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Effect of pharmacologically induced smooth muscle activation on permeability in murine colitis

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Background: **Both intestinal permeability and contractility are altered in inflammatory bowel disease. Little is known about their mutual relation. Therefore, an *in vitro* organ bath technique was developed to investigate the simultaneous effects of inflammation on permeability and smooth muscle contractility in different segments of the colon.**

Methods and materials: **BALB/c mice were exposed to a 10% dextran sulphate sodium drinking water solution for 7 days to induce a mild colitis, while control mice received normal tap water. Intestinal segments were placed in an oxygenated organ bath containing Krebs buffer. Permeability was measured by the transport of the marker molecules ^{3}H-mannitol and ^{14}C-polyethyleneglycol 4000. Contractility was measured through a pressure sensor. Smooth muscle relaxation was obtained by salbutamol and L-phenylephrine, whereas contraction was achieved by carbachol and 1-(3-chlorophenyl)-biguanide.**

Results: **The intensity of mucosal inflammation increased throughout the colon. Also, regional differences were observed in intestinal permeability. In both normal and inflamed distal colon segments, permeability was diminished compared with proximal colon segments and the non-inflamed ileum. Permeability in inflamed distal colon segments was significantly decreased compared with normal distal segments. Pharmacologically induced relaxation of smooth muscles did not affect this diminished permeability, although an increased motility positively affected permeability in inflamed and non-inflamed distal colon.**

Conclusions: **Inflammation and permeability is inversely related. The use of pro-kinetics could counteract this disturbed permeability and, in turn, could regulate the disturbed production of inflammatory mediators.**

Key words: Dextran sulphate sodium, Mice, Inflammation, Intestinal permeability, Intestinal motility

Introduction

Human inflammatory bowel disease (IBD) represents Crohn's disease and ulcerative colitis. Despite extensive research, the aetiology of human IBD remains unclear. Genetic, environmental and immunologic factors are probably all involved in the pathogenesis of IBD.[1–3]

The gut wall of healthy subjects will normally provide both barrier and transport functions towards luminal molecules. In human IBD, an impaired barrier function has been observed.[4–7] A defective barrier function increases the exposure of the mucosa to the gut contents, including bacteria and antigens, which may induce intestinal inflammation. It remains to be defined whether this defective barrier function occurs as the primary or secondary event, and which mechanisms and pathways are involved.[8,9]

In ulcerative colitis, altered gastrointestinal motility has been described.[10,11] Several clinical studies have shown that ulcerative colitis patients have a decrease in motility, which may increase their diarrhoeal symptoms.[10,11] According to Collins,[12] studies in animal models clearly indicate a causal relationship between the presence of mucosal inflammation and altered sensory-motor function. Moreover, Snape and Kao[10] observed that a decrease in colonic smooth muscle myoelectric and contractility response occurred in animals with experimental colitis. In 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis in rats, inflammation-induced changes in smooth muscle function were observed that might contribute to the abnormal motility associated with IBD.[13]

Information on the intestinal barrier function can be obtained by monitoring urinary excretion of orally administered test markers. Several intestinal perme-

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ability tests are operational in clinical practise and research.

Ideal intestinal permeability markers should be biochemically inert and cross the intestinal epithelium by non-mediated diffusion through defined pathways.[14,15] Frequently used markers are the cylindrical polymer polyethylene glycol (PEG) 400 with a cross-sectional diameter of 5.3 Å, the globular-shaped sugar alcohol mannitol with a cross-sectional diameter of 6.7 Å, and ^{51}Cr-labeled ethylenediamine tetraacetic acid with a cross-sectional diameter of approximately 11 Å.[16–18] Instead of PEG 400, which has a similar size as mannitol, PEG 4000 has also been used, with a cross-sectional diameter of more than 10 Å.

The relation between colonic inflammation, inflammatory mediators, altered permeability and changed motility is not yet fully elucidated. Our aim was to investigate the effects of two different agonists on smooth muscle relaxation, namely salbutamol (β_2-agonist), and l-phenylephrine (α_1-agonist), and the effects of two different smooth muscle contracting agonists, namely carbachol (muscarinic receptor agonist) and 1-(3-chlorophenyl)-biguanide (5-HT_3-agonist). Contractile activity and transmural permeability were measured simultaneously in an organ bath as described previously.[19] The dextran sulphate sodium (DSS) model to induce a mild colitis in mice, as described by Okayasu *et al*.,[20] has been modified and extensively investigated as described previously.[21–25]

Materials and methods

Animals

The experiments were conducted with female adolescent BALB/c mice (20–22 g; IFFA Credo, France). Animals were kept individually on chopped wood bedding in polystyrene cages under a 12 h day/night cycle at 20–22°C. Mice were permitted free access to a standard mouse chow (Hope Farms, Woerden, The Netherlands) and 10% (w/v) DSS supplemented or normal tap water. The experiments were carried out after approval of the Ethics Committee for the use of experimental animals of the Erasmus university Medical Centre (protocol 118-00-02).

Experimental design

Mice received 10% DSS (molecular weight 500 kDa, sulphur content 17 ± 1%; Pharmacia Biotech AB, Uppsala, Sweden) added to their drinking water to induce colitis. Controls received normal tap water throughout the whole study. Animals were killed by cervical dislocation after drinking DSS for 7 days. Before sacrifice, mice were 20 h deprived from food. Immediately after sacrifice, the intestinal segments were used for *in vitro* permeability and contractility experiments. The effect of smooth muscle relaxation and contraction, induced by various receptor agonists, on intestinal permeability was tested. In the first series of experiments, salbutamol and 1-(3-chlorophenyl)-biguanide were used to induce smooth muscle relaxation and contraction, respectively, while L-phenylephrine and carbachol were used for the same purpose in another series of experiments.

Drugs

Salbutamol (a specific β_2-adrenoceptor agonist, 10^{-2} M; generous gift of Glaxo Wellcome, UK), 1-(3-chlorophenyl)-biguanide (a specific 5-HT_3 receptor agonist, 10^{-4} M; lot number 3067A, ICN, Aurora, OH, USA), L-phenylephrine (an α_1-adrenoceptor agonist, 3×10^{-4} M; lot number 115H0665, Sigma, St Louis, MO, USA) and carbachol (a muscarine receptor agonist, 10^{-1} M; lot number 35H2516, Sigma) were used. All compounds were dissolved in distilled water and stored at 4°C until use.

Tissue preparation

After the mice were killed, the intestines were removed from the abdominal cavity. One segment of the ileum adjacent to the caecum, a proximal colon and a distal colon segment were taken and immediately immersed in standard Krebs buffer (pH 7.4), containing 118 mM of NaCl, 4.7 mM of KCl, 2.5 mM of $CaCl_2 \cdot 2H_2O$, 1.2 mM of $MgSO_4 \cdot 7H_2O$, 1.2 mM of KH_2PO_4, 25 mM of $NaHCO_3$ and 8.3 mM of glucose. The experimental *ex vivo* model used in this study has been described previously.[19] In short, each end of an intestinal segment was directly cannulated with stainless-steel cannulas and mounted horizontally in a 5-ml double-walled Perspex organ bath. The organ baths, warmed to 37°C and filled with standard Krebs buffer, were continuously gassed with carbogen (95% O_2 and 5% CO_2). Next the intestinal lumen was filled with standard Krebs buffer, containing the marker molecules ^{3}H-mannitol (NEN Life Science Products, Hoofddorp, The Netherlands) and ^{14}C-PEG 4000 (Amersham Life Science, Hertogenbosch, The Netherlands); about 500,000 dpm/ml for each labelled marker. The distal cannula was connected to a low-pressure sensor (Dépex, De Bilt, The Netherlands), which measures the pressure difference between a passive port, set at 15 mmHg, and the active port. The signal was recorded by Multiple Channel Registration computer-software. The pressure detection range was 15 ± 9 mmHg.

Permeability measurements *in vitro*

Permeability measurements started at $t = 0$ by replacing the organ bath fluid with 5 ml of fresh, carbogenated Krebs buffer. Every 15 min, 2 ml samples were taken in duplicate from the serosal reservoir for marker analysis, directly followed by exchange of the organ bath fluid. This procedure was continued for 60 min. Samples were collected in 20-ml Econo glass vials (Packard Instrument BV, Groningen, The Netherlands) and 8 ml of scintillation fluid (Pico-Fluor 15; Packard Instrument BV) was added. Each vial was counted for radioactivity by a multi-channel β liquid scintillation counter (1500 TRI-CARB Liquid Scintillation Analyser; Packard Instrument BV). The ^{14}C-PEG 4000/^{3}H-mannitol (P/M) ratio was calculated at each time point and standardised with the P/M ratio of the filling buffer at $t = 0$ to perform statistical analysis. Due to the marked difference in molecular size between PEG 4000 and mannitol, a diminished P/M ratio also means a diminished permeability.

Contractility measurements *in vitro*

Running parallel with the permeability measurements, contractility was monitored by measuring the intraluminal pressure (mmHg) of each intestinal segment. At $t = 0$ min, a control period started by the application of saline was monitored. At $t = 15$ min, smooth muscle relaxation was induced in segments of controls and DSS-treated mice by adding a single dose of 50 µl of salbutamol (10^{-2} M) to the organ bath directly after buffer exchange. At $t = 30$ min, smooth muscle contraction was induced following the same procedure with a single dose of 50 µl of 5-HT_3 agonist (1-(3-chlorophenyl)-biguanide, 10^{-4} M). In an additional series of experiments, we tested contractility in the same way and at the same time-points with single doses of 50 µl of L-phenylephrine (3×10^{-4} M) and 50 µl of carbachol (10^{-1} M) to induce relaxation and contraction, respectively. After the experiment, the recordings of the intraluminal pressure were evaluated and intestinal contractility was expressed as the number of contractions per 15 min interval.

Macroscopy and histology

Upon sacrifice, the animal and the removed intestines were macroscopically examined. Signs of inflammation were scored in a blind fashion. The macroscopic score ranged from 0 to 12, which represents the sum of scores for diarrhoea (0 = solid stool consistency, 1 = semi-solid stool consistency, 2 = watery stools), bloody stool (0 = none, 1 = positive), appearance of the skin (0 = shining, 1 = dull, 2 = hair loss), locomotion (0 = lively, 1 = languid), colon colour (0 = pink, 1 = light red, 2 = dark red), distension (0 = none, 1 = distinctly), appearance of the colon (0 = normal, 1 = thickened) and weight gain (0 = > 5%, 1 = 0–5%, 2 = weight loss).

Histological analysis was performed with pieces of the ileum and of the proximal and distal colon. The pieces were fixed in a pH 7-buffered 3.6% formaldehyde solution (Lansberg-Rotterdam BV, Uden, The Netherlands) and embedded in paraffin wax. Sections cut at 5 µm thickness and stained with haematoxylin and eosin were examined under a light microscope using a 250 × magnification.

Statistical analysis

The data are presented as mean ± standard error of the mean. The statistical significance was determined by non-parametric methods. $p < 0.05$ was considered statistically significant.

Results

DSS-induced inflammation

After drinking a solution of 10% dextran sodium sulphate for 7 days, BALB/c mice developed clinical signs of colonic inflammation (Table 1). The overall macroscopic score in the DSS-treated mice was significantly increased compared with controls ($p < 0.001$). This was mainly reflected by the occurrence of bloody stools, diarrhoea and weight loss. Bloody stools appeared in 83% of the DSS-exposed animals versus none of the controls and diarrhoea was present in 92% of the DSS-treated mice. In addition, during DSS exposure animals lost a considerable amount of weight, while controls slightly gained weight.

The degree of inflammation reflected by histology showed regional differences between the intestinal segments. The ileum was not affected by DSS, whereas aborally an increasing damage in inflamed colonic segments was observed.

Intestinal contractility

Recording of the intestinal motility resulted in expected contractility patterns in the control and inflamed segments of the small intestine and colon during pharmacological exposure (tracings not shown). Smooth muscle contractility was measured as the intraluminal pressure, while the agents were added at the serosal side of the intestinal segments to influence smooth muscle activity. Relaxation was registered as a pressure decline, and contraction as a pressure rise. In the intestinal segments of both control and DSS-treated mice, final concentrations of 3×10^{-6} M L-phenylephrine attenuated the smooth

Table 1. Macroscopic degree of inflammation in DSS-induced colitis in mice

Group	*n*	Macroscopic score (mean ± SEM)	Bloody stools	Diarrhoea	% weight change (mean ± SEM)
Control	12	1.6 ± 0.3	0/12	1/12	+ 1.4 ± 0.6
DSS	12	6.5 ± 0.4*	10/12*	11/12*	− 13.6 ± 1.8*

muscle contractility, while addition of 10^{-3} M carbachol induced strong contractions.

In an additional experiment, 10^{-4} M salbutamol and 10^{-6} M 1-(3-chlorophenyl)-biguanide were used with the same purpose. Salbutamol suppressed smooth muscle contractility whereas biguanide induced contractions (Table 2).

The exact amount of circular smooth muscle relaxation or constriction could not be derived from the pressure transducers. The intestinal activity of each segment, however, could be expressed as the number of spontaneous contractions during pharmacological exposure (Figs. 1 and 2, top panels). In the ileum, which served as the ultimate control, the frequency of contraction was low compared with the proximal and distal colon segments. An increased contractility was found in the presence of either carbachol or biguanide. No change in the frequency of contraction was observed in the inflamed colon compared with control segments during any pharmacological exposure. In the proximal colon of control mice, phenylephrine significantly reduced the number of contractions compared with the saline exposed time interval. Subsequently, carbachol exposure significantly increased the number of smooth muscle contractions.

Intestinal permeability

Simultaneously with contractility, the *ex vivo* permeability was measured from the luminal side towards serosal side of the intestinal wall with ^{14}C-PEG 4000 and ^{3}H-mannitol molecules. To reach the serosal side of the intestinal wall, both markers had to cross the epithelium, the lamina propria, and also the adjacent circular and longitudinal muscle layers of the muscularis externa and the serosa. Transmural permeation is expressed as the P/M ratio of the large PEG 4000 and the small mannitol molecule (Figs. 1 and 2, bottom panels).

In the ileum, exposure to phenylephrine, carbachol, salbutamol and biguanide have no effects on the P/M ratios both in controls and DSS-exposed mice. The P/M ratio of the ileum segments, however, was higher compared with the proximal and distal colon segments as a result of a greater transport of mannitol in the colon.

In the inflamed distal colon, permeability was significantly decreased after both phenylephrine-induced relaxation and carbachol-induced contraction (Fig. 2, right bottom panel), compared with saline-exposed interval and control measurements (Fig. 3).

Discussion

Currently, several animal models are used in inflammatory bowel disease research. In 1990, Okayasu *et al*.[20] developed a method to induce colitis in mice by the administration of DSS (MW 54 kDa) via their drinking water. The DSS model used in our study was adapted from this method. We used a DSS variant with a higher molecular mass (500 kDa) and observed comparable macroscopic and histologic effects. DSS colitis is easily induced and the DSS model is known as a reproducible method in which colitis appears with changes corresponding well to those of human ulcerative colitis[20,26]. In our hands, administration of 10% DSS to BALB/c mice during 7 days induced a mild colitis. This was mainly reflected by the appearance of bloody stools, diarrhoea and weight loss. Histologic examination of the intestinal tissue showed a focal and superficial inflammation. An influx of inflammatory cells in the lamina propria and disturbed crypt architecture was observed in the colon, while the ileum was histologically not affected by DSS exposure.

In general, alterations in intestinal permeability are studied in flat tissue sheets mounted in Using chambers[27] or with *in vivo* perfusion models,[28] while alterations in contractility are mostly examined with tissue strips vertically mounted in organ baths.[22,29] In the *ex vivo* method used for our study, permeability was monitored transmurally from the luminal towards the serosal side. Transmural permeability has

Table 2. Overview of contractile activities and number of movements of colonic segments induced by pharmacological intervention

	Salbutamol (β_2)	Phenylephrine (α_1)	Carbachol (muscarine)	Biguanide (5-HT$_3$)
Contractility	Relaxation	Relaxation	Contraction	Contraction
Movements	Decrease	Decrease	+	No effect/increase

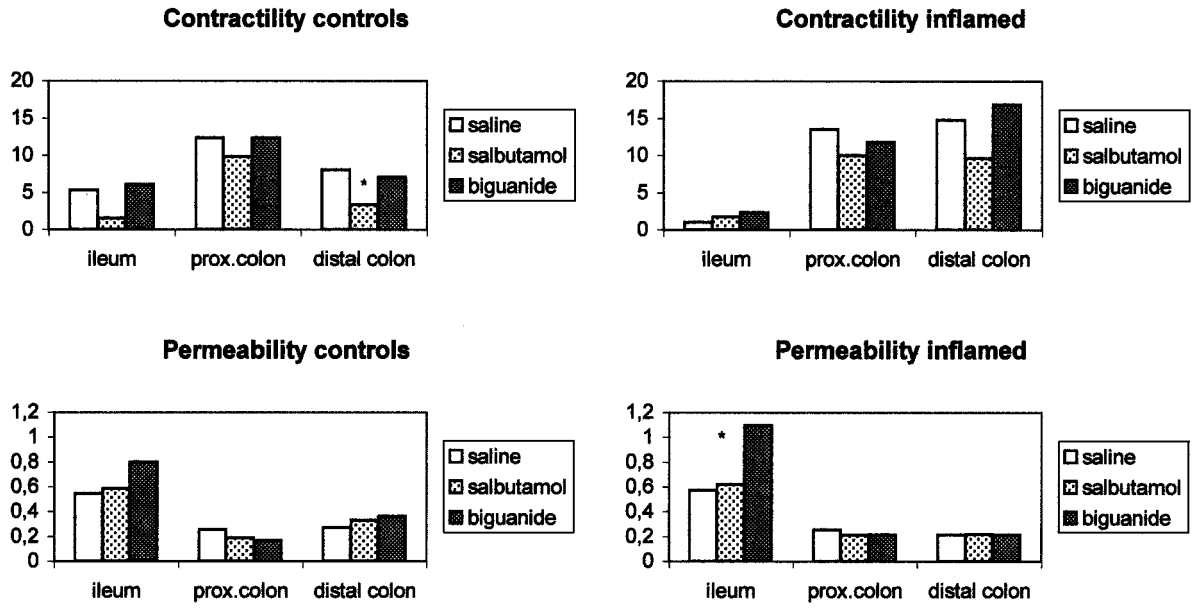

FIG. 1. Mean effects of salbutamol (10^{-4} M) and biguanide (10^{-6} M) on contractility measured as the number of movements in intestinal segments from the ileum, proximal colon and distal colon in control mice ($n = 6$; top left panel) and DSS-induced inflammation ($n = 6$; top right panel), and on permeability determined by the PEG 4000/mannitol ratio in the same intestinal segments of control mice (bottom left panel) and DSS-induced inflammation (bottom right panel). * $p < 0.05$ versus saline.

been described in the isolated everted sac model.[30,31] Intestinal loops are everted and filled with fluid. In this method, the mucosal to serosal transport of permeability markers is measured to assess changes in the intestinal barrier function. In our model the intestinal segments are not everted, but directly filled with buffer containing the marker molecules. The large PEG 4000 molecule was used to mimic the macromolecular permeability of dietary antigens and bacterial products. Seidman *et al*.[32] have shown that PEG 4000 is a suitable permeability marker, which is neither degraded by intestinal bacteria nor metabolised after absorption in the intestine. PEG 4000 and mannitol are both used as paracellular permeability probes.[33,34] Large molecules like PEG 4000 are thought to diffuse via large pores at the crypt membrane, while the permeation pathway of small molecules can be both via small pores at the villus

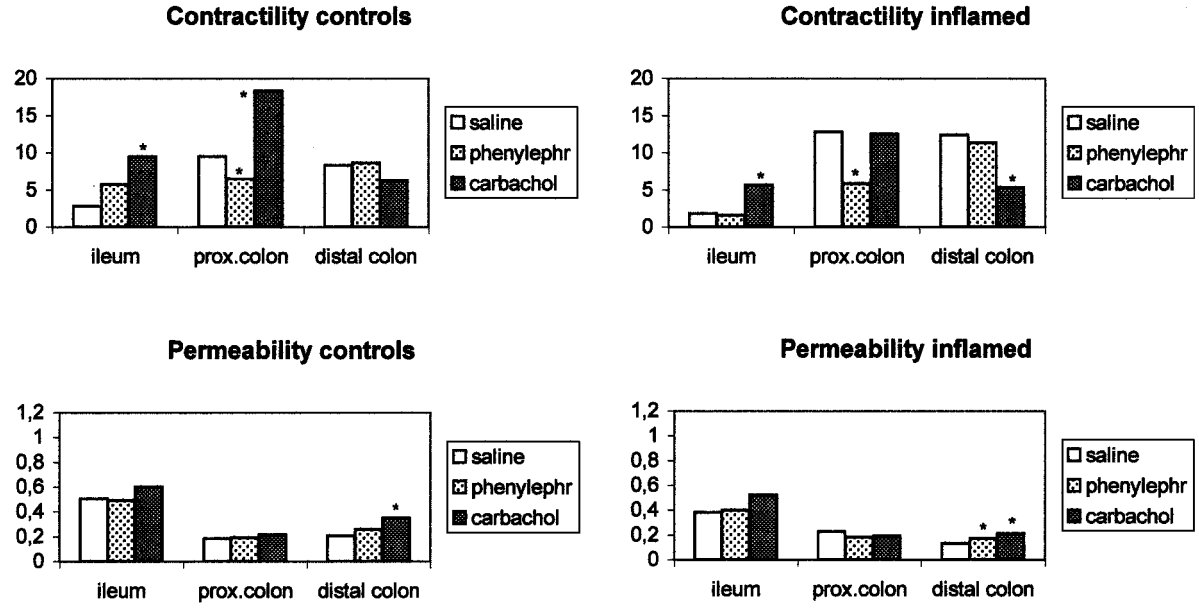

FIG. 2. Mean effects of phenylephrine (3×10^{-6} M) and carbachol (10^{-3} M) on contractility measured as the number of movements in intestinal segments from the ileum, proximal colon and distal colon in control mice ($n = 6$; top left panel) and DSS-induced inflammation ($n = 6$; top right panel), and on permeability determined by the PEG 4000/mannitol ratio in the same intestinal segments of control mice (bottom left panel) and DSS-induced inflammation (bottom right panel). * $p < 0.05$ versus saline.

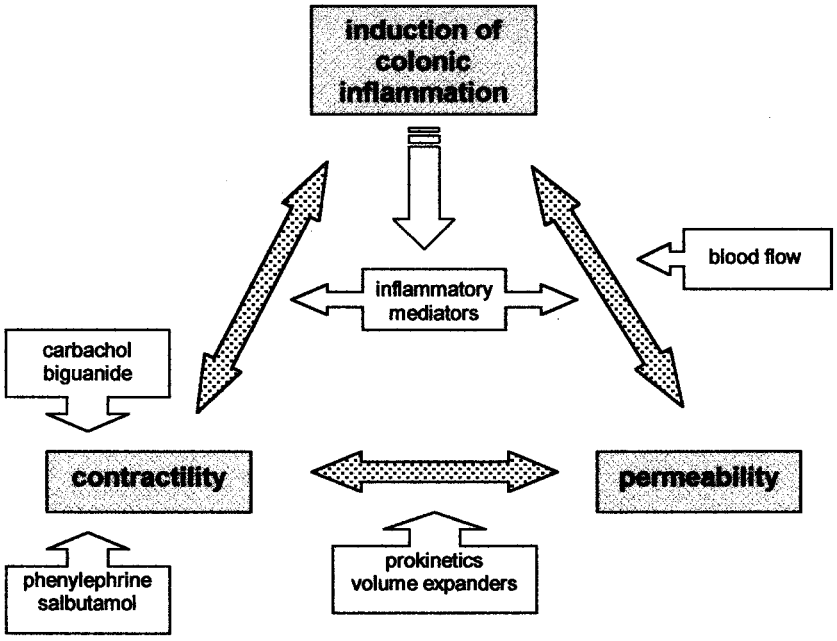

FIG. 3. Suggested interplay between colonic inflammation, contractile activity and permeability.

top or also at the larger pores of the crypt membrane.[13,15] Thus, inflammatory damage of the intestinal barrier will result in an altered P/M ratio.[14]

In the present study, a significantly decreased P/M ratio was demonstrated in the inflamed distal colon compared with control. In the less inflamed proximal segment, permeability was not altered. Pharmacological-induced relaxation and contraction of smooth muscles had minor effects on permeability. Only a significant increase in permeability was observed after application of carbachol to both inflamed and control distal colon segments. In earlier studies we found an aboral increase of longitudinal smooth muscle contraction induced by carbachol in non-inflamed colon, whereas this was abolished in inflamed colonic tissue.[22] In the experimental set-up of the herewith-used model, smooth muscle activity is mainly resulting from the circular instead of the longitudinal intestinal muscle layer.[22] Although for all compounds used a clear relaxation or contraction was observed through registration of the intraluminal pressure, the frequency of contraction was less influenced by these agonists. In general, from our observations it could be concluded that pharmacologically induced reduction in motility, reflected by the number of movements, circular and longitudinal smooth muscle relaxations, does not directly alter intestinal permeability. On the other hand, the build-up pressure by constrictive agonists such as carbachol and biguanide slightly increased permeability in distal colon segments.

Intestinal permeability is not only influenced by the epithelial barrier, but also by the endothelial barrier.[35] In our experimental set-up the latter pathway is not involved, however, as blood flow in the isolated intestinal segment is absent. Recently, we investigated intestinal blood flow in BALB/c mice by means of radioactive microspheres.[36] Interestingly, there was a stepwise decline in the intestinal blood flow in control mice from the upper small intestine down to the distal colon. In DSS-induced inflammation, however, blood flow in the middle and distal colon was increased compared with control mice. Inflammatory mediators locally formed[19,21,23,24] could be cleared more rapidly by an increased blood flow, considering systemic effects will be negligible. Attributive to this self-regulating effect, the additional use of pro-kinetics or volume expanders could be beneficial to counteract the disturbed permeability and, in turn, could regulate the influx and activation of inflammatory cells and the subsequent production of proinflammatory mediators, possibly resulting in a recovered colitis.

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