2006; **130 (4, suppl 2)**: A144 ***IF: 12.457***

VI. **Róka R**, Ait-Belgnaoui A, Fioramonti J, Bueno L, Wittmann T. Proteinase-activated receptor-2 agonist induced visceral hyperalgesia is prevented by dexamethasone via regulation of colonic mast cells in rats. *Z. Gastroenterol* 2006; **43**: 440A ***IF: 1.293***

VII. Rosztóczy A, **Róka R**, Izbéki F, Molnár T, Nagy F, Lonovics J, Garcia-Villar R, Fioramonti J, Bueno L., Wittmann T. Increased fecal serine-protease activity in patients with diarrhea-predominant irritable bowel syndrome. *Z. Gastroenterol* 2006; **43**: 441A

IF: 1.293

VIII. **Róka R**, Ait-Belgnaoui A, Salvador-Cartier C, Waget A, Fioramonti J, Wittmann T, Bueno L. Dexamethasone treatment prevents proteinase-activated receptor-2 agonist induced visceral hyperalgesia, but does not influence proteinase-activated receptor-2 agonist induced increase in colonic permeability in rats. *Gut* 2006; **55 (Suppl II):** A202

IF: 9.002

Sum of impact factors (IF) for base references: ***12.340***

Sum of impact factors (IF) for references related to the thesis: ***61.299***

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1. INTRODUCTION

Proteases represent 2% of the human genom and are present in great amount in the gastrointestinal tract. Trypsin is released in its proactive form trypsinogen in the upper gastrointestinal tract lumen and in the pancreatic duct, for physiological digestive purposes. On mucosal surfaces, a balance between proteolytic activity and the presence of protease inhibitors such as pancreatic secretory trypsin inhibitor is constantly present. In the upper intestine the balance between proteolytic activities in the lumen and the presence of protease inhibitors at mucosal surfaces both efficient digestive processes and mucosal protection. Trypsinogen can also be synthesized by several different extrapancreatic cell types including the endothelium. Tryptase, which is expressed by almost all subsets of human mast cell, is released upon mast cell degranulation. Proteases of the coagulation cascade such as thrombin, factor VIIa and Xa are also potentially present in the gastrointestinal tract during inflammation or tissue trauma. Cathepsin G, which is released upon neutrophil activation, is also present in the gut associated with inflammatory conditions. Finally, mucosal surfaces are constantly exposed to bacterial products, particularly to bacterial proteases (81). Thus the importance of signalling pathways activated by proteases appears to be particularly relevant for the physiology and pathophysiology of the gastrointestinal tract.

Proteases, in addition to their digestive role in protein degradation, play a role as signalling molecules regulating cell functions by cleaving protease-activated receptors (PARs). PARs belong to a family of seven transmembrane domain G-protein-coupled receptors that are activated by cleavage of their N-terminal domain by a proteolytic enzyme (59). The unmasked new N-terminal sequence acts as a tethered ligand that binds and activates the receptor itself. Four PARs have been identified in human tissues: PAR_1, $_{-2}$, $_{-3}$ and $_{-4}$. PARs are expressed throughout the gastrointestinal tract on several cell types, as enterocytes, mast cells, smooth muscle cells, myenteric neurons, endothelial cells and on colonic epithelial cells (45). PARs are activated by a variety of proteases, such as digestive enzymes (trypsin and trypsinogen), proteases released from mast cells and neutrophils, and by proteases of the coagulations cascade. Proteases of human pathogen Porphyromonas gingivalis activate PARs on human oral epithelial cells, neutrophils and platelets (10, 48, 49, 50). Even though resident colonic bacteria release considerable amount of proteases, no studies have already evaluated the effects of commensal intestinal bacterial flora on PAR activation.

PAR_2 is strongly expressed in the gastrointestinal tract, particularly in the colon, and is located both on apical and basolateral sites of colonic epithelial cells. Activation of PAR_2 has been shown to provoke chloride secretion. In pig ileum and mouse colon, PAR_2 induced chloride secretion depends on eicosanoids release and submucosal neurone activation (21, 32). However, results in rat jejunum suggest that nerve activation is not involved but prostaglandin release is a major mediator of induced chloride secretion (82). Stimulation of chloride secretion is associated with fluid transport and results in diarrhoea which constitutes an important clinical symptom of inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). In animal models, intracolonic infusion of PAR_2 agonist activated spinal afferent neurons and produced a delayed rectal hyperalgesia (17), colonic PAR_2 activation caused delayed facilitation of capsaicin-evoked visceral nociception (41), and activation of PAR_2 in enteric nerves by mast cell tryptase caused neuronal hyperexcitability (67). In vivo, intracolonic activation of PAR_2 leads to colonic inflammation in mice and increases paracellular permeability with bacterial translocation into peritoneal organs. PAR_2 activation results in down-regulation of the receptor at the apical sites of colonocytes followed by an up-regulation prominently in the intracellular compartments in crypts (14). In mice, intracolonic infusion of low dose PAR_2 activating peptide (SLIGRL) increases colonic paracellular permeability by a direct MLCK-dependent mechanism (13). PAR_2 activation induced colitis is dependent on sensory neuron activation, substance P and CGRP release (15, 56). Over-expression of PAR_2 was observed in colonic biopsies from IBD patients, suggesting a pathophysiological role of PAR_2 in the development of colonic inflammation (42). In spite of growing evidences of the important role of PAR_2 in pathological conditions, the physiological role of PAR_2 located on colonocytes remains unclear. Understanding the functional role of PAR_2 in the colon represents an important challenge that could lead to define new pharmacological targets in gastrointestinal diseases in general and in particular for pathological conditions such as inflammatory and visceral pain disorders.

Irritable bowel syndrome is a chronic gastrointestinal disorder characterized by continuous or remittent abdominal pain, bloating and altered defecation. Population-based prevalence of IBS has been reported up to 28%, and IBS symptoms are major reasons for primary care visits and consultation with gastroenterologists (20, 23). In spite of intense research, the pathogenesis of IBS remains partially understood, and no specific and universally effective patient management has been developed. Altered colonic motor function, visceral hypersensitivity, changes in neural transmission within the gut, alterations of spinal and

supraspinal sensory afferent system, and low-grade inflammation of the intestinal mucosa may all play a role in the development of IBS (5). There is growing evidence that this low-grade inflammation is an important factor in the pathogenesis of IBS, particularly to initiate symptoms developed after gastrointestinal infection (26, 74).

Although PARs are potentially involved in the development of visceral hypersensitivity in IBS and proteolytic enzymes from both endogenous and exogenous origin are present in high concentrations in the gut, no study has yet previously evaluated the role of colonic luminal proteases in IBS pathogenesis. A recent preliminary work, however, demonstrated an increased trypsin-like proteolytic activity in colonic biopsies from IBS patients. This protease activity was able to cause hyperalgesia when injected into mouse paws thorough a mechanism involving PAR_2 activation (83). Moreover, therapeutic modification of PAR function may be beneficial for the relief of IBS symptoms. However, the lack of PAR_2 antagonists had not permitted until now to confirm a beneficial effect of blocking PAR_2 activation in the therapy of IBD or IBS. The putative role for gut microinflammation in IBS patients has been largely discussed and is supported by numerous studies (19). Although corticosteroids are potent inhibitors of inflammatory processes, and are widely used in the treatment of inflammatory bowel disease, only one study exists on corticosteroids in patients with post-infectious IBS which suggests that prednisolone is not likely to be an effective treatment for IBS symptoms (25).

2. AIMS

With this in mind, the aims of the studies summarized in the thesis were to investigate role of proteases and PAR_2 in the regulation of physiological colonic barrier function and in the pathogenesis of visceral hypersensitivity under experimental, as well as under clinical conditions.

2.1. The aims of the animal experiments:

1. To evaluate if intraluminal proteases and particularly serine proteases play a role in the physiological regulation of colonic paracellular permeability in mice.
2. To clarify the role of bacterial proteases in the regulation of colonic barrier function.

3. To assess the role of mast cell proteases in colonic PAR_2 activation.
4. To investigate if dexamethasone treatment prevents PAR_2 agonist-induced visceral hyperalgesia in rats.
5. To evaluate the role of PAR_2 and colonic mast cells in the effect of dexamethasone on visceral hypersensitivity.

2.2. The aim of the human investigations:

To examine whether colonic luminal proteases are elevated in IBS patients, as reflected by stool analysis.

3. MATERIALS AND METHODS

3.1. Animal experiments

3.1.1. Experiments in mice

3.1.1.1. Animals

Male Swiss 3T3 and C57BL6 mice were obtained from Janvier (Le Genest St-Isle, France). Mice were housed in polycarbonate cages in a light (12h/12 h cycle)- and temperature-controlled room (20-22 ºC) and were fed standard pellets (UAR pellets; Epinay, France). Water was provided ad libitum. The experimental protocols described in this study were approved by the local Institutional Animal Care and Use Committee (INRA, Toulouse).

3.1.1.2. Experimental protocol

For assessing the effect of protease inhibitors on colonic paracellular permeability, 5 groups of Swiss 3T3 mice (n=8-8) received an intracolonic infusion (250 µl/h) of a mixture of water soluble protease inhibitors with a broad specificity for the inhibition of serine-, cysteine-, aspartic-, and metalloproteases (100 µg/ml, Proteases Inhibitor Cocktail for General Use, Sigma, France), cysteine-protease inhibitor (100 µg/ml, Cystatin, Sigma, France), serine-protease inhibitor (100 µg/ml, Aprotinin, Sigma, France), matrix-metalloprotease inhibitor (100 µg/ml,

Galardin, Sigma, France) or saline for 5 hours. Paracellular permeability was assessed during the last 2 hours of protease inhibitor treatment.

Four groups of Swiss 3T3 mice and 6 groups of C57BL6 mice were used for studies on antibiotic treatment, respectively. Three groups of Swiss mice (n=8-8) and 3 groups of C57BL6 mice (n=8-8) were treated for 12 days with antibiotics (0.5 g/l ampicillin and 1 g/l neomycin in drinking water). In preliminary experiments, we verified that these antibiotics had no direct effect on colonic paracellular permeability in Ussing chambers (data not shown). One group of Swiss (n=8) and 3 groups of C57BL6 mice (n=8-8) were used as control. One group of antibiotic treated (n=8) and 1 control group of Swiss 3T3 mice (n=8) were sacrificed at day 12 for measurement of serine-protease activity in colonic luminal contents and immunochemistry of PAR_2 in colonic mucosa. In 2 groups of antibiotic treated Swiss 3T3 mice (n=8-8) immunochemistry of PAR_2 was performed after the animals received daily intracolonically (50 µl) trypsin (400 U/mouse) or saline for 2 days under the antibiotic treatment. Two groups of antibiotic treated (n=8-8) and 2 control groups of C57BL6 mice (n=8-8) were sacrificed at day 12 for in vitro measurement of colonic permeability in Ussing-chambers, in basal conditions and after administration of PAR_2 activating peptide (SLIGRL) or mast cell degranulator (compound 48/80). Further 1 group of antibiotic treated (n=8) and 1 control group of C57BL6 mice (n=8) were sacrificed at day 12 for measurement of mouse mast cell protease-1 (MMCP-1) content of colonic mucosa.

3.1.1.3. Procedure of intracolonic injections

Mice were fasted for 12 h before intracolonic injections. Under mild xylazine/ketamine (10 and 2 mg/mouse, respectively; subcutaneously) anaesthesia, a small polyethylene catheter (0.3/0.07 mm) was inserted intrarectally ending at 4 cm from the anus. Trypsin, saline and protease inhibitors were injected into the distal colon through the catheter.

3.1.1.4. Serine-protease activity in colonic content

Colonic content was obtained by rinsing the entire colon with 1 ml of saline. This solution was transferred to 4 ml of reaction buffer containing 0.15 M NaCl and 20 mM TrisHCl. Coarse particles were removed from this solution by filtration with 0.8 µm size syringe filter (Nalgene, Nalge, New York, USA) after centrifugation at 4500 r.p.m. for 10 min at 4 °C. Samples of 25 µl from the supernatant were incubated with 1 ml of reaction buffer and 1 ml of

0.5 % (w/v) azocasein (Sigma, St Quentin Fallavier, France) at 40 °C for 20 min. The reaction was stopped by addition of 1 ml 10 % (w/v) trichloroacetic acid (TCA, Sigma, St Quentin Fallavier, France). After centrifugation at 4500 r.p.m. for 10 min at 4 °C, absorption of the clear supernatant was measured at 366 nm and compared with standard curves obtained from a titration series of azocasein. The protein content of the filtered supernatant of colonic content was assessed with BCA-200 Protein Assay Kit (Pierce, Rockford, USA), with bovine serum albumin as a standard. Serine-protease activity was expressed as units of trypsin activity in mg of total proteins (U/mg).

3.1.1.5. Immunohistochemistry of PAR_2

Colonic samples were fixed for 12h in 4% formalin, dehydrated through graded ethanol and embedded in paraffin. Sections (5µm) were rehydrated and submerged in antigen retrieval solution (citrate buffer, 10mM, pH6, 95 °C, 3 min.). After inhibition of endogenous peroxydases with 0.6% H_2O_2 in 100% methanol for 30 min and incubation in blocking solution (phosphate buffered saline containing 1% bovine serum albumin and 2% goat normal serum), sections were incubated with rabbit PAR2 antibody (supplied by M.D. Hollenberg) (overnight, 4 °C) followed by a biotinylated goat anti-rabbit IgG immune serum (30 min, room temperature) and subsequently with ABC complexes coupled to peroxydase (Vectastain Elite ABC kit, AbCys, Paris, France). Antigen-antibody complexes were revealed using 3-3'- diaminobenzidine (DAB kit, ICN Pharmaceuticals, Costa Mesa, CA). Hemalum was used as a counterstain. As negative controls, sections were treated with the same procedure except for the absence of primary antibody.

3.1.1.6. Immunohistochemical analysis

Immunohistochemical analysis was performed in a blinded fashion using a Nikon 90i microscope. PAR_2 expression was quantified employing the software LUCIA G (version 4.8, Nikon) measuring the mean density per square micrometer of colonic epithelium. All analysis was done on ten fields per sample.

3.1.1.7. In vivo permeability studies

Mice were anesthetized with xylazine/ketamine (10 and 2 mg/mouse, respectively; subcutaneously). To measure colonic paracellular permeability, 0.7 µCi of ^{51}Cr-EDTA (Perkin

Elmer Life Sciences, Paris, France) in 0.5 ml NaCl 0.9% was slowly infused into the colon (2 h, 0.25 ml/h). After 2 hours, mice were sacrificed by cervical dislocation and colons were removed. Then, colons and remaining bodies were placed in separate counting tubes in gamma-counter (Packard Cobra II, Packard Bioscience, Meriden, Connecticut, USA). The permeability was expressed as the ratio between body and total (body plus colon) radioactivities.

3.1.1.8. In vitro permeability studies

C57BL6 mice were sacrificed by cervical dislocation. The distal colon was removed and two colonic strips from each mouse were mounted in Ussing-type chambers (Easymount, Physiologic Instruments, San Diego, USA) having a flux area 0.3 cm^2. Both sides of each colonic sheet were bathed in 5 ml of Krebs solution which was maintained at 37 ºC and oxygenated continuously with 5% CO_2 in O_2. Permeability was assessed by measuring mucosal to serosal fluxes of fluorescein isothiocyanate (FITC)-Dextran across the colonic strip. For assessment of PAR_2 responsiveness, the FITC-Dextran flux was determined 1 hour after administration of SLIGRL or its vehicle (saline). For measurement of the effect of mast cell degranulation, the FITC-Dextran flux was evaluated 1 hour after administration of compound 48/80 or its vehicle (water). In brief, after a 20 minutes equilibrium period 550 µl of buffer solution on the mucosal side was replaced by 500 µl of FITC-Dextran (10000 MW, 0.022g) and 50 µl of SLIGRL (25 µM), saline, compound 48/80 (10 µg/ml) or water. After 30 and 60 minutes 800 µl solution from the serosal side was removed and fluorescence was measured on fluorimeter (Luminescence Spectrometer LS 50 B, Perkin Elmer, USA). The FITC-Dextran flux was expressed as quantity of FITC-Dextran that crossed 1 cm^2 in 1 hour (nmol/h/cm^2).

3.1.1.9. ELISA for mouse mast cell protease-1

C57BL6 mice were sacrificed by cervical dislocation. The distal colon was removed and the colonic mucosa was carefully removed with a dissector. The mucosal samples were homogenised in RIPA buffer and centrifuged at 10000 g at 4 °C. Supernatants were used for the MMCP-1 and total protein content measurements. MMCP-1 Elisa assays were performed with Mouse Mast Cell Protease-1 ELISA Kit (Moredun, Midlothian, Great-Britain). Dynatech M129B 96-well plates were coated for 24 hours at 4 °C with 2 µg/ml of affinity-purified sheep anti-MMCP-1 Ig in carbonate buffer (pH 9.6). Wells were washed (6x) with PBS, 0.05 % (v/v) Tween 20. Standards (0.25-12 ng/ml of purified MMCP-1) and samples were applied (50

µl/well), diluted as appropriate in PBS containing 4% BSA and 0.05% (v/v) Tween 20. After incubation in room temperature for 1.5 h, plates were washed as above and incubated for 1 h at room temperature with 50 µl/well of rabbit anti-MMCP-1-horse radish peroxidase (HRP) conjugate (diluted as appropriate in PBS containing 4% bovine serum albumin (BSA) and 0.05 % (v/v) Tween 20). Plates were washed and incubated with for 25 min at room temperature with 50 µl/well ortho-phenylene diamine (OPH)/H_2O_2 substrate. The reaction was stopped with 25 µl/well 2.5 M H_2SO_4. Plates were read at 492 nm on Microplate Reader. The standard curve was generated and sample concentrations were calculated using software. Protein concentration was determined with a commercial kit (BCA-200 Protein Assay Kit). MMCP-1 content of colonic mucosa was expressed as ng of MMCP-1 per gram of total proteins.

3.1.2. Experiments in rats

3.1.2.1. Animals

Male Wistar rats weighing 200-250 g were obtained from Janvier (Le Genest St-Isle, France). Rats were housed in polycarbonate cages in a light (12h/12 h cycle) and temperature-controlled room (20-22 ºC) and were fed standard pellets. Water was provided ad libitum. The experimental protocols described in this study were approved by the local Institutional Animal Care and Use Committee (INRA, Toulouse).

3.1.2.2. Experimental protocol

3.1.2.2.1. Visceral sensitivity studies

The effect of dexamethasone on PAR_2-induced visceral hyperalgesia was assessed in 4 groups of rats (n=8-8) surgically prepared for electromyography and intracolonic injections. Rectal distension and abdominal contraction recording was performed 24 hours after intracolonic infusion of SLIGRL (200 µg) or distilled water (vehicle) at a rate of 0.25 ml/20 min in rats previously injected intraperitoneally (ip.) daily with saline (groups 1 and 2) or dexamethasone (1mg/kg) (groups 3 and 4) for 4 days. In 2 other groups of rats (n=8-8), a mast cell stabilizer, doxantrazole, was injected ip. (1mg/kg) 2 hours before and 6 hours after intracolonic infusion of SLIGRL; rectal sensitivity was tested 18 hours after the last injection of doxantrazole.

3.1.2.2.2. Permeability studies

In 2 other groups of rats (n=8-8) after 4 days of daily intraperitoneal treatment with dexamethasone or vehicle, the effect of SLIGRL and the mast cell degranulator (compound 48/80) on the paracellular permeability of colonic strips was measured in Ussing chambers.
Finally, in 2 other groups of rats (n=8-8) after 4 days of intraperitoneal treatment with dexamethasone or vehicle, colonic samples were collected for mucosal rat mast cell protease-II (RMCP-II) assay, immunohistochemistry for PAR_2 and mast cell count.

3.1.2.3. Surgery and electromyography

Rats were surgically prepared for electromyographic recording and intracolonic injections as previously described (17). Briefly, a polyethylene catheter (OD= 0.7 mm, ID= 0.3 mm) was implanted into the proximal colon, 1 cm from the caecocolonic junction, attached to the abdominal muscle wall. This catheter was used to perform intracolonic injections of PAR_2 activating peptide. Rats were also equipped with 3 groups of 3 electrodes of NiCr wire (60 cm length, 80 μm diameter), implanted bilaterally in the abdominal external oblique musculature superior to the inguinal ligament. The catheter and electrodes were exteriorized on the back of the neck and protected by a glass tube attached to the skin. Electromyographic recordings began 5 days after surgery. The electrical activity of abdominal striated muscles were recorded with an electromyograph (Mini VIII, Alvar, Paris, France) using a short time-constant (0.03 seconds) to remove low-frequency signals (<3 Hz) and a paper speed of 3.6 cm/min.

3.1.2.4. Rectal distension procedure

Rectal distension was used as a noxious stimulus to evaluate visceral hyperalgesia. Rats were placed in plastic tunnels (6 cm diameter, 25 cm length) and were trained to stay in tunnels and to undergo rectal distension procedure during the 3 days preceding the experiments. This training period minimalized stress reactions during experiments. The balloon of an arterial embolectomy catheter (2 mm diameter, 2 cm length, Fogarthy, Edwards Laboratories, Santa Ana, USA) was inserted into the rectum at 1 cm from the anus. The balloon was then inflated with water progressively, in 0.4 ml steps, from 0 to 1.2 ml, each step of inflation lasted 5 minutes. To detect possible leakage, the volume of water introduced into the balloon was checked by complete removal with a syringe at the end of the distension period (54).

3.1.2.5. In vitro permeability studies

This procedure is described in chapter 3.1.8. Only flux area in Ussing-type chambers is different from mice experiments, the flux area used in rat permeability studies is 0.5 cm^2.

3.1.2.6. Colonic mucosal RMCP-II assay

The colonic mucosa was carefully removed with a dissector. Mucosal samples were homogenised in RIPA buffer and centrifuged at 10000 g at 4 °C. Supernatants were used for the RMCP-II and total protein assays. RMCP-II Elisa assays were performed with Rat Mast Cell Protease-II ELISA Kit (Moredun, Midlothian, UK). Briefly, coated plates were incubated with monoclonal antibody against RMCP-II raised in mouse at 4 °C for 24 h before use. 30 min incubation with 4% (w/v) Bovine Serum Albumin (BSA) was done before loading standard and unknown samples. The sample incubation was 1.5 h at 37 °C. A sheep anti-RMCP-II and horseradish peroxidase conjugate was added afterwards and incubated for 1 h. Plates were developed using o-phenylenediamine as substrate and read at 492 nm after reaction was stopped. RMCP-II concentration was quantified against RMCP-II standard curve. Protein concentration was determined with a commercial kit (BCA-200 Protein Assay Kit). RMCP-II content of colonic mucosa was expressed as ng of RMCP-II per mg of total proteins.

3.1.2.7. Measurement of colonic PAR_2 expression by immunohistochemistry

Colonic samples were fixed 12h in 4% formalin, dehydrated through graded ethanol and embedded in paraffin. Sections (5µm) were rehydrated and submerged in antigen retrieval solution (citrate buffer, 10mM, pH6, 95°C, 3 min). After inhibition of endogenous peroxydases with 0,6% H_2O_2 in 100% methanol for 30 min and incubation in blocking solution (phosphate buffered saline containing 1% BSA and 2% goat normal serum), sections were incubated with rabbit PAR_2 antibody (supplied by M.D. Hollenberg, Calgary, Canada) (overnight, +4°C) followed by a biotinylated goat anti-rabbit IgG immune serum (30 min, room temperature) and subsequently with ABC complexes coupled to peroxidase (Vectastain Elite ABC kit, AbCys, Paris, France). Antigen-antibody complexes were revealed using 3-3' diaminobenzidine (DAB kit, ICN Pharmaceuticals, Costa Mesa, USA). Hemalum was used as a counterstain. As negative controls, sections were treated with the same procedure except for the absence of primary antibody.

Immunohistochemical analysis was performed in a blinded fashion using a Nikon 90i microscope. PAR_2 expression was quantified with the software LUCIA G (version 4.8, Nikon, France) measuring the mean density per square micrometer of colonic mucosa. All analyses were done on ten fields per sample.

3.1.2.8. Colonic mucosal mast cell count

Specimens were fixed in buffered formalin 10% and incubated 24 h in 30% sucrose at 4°C. Samples were embedded in Tissue Tek medium (Euromedex, Souffelweyersheim, France) and frizzed in isopentan at -45°C. Cryostat sections (7 µm) were post-fixed with acetone (10 min., -20°C) and hydrated in PBS-Tween. After incubation in blocking solution (PBS containing 1% bovine serum albumin and 2% donkey serum), sections were incubated (overnight, 4°C) with sheep anti-RMCP II antibodies (1/500) (Moredun, Midlothian, UK) followed by incubation for 1h 30 min at room temperature with Alexa fluor 594-conjugated IgG donkey anti-sheep (1/2000). After each incubations, sections were rinsed in PBS-Tween. Sections were mounted in fluorostab medium (MP Biomedicals, Vannes, France) and examined under a Nikon 90i fluorescence microscope. The number of mast cells was determined with the software Lucia G (version 4.8, Nikon). Results were expressed per square millimetre of colon mucosa. All analysis was done on three fields of six control rats and six treated rats.

3.2. Human investigations

3.2.1. Patients

Thirty eight patients with IBS fulfilling the Rome II criteria participated in the study (aged 24-75 year; 31 females, 7 males). Seventeen were diarrhea-predominant IBS patients (IBS-D, age: 20-75; 14 females, 3 males; bowel movements/week: 23.8±3.7; abdominal pain /VAS/: 56.6±6.7 %), 14 were constipation-predominant IBS patients (IBS-C, age: 29-37; 13 females, 1 male; bowel movements/week: 1.4±0.1; abdominal pain /VAS/: 47.7±7.9), and 7 patients had alternating bowel habits (IBS-A, age: 45-69; 5 females, 2 males; bowel movements/week: 6.7±0.28; abdominal pain /VAS/: 50.0±10.8 %). Organic gastrointestinal disorders were excluded by detailed blood and stool analyses, serological assays for celiac disease, lactose- and

lactulose-hydrogen breath test, abdominal ultrasound and colonoscopy. Fifteen patients with clinically and endoscopically active ulcerative colitis (UC) were also enrolled (aged 18-79 years; 10 females, 5 males; bowel movements/week: 23.4±4.5), and 15 gastrointestinal symptom-free subjects were included as controls (aged 30-65 years; 12 females, 3 males; bowel movements/week: 6.8±0.1). Additionally, 5 subjects with acute infectious diarrhoea (ID, aged 69-85 years; 4 females, 1 male) were investigated. No patients or healthy subjects were taking drugs likely to alter gastrointestinal functions.

The study was approved by the Ethical Committee of University of Szeged. All participants gave informed consent.

3.2.2. Procedures

3.2.2.1. Sample preparation

Stools from IBS, UC and ID patients, and from healthy controls were studied. Samples were collected and transported within 1 h after defecation to the First Department of Medicine in Szeged, Hungary, frozen upon arrival, and stored at -20°C. After recollection of the entire set for the study, frozen samples were shipped in dry ice to INRA, in Toulouse, France, for assays.

Upon arrival, stool samples were thawed at 4 °C. One spot (1-2 g) from each stool sample was collected, transferred to 4 ml of reaction buffer containing 0.15 M NaCl and 20 mM Tris-HCl and homogenized. Coarse particles were removed from this solution by filtration with 0.8 µm size syringe filter (Nalgene, Nalge, New York, NY) after centrifugation at 4500 rpm. for 10 min at 4°C. Supernatants were used for serine-protease activity and mast cell tryptase assays. For the assay of elastase, fecal proteases were extracted using sample preparation tubes (Quick-Prep, Schebo-Tech, Giessen, Germany).

3.2.2.2. Serine protease activity assay

Aliquots (25 µl) of supernatants of fecal homogenates were incubated with 1 ml of reaction buffer and 1 ml of 0.5 % (w/v) azocasein (Sigma) at 40 °C for 20 min. The reaction was stopped by adding 1 ml 10% (v/v) trichloroacetic acid (TCA, Sigma). After centrifugation at 4500 rpm for 10 min at 4°C, absorption of the clear supernatant was measured at 366 nm, and compared with standard curves obtained from a titration series of azocasein. Protein concentration of the filtered supernatant of colonic content was assessed with BCA-200 Protein

Assay Kit, with bovine serum albumin as a standard. Serine-protease activity was expressed as units of trypsin activity per mg of protein. Two different serine-protease inhibitors (aprotinin and soybean tryptase inhibitor) were used to confirm assay specificity.

3.2.2.3. Mast cell tryptase activity measurement

Aliquots (10 µl) of supernatant of fecal homogenates were added to 200 µl of buffer (50 mmol/l Tris-HCl, pH 7.6, 120 mmol/l NaCl, 20 µg/l heparin) containing 0.5 mmol/l substrate of mast cell tryptase (tosyl-glycine-proline-arginine-pNitroanilide) and incubated at room temperature for 17 h. Substrate cleavage was measured using a microplate reader (absorbance 415 nm) and normalized to protein content of supernatant. Concentrations were automatically calculated in comparison with a standard curve. A specific mast cell tryptase inhibitor (nafamostat mesylate) was used to confirm assay specificity.

3.2.2.4. Pancreatic elastase-1 detection

Pancreatic elastase-1 was assayed immunologically by ELISA (Schebo-Tech, Giessen, Germany) using a monoclonal antibody against specific epitopes of human pancreatic elastase. The antigen-antibody complex was revealed by adding a conjugate peroxidase (POD)/streptavidin that binds the biotinylated antibody. The concentration of oxidized ABTS (2,2'-azoni-bis /3-ethylbenzothiazolin-6-sulphonic acid/), a POD substrate, was determined by spectrophotometry in a microplate reader at 405 nm as reference wavelength. Concentrations were automatically calculated in comparison with a standard curve.

3.2.2.5. Secretory leukocyte protease inhibitor (SLPI) measurement

Human SLPI was measured immunologically by ELISA (R&D Systems, Minneapolis, MN) using a monoclonal antibody against specific epitopes of human SLPI. The antigen-antibody complex was revealed by adding a conjugated horseradish peroxidase that binds the antibody. The concentration of oxidized chromogen (tetramethylbenzidine), a peroxidase substrate, was determined by spectrophotometry in a microplate reader at 450 nm as reference wavelength. Concentrations were automatically calculated in comparison with a standard curve.

3.3. Statistical analysis

Data are presented as means ± SEM. Analyses were done by running GraphPad Prism 4.0 (GraphPad, San Diego, CA). In animal experiments between-group comparisons were performed by Student's unpaired *t* test, multiple comparisons within groups were performed by repeated measures one-way ANOVA, followed by Student's unpaired *t* test. In human investigations multiple comparisons within groups were performed by repeated measures one-way ANOVA, followed by Kruskal-Wallis test. Correlations were analysed using the Spearman rank correlation test. Statistical significance was accepted at $p<0.05$.

4. RESULTS

4.1. Animal experiment

4.1.1. Experiments in mice

4.1.1.1. Effect of protease inhibitors and antibiotic treatment on gut paracellular permeability (in vivo)

In anesthetized mice (n=8) basal gut permeability measured for 2 hours after intracolonic saline administration was 2.2±0.1% of total ^{51}Cr-EDTA recovered. Intracolonic administration of mixture of anti-proteases reduces this basal value to 1.1±0.2% (n=8). Aprotinin, a serine protease inhibitor, reduced the basal colonic permeability to 1.1±0.1% (n=8). Galardin, a matrix metalloprotease inhibitor, significantly decreased colonic paracellular permeability (n=8) compared with saline perfusion (0.9±0.2 vs. 2.2±0.1%; $p<0.01$). In contrast, only cystatin, a cysteine protease inhibitor, had no effect on colonic paracellular permeability (2.1±0.2%) (n=8) (Figure 1).

In mice treated with antibiotics (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) during 12 days, the colonic paracellular permeability was not significantly changed as compared with control animals (1.1±0.1 vs. 1.0±0.1%) (n=8-8) (Figure 2).

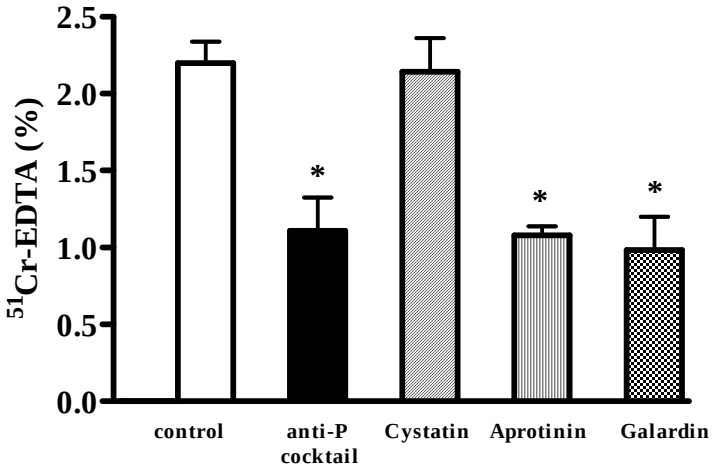

Figure 1. Effect of intracolonic infusion of anti-protease cocktail, selective cysteine- (cystatin), serine- (aprotinin) and matrix metalloprotease inhibitors (galardin) on colonic paracellular permeability. Inhibitors were infused at a dose 250 µg/ h/mouse (n=8-8). Values are means ± SEM. * p< 0.01, significantly different from controls (saline infusion).

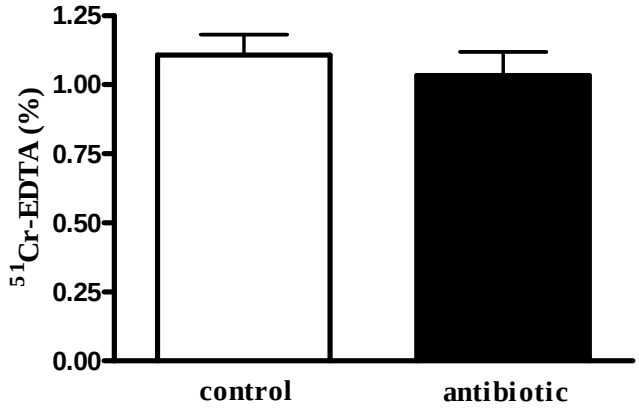

Figure 2. Effect of antibiotic treatment (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) on colonic paracellular permeability (in vivo) (n=8-8). Values are means ± SEM.

4.1.1.2. Effect of antibiotic treatment on serine protease activity of colonic contents

In fasted control Swiss mice (n=8), the serine protease activity of colonic content was 48.4±9.5 U/mg of total proteins. A 12 days oral antibiotic treatment (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) reduced the serine protease activity of colonic content to 9.6±7.0 U/mg of total proteins ($p<0.01$) (n=8) (Figure 3).

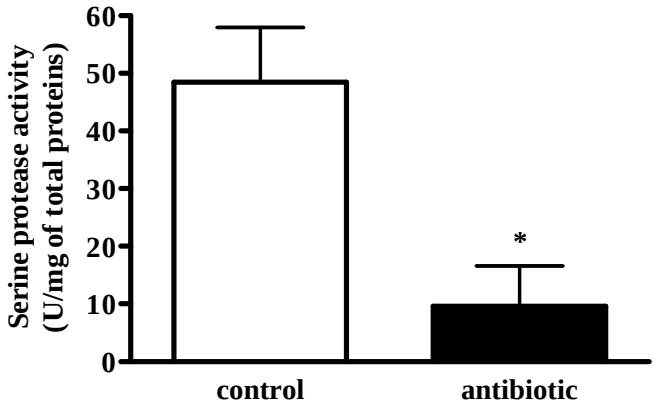

Figure 3. Effect of antibiotic treatment (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) on serine protease activity of colonic content (n=8). Values are means ± SEM. * p< 0.01, significantly different from non-treated controls (n=8).

4.1.1.3. Effect of antibiotic treatment on colonic PAR_2 immunohistochemistry

In control animals (n=8) basal PAR_2 immunochemistry showed prominent immunostaining of colonocytes (optical density: 0.3±0.1). In antibiotic treated mice (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) (n=8), PAR_2 immunostaining was significantly attenuated (optical density: 0.2±0.1) as compared with controls ($p<0.01$). Under antibiotic treatment, 2 days of daily intracolonic administration of trypsin restored the PAR_2 immunoreactivity (optical density: 0.3±0.1) (n=8) (Figure 4 A and B).

4.1.1.4. Effect of antibiotic treatment on gut permeability (in vitro)

Control values of dextran sulphate flux was significantly increased after application of PAR_2 agonist, SLIGRL to the mucosal site (1.4±0.5 vs. 4.1±0.7 nmol/h/cm^2; $p<0.05$) (n=8). Colonic strips collected from animals treated with antibiotics (0.5 g/l ampicillin and 1 g/l neomycin in drinking water, n=8) exhibited similar dextran sulphate flux as the strips collected from controls (1.4±0.1 nmol/h/cm^2), however 12 days antibiotic treatment significantly reduced the effect of SLIGRL on colonic permeability (2.1±0.4 nmol/h/cm^2) (Figure 5). Administration of compound 48/80 resulted a significantly higher increase ($p<0.05$) in colonic permeability in control animals (5.9±0.6 vs.1.9±0.3 nmol/h/cm^2; $p<0.01$) as compared with the antibiotic treated mice (3.6±0.4 vs. 2.2±0.3 nmol/h/cm^2; $p<0.05$) (n=8-8) (Figure 6).

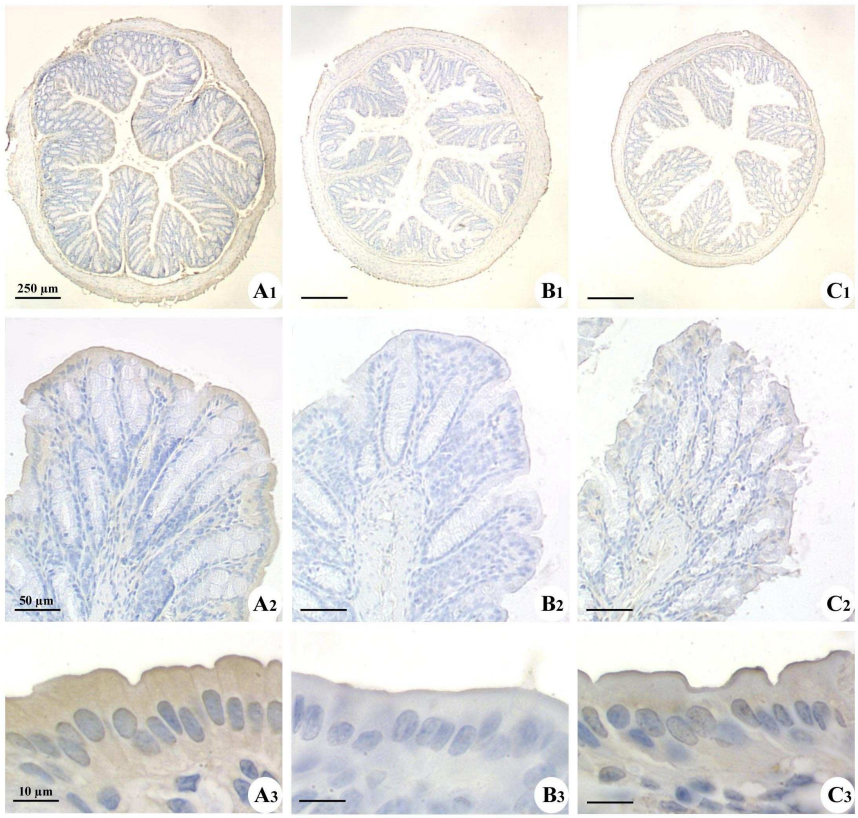

Figure 4 A. Effect of antibiotic treatment (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) on colonic PAR_2 immunostaining. PAR_2 immunoreactivity (brown) in colonic sections of control animals (n=8) (A), antibiotic treated animals (n=8) (B) and animals received daily intracolonic trypsin injections during the antibiotic treatment (n=8) (C).

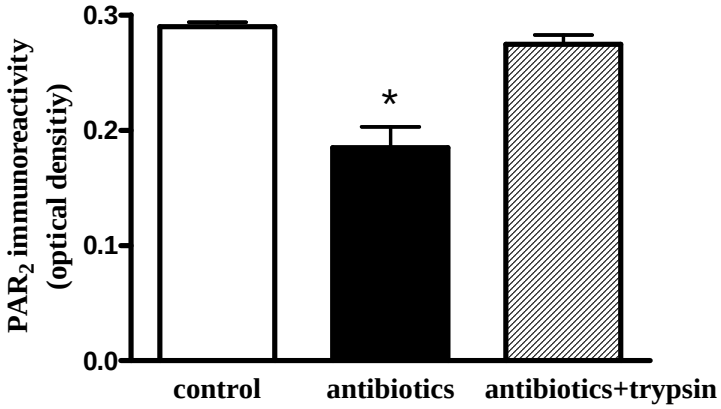

Figure 4 B. Effect of antibiotic treatment (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) on colonic PAR_2 immunohistochemistry (n=8). Data (means ± SEM) are expressed as total number of grey levels per square micrometer of colonic mucosa. * $p < 0.01$, significantly different from control mice (n=8) and animals received daily intracolonic trypsin injections during the antibiotic treatment (n=8).

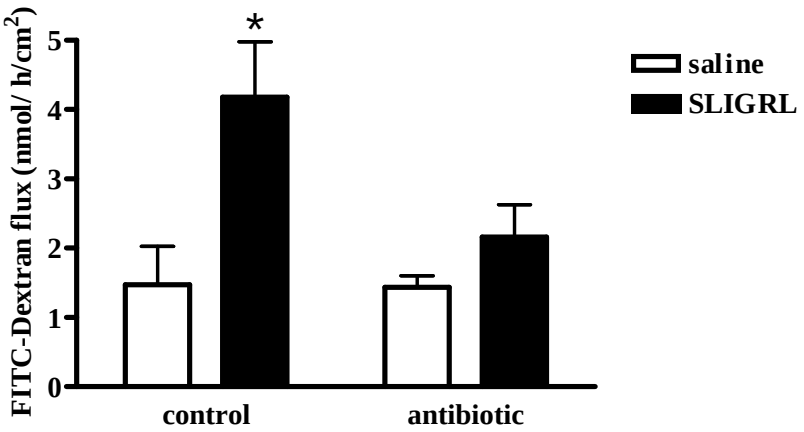

Figure 5. FITC-dextran flux and the effect of SLIGRL on colonic permeability in control animals and after antibiotic treatment (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) (n=8-8). Values are means ± SEM. * p< 0.05, significantly different from non-treated controls.

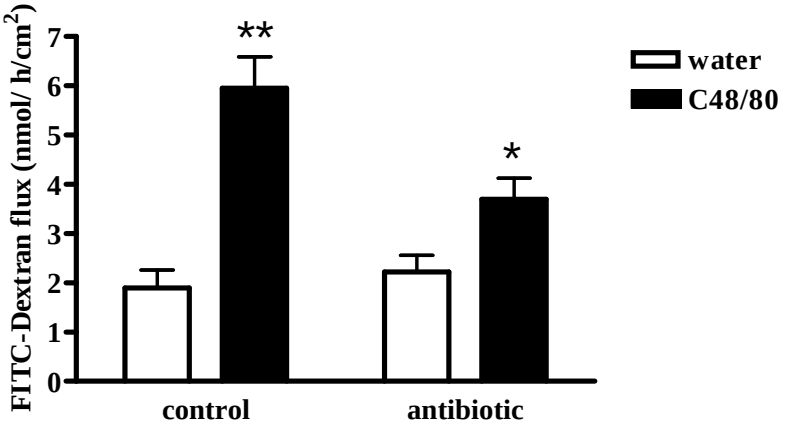

Figure 6. FITC-dextran flux and the effect of compound 48/80 on colonic permeability in control animals and after antibiotic treatment (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) (n=8-8). Values are means ± SEM. * *p< 0.01, significantly different from non-treated controls. * p< 0.05, significantly different from antibiotic treated controls and C48/80 group of non-treated animals.

4.1.1.5. Effect of antibiotic treatment on MMCP-1 content of colonic mucosa

In control mice (n=8), the MMCP-1 content of colonic mucosa was 4.6±0.7 ng/g of total proteins. After 12 days of antibiotic treatment (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) (n=8), MMCP-1 content was not significantly changed (4.1±0.9 ng/g of total proteins) as compared with control values (Figure 7).

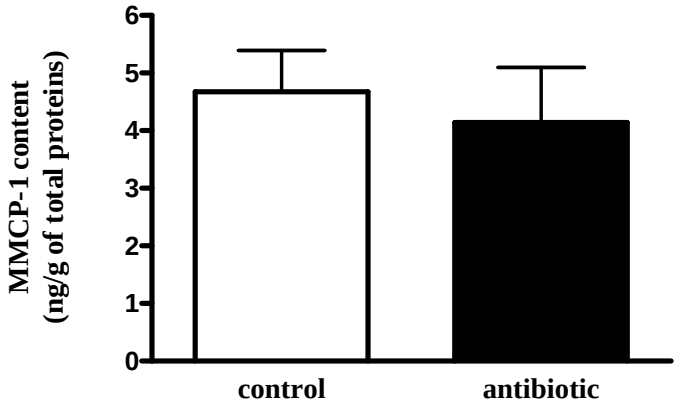

Figure 7. MMCP-1 content in the colonic mucosa in control animals and after antibiotic treatment (0.5 g/l ampicillin and 1 g/l neomycin in drinking water) (n=8-8). Values are means ± SEM.

4.1.2. Experiments in rats

4.1.2.1. Effect of dexamethasone and doxantrazole on SLIGRL-induced visceral hyperalgesia

In untreated rats (control groups, n=8-8), gradual rectal distension increased the frequency of abdominal contractions in a volume-dependent manner and this increase became significant when the volume of distension reached 0.8 ml. SLIGRL (200µg/kg) infused intracolonically (n=8) increased significantly the number of abdominal contractions obtained for all volumes of distension compared with control rats (1.2 ml: 60.1 ± 9.0 vs. 37.8 ± 4.1; $p<0.01$) (n=8-8) (Figure 8 A and B). Dexamethasone treatment (1mg/kg; ip. 4 days, n=8) suppressed the effect of SLIGRL on abdominal contractions for all volumes of distension (1.2 ml: 25.3 ± 7.2 vs. 60.1 ± 9.0, $p<0.01$) (Figure 8 A). Similarly, the mast cell stabilizer doxantrazole (10 mg/kg; ip., n=8) reduced SLIGRL-induced enhancement of abdominal contraction in response to rectal distension (1.2 ml: 40.0 ± 4.1 vs. 60.1 ± 9.0, $p<0.01$) (Figure 8B). Dexamethasone and doxantrazole treatment *per se* had no effect in abdominal response to rectal distension (data not shown).

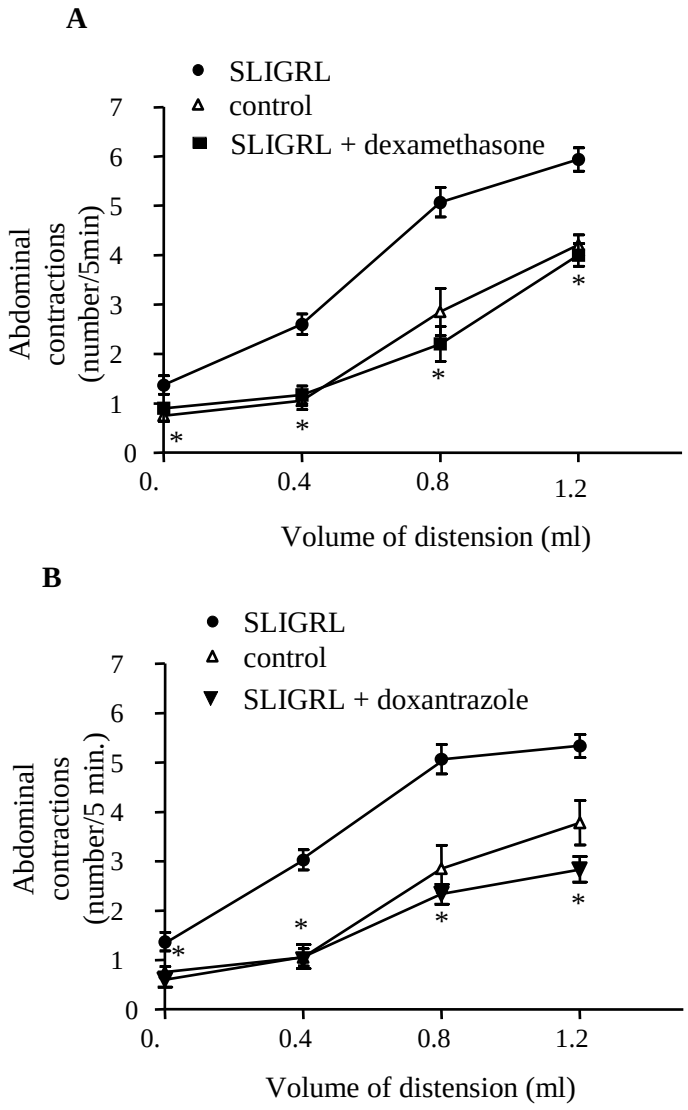

Figure 8. Comparative effect of dexamethasone (A) and doxantrazole (B) on SLIGRL-induced increase of abdominal contractions in response to rectal distension in male rats (n=8-8). Values are means ± SEM. * $p<0.01$ significantly different from SLIGRL treated groups.

4.1.2.2. Permeability responsiveness of colonic mucosa to PAR_2-agonist after dexamethasone treatment

In Ussing chambers, values of FITC-dextran flux were significantly increased after SLIGRL administration (3.39±0.98 vs. 0.86±0.20 nmol/h/cm^2; $p<0.05$) in vehicle-treated rats (n=8). Colonic strips collected from rats treated with dexamethasone (n=8) exhibited slightly, but not significantly increased FITC-dextran flux as compared with strips collected from controls (1.53±0.33 nmol/h/cm^2; $p>0.05$) (n=8). However, four days of dexamethasone treatment did not influence the effect of PAR_2-agonist on colonic permeability (3.44±0.85 nmol/h/cm^2) (Figure 9).

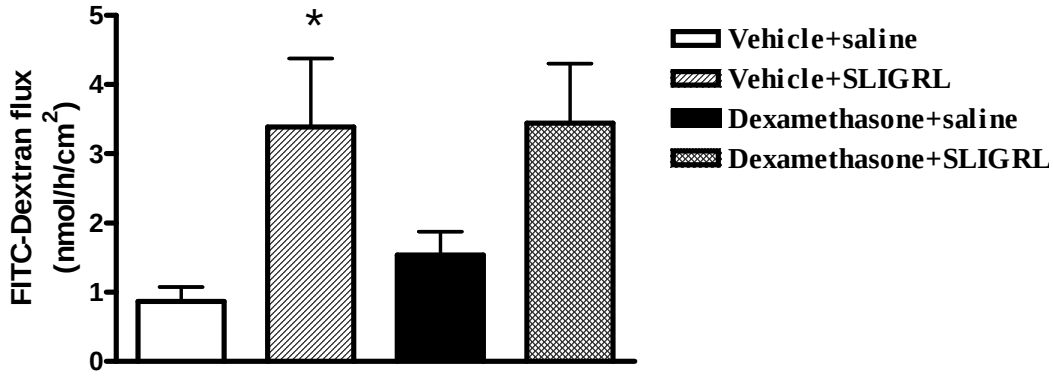

Figure 9. Basal FITC-dextran flux and the effect of SLIGRL on colonic permeability in vehicle and in dexamethasone treated rats (n=8-8). Values are means ± SEM. * p< 0.05, significantly different from basal value in vehicle treated control

4.1.2.3. Colonic PAR_2 immunohistochemistry after dexamethasone treatment

Four days of dexamethasone treatment (n=8) did not influence the colonic mucosal PAR_2 expression as compared with vehicle-treated rats (n=8). No significant difference was observed in PAR_2 immunoreactivity (optical density) between the vehicle-treated controls (0.30±0.01) and dexamethasone-treated animals (0.31±0.01) (Figure 10 A and B).

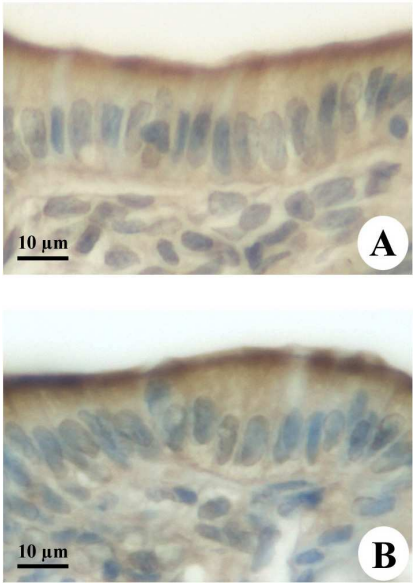

Figure 10 A. Effect of 4 days dexamethasone treatment on colonic PAR_2 expression. PAR_2 immunoreactivity (brown) in colonic sections of (A) vehicle treated control animals (n=8) and (B) dexamethasone treated rats (n=8).

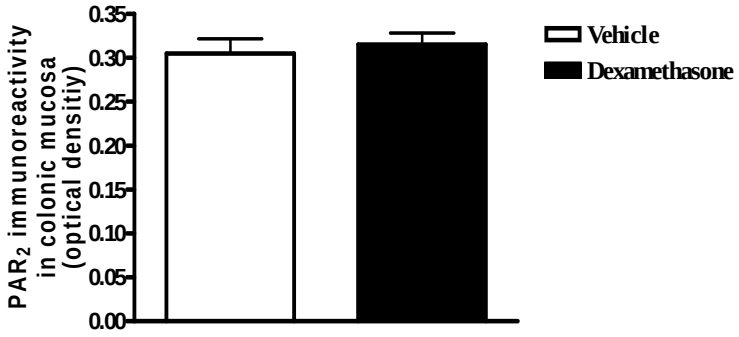

Figure 10 B. Effect of 4 days dexamethasone treatment on colonic PAR_2 expression in colonic sections of vehicle treated control animals (n=8) and dexamethasone treated rats (n=8). Quantification of PAR_2 immunoreactivity in colonic mucosa. Data are expressed as optical density, as the total number of grey levels per square micrometer of colonic mucosa.

4.1.2.4. Permeability responsiveness of colonic mucosa for mast cell degranulator

In Ussing chambers, control values of FITC-dextran flux significantly increased after compound 48/80 administration to the mucosal site (2.45±0.46 vs.0.58±0.16 nmol/h/cm^2; $p<0.01$) of strips collected from vehicle-treated animals (n=8). Dexamethasone treatment (n=8) resulted in a slight, but not statistically significant increase in dextran flux (1.64±0.60 nmol/h/cm^2; $p>0.05$). Four days of corticosteroid treatment diminished the significant effect of mast cell degranulator on colonic permeability observed in control rats (1.67±0.97 nmol/h/cm^2; $p<0.01$) (Figure 11).

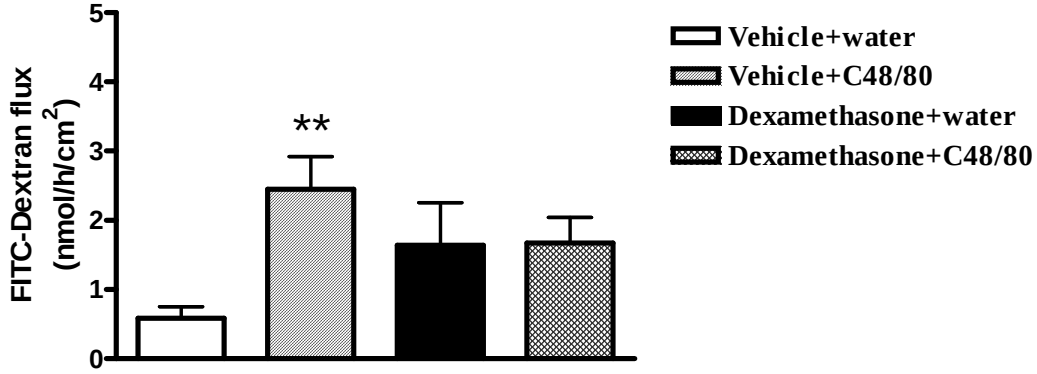

Figure 11. Basal FITC-dextran flux and the effect of compound 48/80 on colonic permeability in vehicle and in dexamethasone treated rats (n=8-8). Values are means ± SEM. **p< 0.01, significantly different from basal value in vehicle treated control.

4.1.2.5. RMCP-II content in colonic mucosa after dexamethasone treatment

In vehicle-treated rats (n=8), the RMCP-II content of colonic mucosa was 0.25±0.02 ng/mg of total protein. After 4 days of dexamethasone treatment (n=8), RMCP-II content was significantly decreased (0.17±0.01 ng/mg of total proteins, $p<0.05$) as compared with control values obtained from vehicle treated animals (Figure 12).

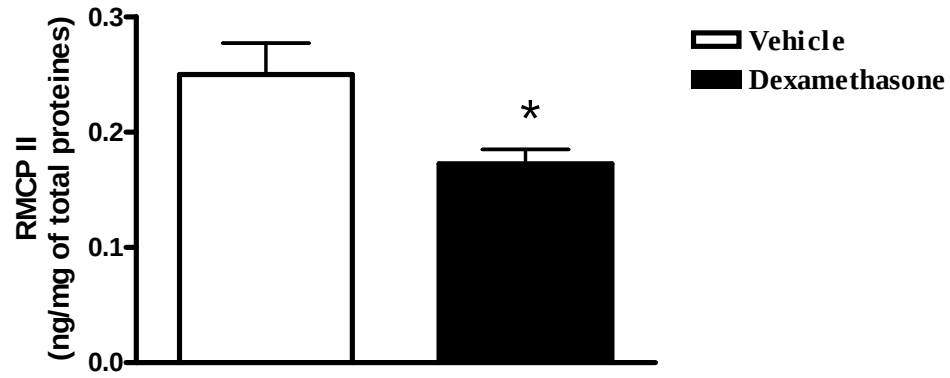

Figure 12. Effect of dexamethasone treatment on colonic mucosal RMCP-II content (n=8). Values are means ± SEM. * $p< 0.05$, significantly different from vehicle treated controls (n=8).

4.1.2.6. Colonic mucosal mast cell count after dexamethasone treatment

In vehicle-treated rats (n=8), the number of mast cell per mm^2 of colonic mucosa was 147.3±14.4. After 4 days of dexamethasone treatment (n=8), the mast cell number in the colonic mucosa was significantly reduced to 15.0±2.8 mast cell/mm^2 ($p<0.01$) (Figure 13 A and B).

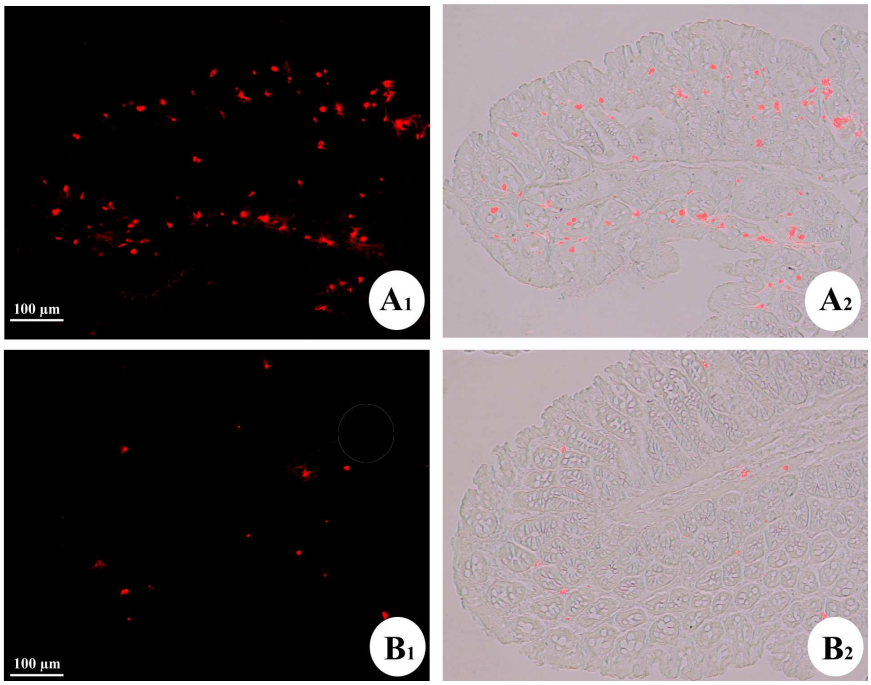

Figure 13 A. Effect of dexamethasone treatment on colonic mucosal mast cell number. Mast cell immunoreactivity (red) in colonic sections of (A_1 and A_2) vehicle treated control animals (n=8) and (B_1 and B_1) dexamethasone treated rats (n=8).

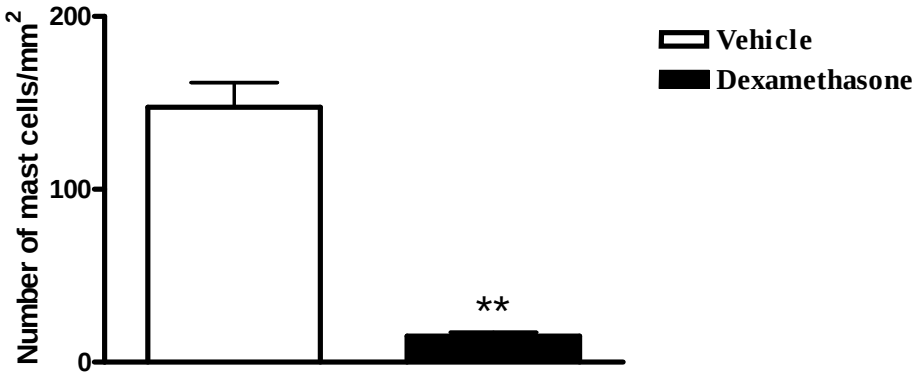

Figure 13 B. Quantification of mast cell immunoreactivity in colonic mucosa after dexamethasone treatment (n=8). Data are expressed as number of mast cells per square millimetre of colon mucosa. Values are means ± SEM. **p<0.01, significantly different from vehicle treated controls (n=8).

4.2. Human investigations

4.2.1. Fecal serine-protease activity

In controls (n=15), the fecal serine-protease activity was 16.4±5.0 U/mg protein. Fecal serine-protease activity was significantly greater ($p<0.001$) in IBS-D patients (67.9±10.8 U/mg protein) (n=17) and UC patients (67.4±15.3 U/mg protein) (n=15), than in controls, IBS-C patients (16.0±7.0 U/mg protein) (n=14) and IBS-A patients (16.4±7.1 U/mg protein) (n=7). No detectable serine protease activity was observed in fecal samples of patients with acute infectious diarrhoea (n=5), therefore no additional enzyme content and activity measurements were performed in this group of patients (Figure 14.).

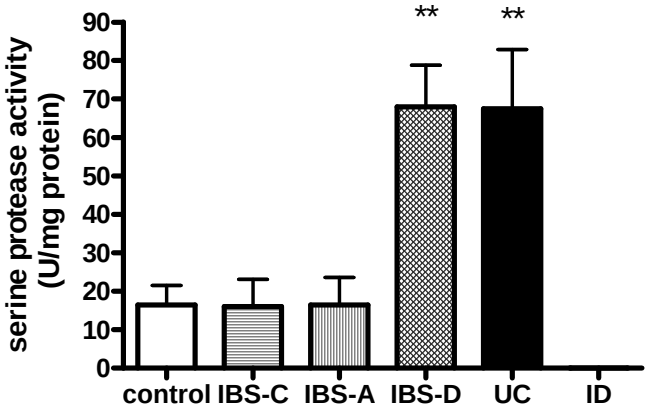

Figure 14. Fecal serine-protease activity in healthy controls (n=15), in IBS patients with constipation (IBS-C, n=14), alternating bowel habits (IBS-A, n=7) and diarrhea (IBS-D, n=17), in patients with ulcerative colitis (UC, n=15) and with infectious diarrhoea (ID, n=5). Values are means ± SEM. ** $p<0.001$, significantly different from controls, IBS-C, IBS-A and ID patients.

Fecal serine-protease activity did not correlate with the frequency of bowel movements in any of the studied groups of patients (Table 1.).

Groups	Fecal serine-protease	Bowel movements/week	Correlation analysis parameters	
	U/mg protein	mean±SEM	r	p
control	16.4 ± 5.0	6.86 ± 0.09	-0.17	0.54
IBS-C	16.0 ± 7.0	1.46 ± 0.14	0.10	0.73
IBS-A	16.4 ± 7.1	6.71 ± 0.28	0.12	0.78
IBS-D	67.9 ± 10.8	23.88 ± 3.77	0.15	0.55
UC	67.4 ± 15.3	23.40 ± 4.54	0.28	0.30

Table 1. Lack of correlation between fecal serine-protease activity and frequency of bowel movements in the different groups under study: healthy controls (n=15), in IBS patients with constipation (IBS-C, n=14), alternating bowel habits (IBS-A, n=7) and diarrhea (IBS-D, n=17), or in patients with ulcerative colitis (UC, n=15). r : correlation coefficient; p : statistics

4.2.2. Fecal mast cell tryptase activity

No significant difference was observed in fecal mast cell tryptase activity between any IBS subgroup, UC patients and controls (Figure 15.).

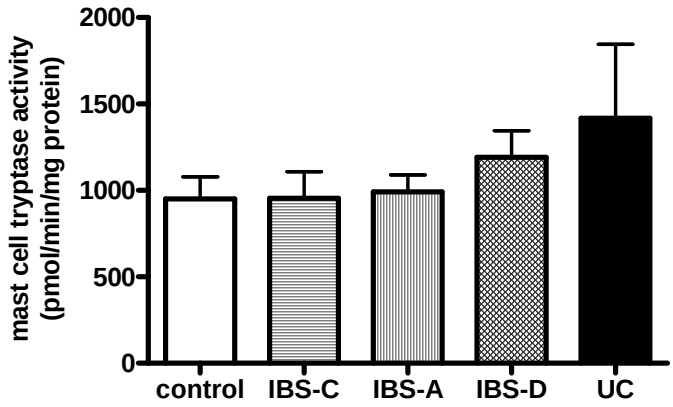

Figure 15. Fecal mast cell tryptase activity in healthy controls (n=15), in IBS patients with constipation (IBS-C, n=14), alternating bowel habits (IBS-A, n=7) and diarrhea (IBS-D, n=17), in patients with ulcerative colitis (UC, n=15).

4.2.3. Fecal pancreatic elastase-1 concentration

No significant difference was observed in fecal pancreatic elastase-1 concentration between any IBS subgroup, UC patients and controls (Figure 16.).

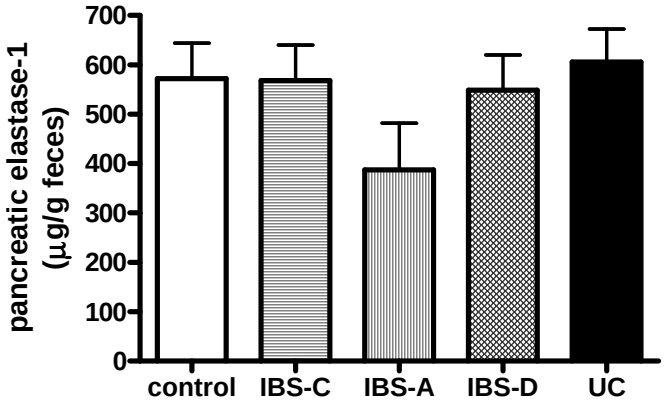

Figure 16. Fecal pancreatic elastase-1 concentration in healthy controls (n=15), in IBS patients with constipation (IBS-C, n=14), alternating bowel habits (IBS-A, n=7) and diarrhea (IBS-D, n=17), in patients with ulcerative colitis (UC, n=15).

4.2.4. Fecal SLPI concentrations

Additionally, no significant difference was found in fecal SLPI concentrations between IBS-D and controls (Figure 17.).

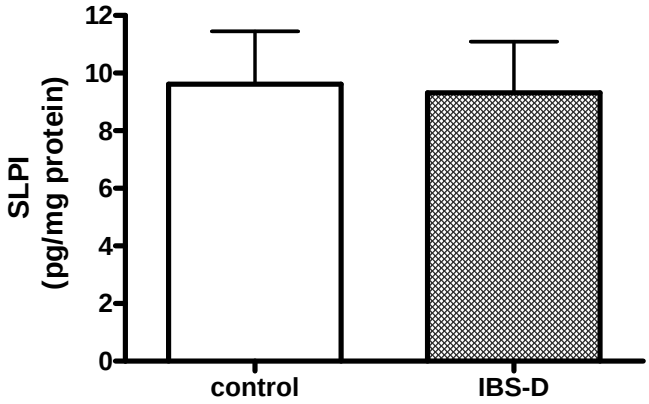

Figure 17. Fecal human secretory leukocyte protease inhibitor (SLPI) concentration in healthy controls (n=15) and in IBS patients with diarrhea (IBS-D, n=17).

5. DISCUSSION

The present observations aimed at the role of proteases and PAR_2 in the regulation of physiological colonic barrier function and in the pathogenesis of visceral hypersensitivity in IBS patients. Of all the body system, the gastrointestinal tract is the most exposed to proteases. In addition to their digestive role in protein degradation, they play a role as signalling molecules regulating cell functions by cleaving PARs. PARs are highly expressed in the gastrointestinal tract. Among others, they are present in colonic epithelial cells. Proteases from endogenous or bacterial origin can activate PARs. Trypsin and trypsinogen released from digestive glands and epithelial cells (58), thrombin, factors VIIa and Xa from coagulation cascade (12, 68), and cathepsin G, elastase or proteinase released from neutrophils (79, 80) are potential activators of PARs. Bacteria have both protease and antiprotease activity. Indeed Porphyromonas gingivalis, a pathogen bacterium in the oral cavity, a mediator of periodontal disease, releases arginine-specific protease (gingipain) which can activate PAR_1, PAR_2 and PAR_4 (48, 49). Recent observations support a role of colonic protease activity and PAR_2 in intestinal inflammation (11, 42), however until now the physiological role of intraluminal proteases and PAR_2 in the control of colonic barrier function has been unexplored. A previous study showed that low dose of PAR_2 activating peptide (SLIGRL) infused intracolonically activated PAR_2 and increased paracellular permeability to macromolecules in vivo through calmodulin and subsequent MLCK activation provoking tight junction opening by perijunctional ring myosin phosphorylation (13). Intracolonic administration of higher dose of SLIGRL also caused colonic inflammation partly independently of paracellular permeability (15). Mast cell serine proteases (43, 70), Entamoeba hystolitica cysteine proteases (85) and Bacteroides fragilis metalloprotease (84) are known to increase intestinal permeability. However colonic digestive enzymes are potent activators of PAR_2 (58) and although intracolonic administration of selective PAR_2 agonist increases colonic paracellular permeability (13, 14), no studies have already reported the physiological effect of digestive enzymes on colonic barrier function. Our study is the first to investigate the effect of different luminal proteases on colonic barrier function. We show that intraluminal delivery of protease inhibitors decreases colonic paracellular permeability demonstrating that luminal proteases plays a role in the regulation of colonic barrier function in physiological conditions (Figure 1.).

In the colon, pathogenic and non-pathogenic resident bacteria are considerably responsible for the luminal proteolytic activity. Although substantial proportion of colonic intestinal proteases is of bacterial origin, no evidence has been reported about the effect of colonic bacteria on PAR function. We demonstrate that reduction of colonic bacteria by antibiotic treatment (1) decreases the serine protease activity of the colonic content and the permeability response of colonic strips for PAR_2 agonist coupled with a decrease in PAR_2 expression in colonic mucosa (Figure 3, 4 and 5.). The effect on PAR_2 expression is specific for the protease activity of the colonic content, because daily colonic administration of a serine protease restores PAR_2 expression of colonocytes (Figure 4.). However, in contrast to that observed with serine-protease inhibitors, we did not find any significant change in colonic paracellular permeability after broad spectrum antibiotic treatment, despite the down-regulation of PAR_2 and the reduced protease activity (Figure 2.). A possible explanation for this phenomenon is that changes in PAR_2 expression and in protease activity occur slowly during the 12 days of treatment giving rise to compensatory mechanism regulating colonic paracellular permeability.

Tryptase released from mast cells may activate PAR_2 located on epithelial cells directly on the basolateral site (30). Increased mast cell tryptase release was found in colonic biopsies of ulcerative colitis patients (66), moreover increased mast cell density was observed in colonic biopsy specimens of IBS patients (6, 57, 61). Although mast cells localized in close contact with the external environment play an important role in the response to pathogen intestinal bacteria (3, 65), the effect of non-pathogen resident intestinal microorganisms on mast cell function is not clear. We found, that oral antibiotic treatment decreased colonic mast cell degranulation (Figure 6.); however colonic mucosal mast cell proteinase-1 content remained unchanged (Figure 7.). Through the bidirectional cross-talk between mast cells and PAR_2, the explanation of decreased mast cell degranulation after suppression of colonic flora could be dual. The decreased sensitivity of mast cells to degranulate related to changes in colonic bacterial stimulation could result from a decrease in PAR_2 expression of colonocytes, however the decrease of mast cell degranulation could be the result of decreased PAR_2 responsiveness of mast cells mediated by change of colonic luminal protease activity.

Intestinal permeability plays a key role in the pathogenesis of different gastrointestinal disorders. Increased intestinal permeability has been described in patients with Crohn’s disease, (77) and in symptom-free, healthy relatives of Crohn’s disease patients (64). Increased intestinal

permeability is also a predictor of relapse in patients with Crohn's disease (39). Both post-infectious and sporadic IBS are associated with an increase in intestinal permeability (52). A recent study emphasizes that the increase in intestinal permeability is more pronounced in IBS-D patients than in other subgroups of IBS (24). Furthermore, alterations of visceral sensitivity provoked by acute partial restraint stress depend upon the increase in colonic paracellular permeability in rats (2). Similarly, intracolonic infusion of subinflammatory doses of PAR_2 agonist produces a delayed rectal hyperalgesia that involves increase in intestinal permeability in rats (17). Our data indicate that luminal proteases regulate colonic paracellular permeability in physiological conditions and modification of colonic bacterial flora affects colonocyte PAR_2 expression and sensitivity of mast cells to degranulate. Therefore, it let us to speculate that changes in intraluminal protease activity linked to alterations of colonic microflora and/or proteolytic enzyme secretion may play a role in the genesis of visceral hypersensitivity and IBS symptoms.

There is recent evidence for an important role of colonic mucosal micro-inflammation in the pathogenesis of IBS. IBS symptoms are common in patients in remission from UC (37) and in those having had gastrointestinal infection (34, 55). Increased numbers of inflammatory cells are characteristic in the colonic mucosa of IBS patients (6, 16, 22, 26, 74). Despite of growing evidence for a role of colonic micro-inflammation in the pathogenesis of IBS and the anti-inflammatory effect of corticosteroids, only few studies exist in support of a beneficial effect of steroid treatment a colonic micro-inflammation and, subsequently, in the improvement of IBS symptoms. In an experimental study, dexamethasone treatment normalized postinfective intestinal dysmotility and visceral hyperalgesia in mice after intestinal Trichinella spiralis infection (8). Based on clinical cohort studies, oral corticosteroid therapy can reduce a risk of the prevalence of IBS (36) and the risk of IBS is also reduced by the use of oral steroids in asthma patients (35). Up to now, only one clinical trial with corticosteroids has been performed in a treatment of IBS. In a randomized, double-blind, placebo-controlled trial, 30 mg prednisolone once daily for 3 weeks did not improve symptoms in post-infectious IBS and did not reduce the number of colonic inflammatory cells (25). Mast cells play a key role in maintenance of colonic micro-inflammation and subsequently in the development of IBS symptoms. Increased mast cell number can be detected in coecal and colorectal biopsies from IBS patients (33, 61, 62) and activated mast cells in proximity to colonic nerves correlate with abdominal symptoms in IBS (6). Increased number of mast cells in colonic or rectal biopsies of IBS patients has been recently

confirmed (6, 61, 63) particularly in patients developing IBS after severe gastroenteritis (27). Mucosal mast cell mediators from IBS patients excite rat nociceptive visceral sensory nerves (7). There are contradictory data and also remarkable species differences concerning the effect of corticosteroids on mast cell function. Dexamethasone treatment abolishes intravenous worm antigen-mediated intestinal anaphylaxis in Nippostrongylus brasiliensis infected rats and suppression of the response is associated with depletion of RMCP-II in intestinal mucosa (44). Corticosteroid treatment reduces mast cell number in rectal biopsy specimens of IBD patients; however the reduction of mast cell counts is independent of the degree of inflammation (29). Budesonide and dexamethasone had potent inhibitory effects on the release of cytokines from a human mast cell line (86) however in another study, dexamethasone treatment failed to inhibit the release of mast cell mediators from cultures of human airway, skin and intestinal tissues (18). In our study we show, that a 4 day daily treatment with dexamethasone inhibits PAR_2 agonist-induced rectal hyperalgesia in rats. Using doxantrazole, we validate that this hyperalgesia is linked to mast cell degranulation (Figure 8.). The effect of treatment with corticosteroid on visceral hyperalgesia is coupled with a decrease in the colonic mucosal mast cell number, RMCP-II content and decrease in the degree of mast cell degranulation (Figures 11, 12 and 13.). Therefore, we can hypothesise that the effects of dexamethasone on intracolonic SLIGRL-induced rectal hyperalgesia is linked to its inhibitory effects on mast cells. Based on our findings, one may speculate a beneficial effect of corticoids as well as mast cell tryptase inhibitors in the therapy of IBS (78). Furthermore, 5-aminosalycilic acid is an effective inhibitor of IgE-induced histamine and PGD2 release from human intestinal mast cells (28) and mast cell stabilizers used in allergic diseases, such as sodium cromolyn (31, 72), could also have attenuating effect on symptoms in IBS patients.

Our results indicate a lack of direct action of corticosteroids on intracolonic PAR_2 agonist-induced increase of colonic permeability (Figures 9 and 10.). Furthermore our data establish that mast cell tryptase secretion, which was strongly reduced after dexamethasone treatment, has no regulatory effect on PAR_2 expression on the apical site of colonic epithelial cells. In a previous study in human colonic epithelial cells, it was shown that basolateral administration of mast cell degranulator, compound 48/80 had increasing effect on paracellular permeability mediated by mast cell tryptase, which cleaved PAR_2 on the basolateral part of colonocytes (38). Since in our experiments we did not observe any changes in colonic mucosal PAR_2 expression and paracellular permeability coupled with a significant decrease in the

mucosal mast cell number, tryptase content and degranulation after dexamethasone treatment, we could speculate different interactions between mast cells and PAR_2 in luminal and serosal parts of colonic epithelial cells. Our last finding reinforces the hypothesis of a crucial role of luminal proteases in the regulation of epithelial cell PAR_2 expression and function, already reported in a previous study (83). The lack of effect of dexamethasone on PAR_2 agonist-induced colonic permeability increase suggests that corticoids may be unable to prevent the alterations of intestinal permeability and subsequent mucosal immune stimulation, which mechanism plays an important role in the development of IBS symptoms. However, the only one existing clinical study with prednisolone suggesting the inefficiency of corticosteroids in the treatment of post infectious IBS symptoms was performed in small population and only in one subgroups of IBS patient. Therefore further studies in greater patient population and in other subtypes of IBS are required to define the possible clinical importance of corticosteroids in the treatment of IBS symptoms.

Our human study demonstrates that serine-protease activity is markedly elevated in stools of diarrhea-predominant IBS patients. Similarly, increased serine-protease activity is found in patients with active UC (Figure 14.). Elevated luminal proteases can directly break the mucus barrier, digest the underlying epithelium and subsequently, produce mucosal inflammation and damage. In addition to their digestive role, proteases are signalling molecules regulating cell functions by cleaving PARs, which are highly expressed throughout the gastrointestinal tract. These receptors are present, among other, in colonic epithelial cells, and are particularly dense at the apical site (83). In rats, intracolonic but not systemic infusion of a PAR_2-agonist produced delayed rectal hyperalgesia (17). In mice, colonic PAR_2 activation caused delayed facilitation of capsaicin-evoked visceral nociception (41); stimulation of colonic PAR_2 with endogen activator, as well as PAR_2 activating peptide, resulted delayed development of colonic hypersensitivity to capsaicin in wild type but not in PAR_2 KO mice (40). In guinea-pigs, activation by mast cell tryptase of PAR_2 located at the enteric nerves caused neuronal hyperexcitability (67). PAR_2 could provoke visceral hypersensitivity not only by direct action, but also indirectly by increasing colonic permeability. Thus, intracolonic administration of PAR_2 agonist to mice increased colonic paracellular permeability by direct MLCK-dependent mechanism (13, 53). Moreover, increase in colonic paracellular permeability induced by acute stress in rats, is responsible for concomitant rectal hypersensitivity to distension (2). Increased intestinal permeability was found to be an important factor in the development of symptoms in patients

with post infectious IBS (74) or presenting other types of IBS (52), particularly for IBS-D patients and at colonic level (24). Recently, a preliminary study demonstrated an increased trypsin-like proteolytic activity in colonic biopsies from IBS patients. This protease activity was able to cause hyperalgesia when injected into mouse paws, through a mechanism involving PAR_2 activation (83). Furthermore, activation of PAR_2 has been shown to provoke chloride secretion (21, 32, 47, 82). Stimulation of chloride secretion is a crucial pathophysiological factor in the development of diarrhoea which is an important clinical symptom of one subtype of irritable bowel syndrome. Our study is the first, so far, to show that an increased fecal serine-protease activity is characteristic in diarrhea-predominant IBS patients, and we suggest that this enhanced protease activity may be a pathogenic factor either by a direct action or, more probably, through PAR_2 activation.

Since micro-inflammatory alterations of the colonic mucosa have been reported in IBS patients, we compared their fecal protease contents to that of UC patients who had macroscopically evident colonic inflammation. We observed similarly elevated fecal serine-protease activities in patients with UC and in patients with diarrhea-predominant IBS as a possible marker of colonic inflammation in IBS patients (Figure 14.). Our finding supports the results of earlier clinical studies concerning colonic protease activity and inflammatory bowel disease. Thus, fecal proteolytic activity, alpha-1-antitrypsin and neutrophil elastase were found increased in patients with UC; however only the correlation between proteolytic activity and severity of disease was significant (11). Patients with acute attack of UC had higher levels of casein digestion, pancreatic elastase and granulocytic elastase in fecal samples than both quiescent disease patients and controls (9). There is also growing evidence in favour, not only of a direct pathogenic role of proteases, but also for a role of PARs in IBD. PAR_2 and TNF-α proteins are more expressed in UC colonic samples than in normal tissues. Most of the PAR_2 positive cells are tryptase-positive mast cells (42). In vivo, intracolonic activation of PAR_2 in mice provoked colonic inflammation, at least partly mediated by an increased paracellular permeability leading to bacterial translocation into peritoneal organs (13, 14). Our results agree with previous observations indicating that colonic serine-protease activity had a role in the development of inflammation in UC. According to recent observations concerning PAR expression in IBD, it is tempting to hypothesize that PARs participate in the genesis and severity of colonic inflammation.

It is well established that colonic resident bacteria release considerable amount of serine-, cysteine- and metallo-proteases, and that digestive enzymes are partly degraded by host-proteases and bacterial peptide hydrolases in the colon (51). Therefore, fecal proteolytic activity is influenced by duration of colonic transit, which effect was confirmed in a clinical study in Crohn's disease patients (69). It can be speculated that colonic transit-time could influence fecal protease activity by modifying the exposure of different luminal proteases to bacterial degradation. However, we were unable to find any correlation between serine-protease activity and frequency of bowel movements in any subgroups of IBS patients (Table 1.). Moreover we failed to detect an increased serine-protease activity in feces of patients with acute infectious diarrhoea (Figure 14.). Therefore, we can speculate that the elevated fecal protease activity is the cause rather than the result of frequent bowel movements in patients with diarrhea-predominant IBS. Furthermore, increased serine protease activity was observed only in diarrhea-predominant IBS, and not in patients with constipation. This phenomenon could be explained by the heterogeneity of IBS patients and the involvement of different pathological pathways in different types of IBS. Similar heterogeneity exists in the possible role of micro-inflammation in the development of symptoms. Increased numbers of inflammatory cells were also found in subgroups of IBS patients, particularly in post-infectious IBS, while marked differences in colonic inflammatory cell infiltration were observed between constipation- and diarrhea-predominant IBS patients (16).

The origin of the elevated protease activity in stool of diarrhoea predominant IBS patients has not yet been identified, since we have not observed any change in mast cell tryptase activity and pancreatic digestive enzyme concentration in fecal samples of these patients (Figures 15 and 16.). It has been reported earlier that pathological changes in colonic microflora may be characteristic to certain subgroups of IBS patients (4, 75). Since colonic bacteria release proteases, we can speculate that perturbed bacterial flora may be one of the sources of elevated fecal protease activity. It was also demonstrated that increased neutrophil elastase levels were partly responsible for elevated fecal proteolytic activity in UC patients (11). According to the rising evidence of micro-inflammation in IBS, proteases released by inflammatory cells, except mast cells, may also be responsible for elevated fecal levels of serine-protease activity. A physiological balance exists between proteases and protease-inhibitors, which prevents the proteolytic injury of colonic mucosa in healthy conditions (73). SLPI, an anti-protease with potent inhibitory effect on human trypsin, leukocyte elastase and mast cell chymase, can be

found in biopsies from human colonic mucosa and in human intestinal epithelial cell lines (60, 73). Since we found an elevated fecal serine-protease activity in IBS patients with diarrhea, we investigated the possible role of SLPI in the disturbance of the protease/anti-protease balance in this group of patients. We confirmed that the increase in serine-protease activity in IBS-D patients was not related to changes in the luminal concentration of this proteinase inhibitory factor (Figure 17.). This observation also underlines a potentially major role of inflammatory cells as a source of elevated fecal serine-proteases in IBS-D patients. Since no serine-protease activity was detected in fecal samples collected from our small population of patients with acute infectious diarrhoea, the role of bacteria in the over-production of fecal serine-proteases needs more investigations.

The possible role of elevated luminal proteases in the development of symptoms raises the potential use of protease inhibitors in the treatment of diarrhoea predominant IBS. A potato protein extract containing protease inhibitors almost completely suppressed proteolytic activity in feces, and were successfully applied topically in protease-induced dermatitis (69). Camostat, a serine-protease inhibitor, improved reflux esophagitis after gastrectomy (46) and induced and maintained remission in UC patients (71). These protease inhibitors are safely used in clinical practice, and could represent new agents for IBS therapy, particularly in diarrhea-predominant patients.

6. NEW RESULTS ESTABLISHED IN THE THESIS

1. This study provides new evidence that luminal proteases regulate colonic paracellular permeability in physiological conditions in mice.
2. Oral antibiotic treatment decreases colonocyte PAR_2 expression possibly through the modulation of luminal protease activity in mice.
3. Oral antibiotic treatment reduces mucosal mast cell degranulation, but not the release of mast cell protease into the colonic lumen in mice. This observation suggests a limited role of secreted proteases from mucosal mast cells in the regulation of epithelial cell PAR_2 expression.
4. Dexamethasone treatment prevents intracolonic PAR_2-agonist provoked rectal hypersensitivity in rats.

5. PAR_2-induced rectal hypersensitivity is linked to mast cell degranulation. The effect of corticosteroid treatment on visceral hyperalgesia is accompanied by a reduction of colonic mast cell number, tryptase content and mast cell degranulation. However, dexamethasone treatment is unable to prevent the alterations of colonic permeability triggered by intracolonic PAR_2 activation.
6. Serine-protease activity is markedly elevated in stools of diarrhea-predominant IBS patients. Similarly, increased serine-protease activity is found in patients with active ulcerative colitis.

7. SUMMARY

BACKGROUND: Proteases represent 2% of the human genom and are present in great amount in the gastrointestinal tract. In addition to their digestive role in protein degradation, they act as signalling molecules regulating cell functions by cleaving protease-activated receptors (PARs). PARs belong to a family of seven transmembrane domain G-protein-coupled receptors that are activated by cleavage of their N-terminal domain by a proteolytic enzyme. The unmasked new N-terminal sequence acts as a tethered ligand that binds and activates the receptor itself. Four PARs have been identified in human tissues: PAR_1, $_{-2}$, $_{-3}$ and $_{-4}$. PARs are expressed throughout the gastrointestinal tract on several cell types, as enterocytes, mast cells, smooth muscle cells, myenteric neurons, endothelial cells and on colonic epithelial cells. PARs are activated by a variety of proteases, such as digestive enzymes (trypsin and trypsinogen), proteases released from mast cells and neutrophils, and by proteases of the coagulations cascade. Proteases of human pathogen Porphyromonas gingivalis activate PARs on human oral epithelial cells, neutrophils and platelets. Even though resident colonic bacteria release considerable amount of proteases, no studies have already evaluated the effects of commensal intestinal bacterial flora on PAR activation.

PAR_2 is strongly expressed in the gastrointestinal tract, particularly in the colon, and is mainly located on the apical site of colonic epithelial cells. Activation of PAR_2 has been shown to provoke chloride secretion in mouse colon. In animal models, intracolonic infusion of PAR_2-agonist activated spinal afferent neurons and produced a delayed rectal hyperalgesia, colonic PAR_2 activation caused delayed facilitation of capsaicin-evoked visceral nociception, and activation of PAR_2 in enteric nerves by mast cell tryptase caused neuronal hyperexcitability. In vivo, intracolonic activation of PAR_2 leads to colonic inflammation in mice and increases paracellular permeability with bacterial translocation into peritoneal organs. In mice, intracolonic infusion of low dose PAR_2 activating peptide (SLIGRL) increases colonic paracellular permeability by a direct MLCK-dependent mechanism. In spite of growing evidences of the important role of PAR_2 in pathological conditions, the physiological role of PAR_2 located on colonocytes remains unclear.

Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder characterized by continuous or remittent abdominal pain, bloating and altered defecation. In spite of intense research, the pathogenesis of IBS remains partially understood, and no specific and universally

effective patient management has been developed. Altered colonic motor function, visceral hypersensitivity, changes in neural transmission within the gut, alterations of spinal and supraspinal sensory afferent system, and low-grade inflammation of the intestinal mucosa, may play a role in the development of IBS. There is growing evidence that this low-grade inflammation plays a role in the pathogenesis of IBS, particularly to initiate symptoms developed after gastrointestinal infection. Although PARs are potential receptors involved in the development of visceral hypersensitivity in IBS and proteolytic enzymes from both endogenous and exogenous origins are present at high concentrations in the gut, no study has evaluated the potential role of colonic luminal proteases in IBS pathogenesis. Moreover, therapeutic modification of PAR function may be beneficial for the relief of IBS symptoms. However, the lack of PAR_2 antagonists had not permitted until now to confirm a beneficial effect of blocking PAR_2 activation in the therapy of inflammatory bowel disease or IBS. Although corticosteroids are potent inhibitors of inflammatory processes, and are widely used in the treatment of inflammatory bowel disease, only one study with corticosteroids in patients with post-infectious IBS exists and suggests that prednisolone is not likely to be an effective treatment for IBS symptoms.

AIMS: To evaluate if intraluminal proteases and particularly serine-proteases play a role in the physiological control of paracellular permeability in mice. To clarify the role of bacterial proteases in the regulation of colonic barrier function. To assess the role of mast cell proteases in colonic PAR_2 activation. To investigate if dexamethasone treatment prevents PAR_2 agonist-induced visceral hyperalgesia in rats. To evaluate the role of PAR_2 and colonic mast cells in the effect of dexamethasone on visceral hypersensitivity. To examine whether colonic luminal proteases are elevated in IBS patients, as reflected by stool analysis.

MATERIALS AND METHODS:

Experiments in mice: Colonic paracellular permeability was assessed with ^{51}Cr-EDTA after intracolonic administration of different protease inhibitors (mixture of water soluble protease inhibitors, cystatin, aprotinin, galardin) or saline for 5 hours. After 12 days of oral antibiotic treatment (ampicillin + neomycin), measurements of colonic luminal serine-protease activity, paracellular permeability, mucosal mouse mast cell protease-1 (MMCP-1) content, immunochemistry of PAR_2 and assessment of effects of PAR_2 agonist (SLIGRL) and mast cell degranulator (C48/80) on colonic paracellular permeability in Ussing chambers were performed.

Additionally, immunochemistry was repeated after intracolonic trypsin administration under the antibiotic treatment.

Experiments in rats: Abdominal contractions provoked by rectal distension were recorded in rats equipped with intramuscular electrodes. Changes in visceral hypersensitivity provoked by intracolonic administration of SLIGRL, changes in colonic mucosal rat mast cell protease-II (RMCP-II) content, mast cell count and PAR_2 expression were measured after 4 day treatment with dexamethasone (1mg/day/rat ip.) or its vehicle (water). Effect of mast cell stabilizer (doxantrazole, 1mg/kg ip, 2 hours before and 6 hours after intracolonic infusion of SLIGRL) on SLIGRL induced visceral hyperalgesia was also assessed. Effects of SLIGRL and C48/80 on permeability of colonic strips from vehicle or dexamethasone-treated rats were investigated in Ussing chambers.

Human investigations: Fecal samples of 38 IBS patients, 15 patients with ulcerative colitis (UC), 5 patients with acute infectious diarrhoea and 15 healthy controls were studied. Fecal serine-protease activity was determined photometrically using azocasein as a proteolytic substrate; fecal pancreatic elastase-1 and mast cell tryptase content were measured by ELISA. Fecal secretory leukocyte protease inhibitor concentration was determined by ELISA in control subjects and in patients with diarrhea-predominant IBS.

RESULTS:

Experiments in mice: Colonic infusion of protease inhibitors significantly reduced colonic paracellular permeability. In antibiotic treated mice colonic luminal serine-protease activity was reduced coupled with a decrease in PAR_2 expression, but with no change in colonic paracellular permeability and MMCP-1 content. Trypsin administration restored PAR_2 expression. The increase in paracellular permeability induced by SLIGRL and C48/80 were reduced after antibiotic treatment.

Experiments in rats: Four days of dexamethasone as well as doxantrazole treatment diminished the SLIGRL-induced hyperalgesia for all volumes of distension. This effect of dexamethasone was accompanied by reduced colonic permeability in response to C48/80 and decreased RMCP-II content and mast cell number. Dexamethasone treatment did not influence colonic mucosal PAR_2 expression and permeability responsiveness to SLIGRL.

Human investigations: Fecal serine-protease activity was 3-fold higher in patients with diarrhea predominant IBS, than in controls or IBS patients with either constipation or alternating bowel habits. Fecal serine-protease activity was not correlated with the frequency of bowel movements

in either group. Elevated serine-protease activity was also detected in stools of UC patients. No significant difference was observed in fecal mast cell tryptase and pancreatic elastase concentrations among any of the groups, and in fecal secretory leukocyte protease inhibitor concentration between controls and diarrhea-predominant IBS patients.

CONCLUSIONS:

Luminal proteases regulate colonic paracellular permeability in physiological conditions. Oral antibiotic treatment decreases colonocyte PAR_2 expression possibly through the modulation of luminal protease activity in mice. Furthermore, oral antibiotic treatment reduces mucosal mast cell degranulation, but not the release of mast cell protease into the colonic lumen in mice. This observation suggests a limited role of secreted proteases from mucosal mast cells in the regulation of epithelial cell PAR_2 expression. Since colonic paracellular permeability plays a role in the development of visceral hypersensitivity, one may hypothesize that changes in intraluminal protease activity linked to alterations of colonic microflora and/or proteolytic enzyme secretion may play a role in the genesis of IBS symptoms.

Dexamethasone treatment improves PAR_2 agonist-induced visceral hypersensitivity but does not prevent PAR_2 agonist-induced increase in colonic permeability in rats. This effect is coupled with a reduction of colonic mast cell number and RMCP-II contents. Therefore, one may speculate a beneficial effect of corticoids as well as mast cell tryptase inhibitors in the therapy of IBS.

Fecal serine-protease activity is markedly elevated in patients with diarrhoea-predominant IBS. The possible role of elevated luminal proteases in the development of symptoms raises the potential use of protease inhibitors in the treatment of diarrhoea-predominant IBS.

8. ACKNOWLEDGEMENTS

I am grateful to **Prof. Dr. Tibor Wittmann**, the head of the First Dept. of Medicine, my tutor and scientific adviser; without his support this work could not be done.

I express my gratitude to **Prof. Dr. Lionel Bueno**, my tutor at INRA, who inspired me and supported my experimental work in Toulouse.

I would like to express my deepest appreciation to **Prof. Dr. János Lonovics**, the former head of the First Dept. of Medicine for providing the conditions of experimental and clinical research in his department.

I would like to thank my colleagues and co-authors: **Dr. András Rosztóczy, Dr. Ferenc Izbéki, Dr. Krisztina Gecse, Dr. Ferenc Nagy, Dr. Tamás Molnár, Dr. Ildikó Kovács, Dr. Zsuzsanna Lénárt, Klára Vadászi, Prof. Dr. Jean Fioramonti, Dr. Afifa Ait-Belgnaoui, Dr. Hélène Eutamène, Dr. Julien Demaude, Dr. Nicolas Cenac, Dr. Laurent Ferrier, Dr. Rafael Garcia-Villar, Mathilde Leveque and Christel Salvador-Cartie**r for their help.

Last but not least I would like to express my deepest gratitude **my family members** who are always with me and support my work.

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10. ANNEXES

Annex I.

Annex II.

Annex III.

Annex IV.

Annex V.

Annex VI.

Annex VII.

Annex VIII.

Annex IX.

Annex X.

Annex XI.

Annex XII.