Enteric neurones modulate the colonic permeability response to luminal bile acids in rat colon in vivo

Y Sun, B-M Fihn, H Sjövall, M Jodal

Background: The mechanisms behind microscopic colitis and exacerbations of ulcerative colitis are incompletely understood. It seems highly likely that both luminal antigens and bile are involved. The aim of this study was to test the hypothesis that bile acids increase colonic mucosal permeability by activating enteric neurones.

Method: The effect of 4 mM deoxycholic acid (DCA) on the appearance rate of intravenously administered \(^{3}\)H-mannitol and \(^{14}\)C-urea into the lumen of the proximal and distal rat colon was measured in vivo and expressed as clearance. The nerve blocking agents atropine and hexamethonium were given intravenously, and lidocaine was applied onto the serosal surface of the colon, before and after DCA exposure.

Results: DCA markedly increased clearance of the permeability probes into the lumen in both colonic segments and also the ratio of mannitol/urea clearance, particularly in the distal colon. Pretreatment with atropine, hexamethonium, and lidocaine significantly inhibited the increase in clearance by approximately 65–80% but did not affect the clearance ratio. In the distal colon, the inhibitory effect of lidocaine was not statistically significant. Also, administration of atropine and hexamethonium after DCA exposure significantly inhibited the DCA effect on clearance of the probes.

Conclusion: The results suggest that in vivo, the permeability increase induced by a moderate concentration of bile acid is to a large extent mediated by a neural mechanism involving muscarinic and nicotinic receptors. This mechanism may be a link between the central nervous system and colonic mucosal barrier function, and may be a new target for treatment.

Bile acid malabsorption is a relatively common cause of diarrhoea of unknown origin in Western countries. In some patients, colonic biopsies are normal and in others, either a thickened subepithelial collagen layer or increased lymphocytic infiltration of the surface epithelium is seen. The mechanism through which moderately elevated concentrations of bile acids induce diarrhoea are largely unknown. In animal studies in vivo, moderate to high concentrations of deconjugated bile acids, particularly deoxycholic acid (DCA), damage the epithelium, reduce absorption and/or stimulate secretion, increase epithelial permeability, and enhance mucus secretion. The epithelial damage and thus the increased epithelial permeability has been attributed to the detergent properties of the bile acids. The lesions seem mainly to be localised to the colonic surface epithelium, implying a predominant effect on absorptive epithelial function. In vitro observations also indicate that activation of mucosal mast cells may be responsible for at least part of the tissue lesions.

Studies from our laboratory have previously shown that in the small intestine the major part of the net fluid secretion elicited by DCA was induced via an intramural nervous reflex within the enteric nervous system (ENS). Other mechanisms—for example, inhibited absorption and passive leakage through the damaged part of the epithelium—probably also contribute. In the same segment, we also found that the marked increase in intestinal permeability induced by DCA was mainly neurally mediated; only a minor part was due to a direct effect of bile acids on epithelial cells.

In general terms, the organisation of the ENS in the colon is similar to that in the small intestine, and studies in vitro also indicate similar function. However, in vivo, and in contrast with the findings in the small intestine, we recently found no evidence for involvement of the ENS in the weak antiabsorptive response elicited by 4 mM DCA in the rat colon. In contrast, in the same study, we observed that the reduction in transmucosal potential difference (PD) induced by DCA was attenuated by hexamethonium (nicotinic receptor antagonist), an effect that did not seem to be mediated by any change in electrogenic ion transport. This observation suggested to us that the decrease in PD in vivo may instead reflect a bile induced permeability increase, a response that might, in such cases, be mediated in part by a hexamethonium sensitive neural mechanism. The concept of neural modulation of colonic permeability is also in agreement with the observation that stress, via a cholinergic mechanism, elicits increased para- and transcellular transport of macromolecules both in the small intestine and colon.

The aim of the present study was therefore to directly test the hypothesis that neural mechanisms contribute to the DCA induced increase in epithelial permeability in the rat colon. Colonic clearance of \(^{3}\)H-mannitol and \(^{14}\)C-urea was measured during exposure of the epithelium to 4 mM DCA, with or without administration of the nerve blocking agents atropine, hexamethonium, and lidocaine.

MATERIAL AND METHODS

Animals

Adult male Sprague-Dawley rats, weighing 240–450 g (Möllegard, Denmark) were used. Animals were kept under standardised environmental conditions (22°C, 60% humidity, artificial lightning 06.00 to 18.00 h) in the animal quarters.

Abbreviations: DCA, deoxycholic acid; ENS, enteric nervous system; NFT, net fluid transport; PD, potential difference
for at least seven days prior to the experiments. The experiments were approved by the animal ethics committee at Göteborg University.

Operative procedures
The general setup and operative procedures have been described previously. Briefly, rats were fasted overnight with free access to water before the experiments. Anaesthesia was induced by intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight) and a cannula was placed in the trachea to ensure free airways. The right femoral vein was cannulated for administration of drugs. Systemic mean arterial pressure was continuously recorded by means of a pressure transducer (DPT-600 Single-Use Transducer; Peter von Berg Medizintechnik GmbH, Eglharting, Germany) via a T tube connected to the femoral artery. The T tube was also used to maintain anaesthesia by continuous infusion of chloralose (3.6 mg/ml, 0.02 ml/min) given in a solution used to maintain anaesthesia by continuous infusion of NaCl to prevent dehydration and acidosis during and after surgery. Body temperature of the rat was maintained between 37 and 38˚C using radiant heat from overhead lamps and a heated dissection table.

After tracheotomy, an abdominal midline incision was performed and the colon was isolated with intact vascular and nervous supplies. Orally, the colon was divided at the border to the caecum and aborally, as far down towards the rectum as possible. The colon was divided into two segments of approximately equal size, just orally to the artery that supplies the distal segment. Colonic segments were then flushed with body warm normal saline. The kidneys were extirpated in order to keep plasma concentrations of permeability probes (see below) as constant as possible.

Measurement of net fluid transport
In separate experiments, the effect of atropine and DCA on net fluid transport (NFT) was measured using a volumetric method. At the beginning of each registration period, a known amount of Krebs solution (0.5–0.8 ml) was added to each segment and after one hour the segments were emptied with air. The volumes of the solutions added to and collected from the colonic segments were measured by administering the solution from preweighed syringes and sampling in preweighed tubes.

Measurement of colonic permeability
The two ends of the colonic segments were connected to a perfusion system of silicon tubings. The perfusion system to each of the segments was non-recirculating and contained a roller pump (Ismatec mini-micro 2/6, Zürich, Switzerland) and a reservoir with two compartments. The perfusate was a modified Krebs’ Henseleit solution containing (mM) NaCl 122, KCl 3.5, NaHCO3 25, and KH2PO4 1.2, and was pumped (0.5 ml/min) from one of the compartments through the segment to the other compartment. During perfusion, segments were placed on the abdominal wall and covered with gauze and a thin plastic film.

The two probes 14C-urea (4 µCi, 70 nmol/l) and 3H-mannitol (20 µCi, 360 nmol/l) (NEN Life Science Products, Zaventem, Belgium) were given intravenously and allowed to equilibrate for one hour. Arterial plasma concentrations of the probes were determined three times during the experiment by measurement of 100 µl plasma samples in duplicate and a linear regression curve was calculated to estimate the plasma concentration at any time. Perfusate samples of 3 ml were obtained every 20 minutes from the perfusate after passage through the colon segment, and 9 ml of scintillation fluid (Ultima Gold XR, Packard, Campbell, USA) were added to each sample. The probes were counted in a Packard liquid scintillation analyser (1900 TR) to a standard deviation of 1%. Plasma clearance was expressed as µl(min/g) and was calculated from the appearance of the probes in the perfusate using the equation:

\[ \text{Clearance} = \frac{(C_{\text{per}} \times r)}{(C_{\text{pl}} \times g)} \]

where C is the concentration (dpm/ml) of the perfusate (per) and of plasma (pl), r is the perfusion rate (µl/min), and g is the intestinal wet weight in grams (1 g colon tissue = 8.14 (0.27) and 10.56 (0.42) cm² in the proximal and distal colon, respectively).

Experimental protocol
The same time protocol was followed in most experiments: after one hour of control recording, saline or drug was administered (intervention point), and after another hour, DCA (4 mM) was administered intraluminally. At the intervention point, saline, hexamethonium, atropine, or lidocaine was administered. Saline and atropine 1 ml/kg and 0.25 mg/kg, respectively, were given intravenously. Hexamethonium (10 mg/kg intravenously) was given every 45 minutes to compensate for biological clearance of the drug. Lidocaine was administered serosally by dropping it onto the serosal surface of the segment at a dose of 0.5 mg/10 cm every 10 minutes. In one experimental series, first atropine and then hexamethonium, given as above, were administered after the colon segments had been exposed to 4 mM DCA for one hour.

Drugs
The following drugs were used: atropine, hexamethonium, and DCA from Sigma Chemical Co (St Louis, Missouri, USA), and lidocaine from Astrazeneca Company (Sweden).

Statistics
All values are reported as mean (SEM) and statistical comparisons were made using Wilcoxon’s one sample (or matched pairs) test between different periods in the same group or the Mann-Whitney test (Wilcoxon’s two sample test) for different groups. Values of p<0.05 were considered statistically significant.

RESULTS
Effects of DCA in the absence of nerve blockers
DCA markedly increased the colonic plasma clearance of mannitol and urea in both the proximal and distal segments (fig 1). DCA also increased the clearance ratio of mannitol/urea in both the proximal (from 0.35 (0.04) to 0.48 (0.05); p<0.05) and distal (from 0.26 (0.04) to 0.65 (0.05); p<0.05) colon, the value in the proximal colon being significantly less (p<0.05). In control experiments (no DCA administration), clearances of mannitol and urea as well as the clearance ratio of mannitol/urea in both the proximal and distal segments were constant during the experimental period (fig 1). Mean arterial blood pressure remained stable between 95 and 125 mm Hg during these experiments.

Effects of nerve blockers before DCA
Hexamethonium rapidly reduced mean systemic blood pressure to 70–80 mm Hg but blood pressure then started to rise, eventually stabilising at approximately 80–90 mm Hg before the next dose was given. DCA, atropine, and lidocaine did not affect systemic pressure.

Clearance of mannitol and urea as well as the ratio of mannitol/urea were slightly but significantly decreased by hexamethonium in the proximal colon while corresponding values in the distal colon were only close to statistical significance. In contrast, atropine and lidocaine had no
significant effects on clearance of mannitol and urea in either the proximal or distal segments (figs 2–4).

Effects of DCA after nerve blockade
DCA administered after atropine, hexamethonium, and lidocaine still caused a significantly increase in clearance of mannitol and urea in both the proximal and distal segments. However, the increase in clearance was significantly smaller compared with the effect of DCA per se—that is, reduced to approximately 20% and 33% of mannitol and urea in the proximal and distal segments. After lidocaine, DCA induced a larger increase in clearance of mannitol and urea in the distal colon, respectively. After lidocaine, DCA induced a larger increase in the clearance of mannitol and urea in the distal than in the proximal segment (p<0.05).

Pooling the clearance ratio values for the proximal and distal colon after DCA plus nerve blockers gave ratios of 0.39 (0.02) and 0.62 (0.05) in the proximal and distal colon, respectively (p<0.01). However, there was no significant difference compared with the effect of DCA alone in the respective colon segments.

Effects of nerve blockers after bile acid
In one series of experiments, the nerve blockers were given after DCA. The results showed that atropine in this situation significantly decreased clearance of mannitol and urea in both the proximal and distal colon (fig 5). Compared with the effect of DCA per se, the clearance values were reduced to approximately 45% of mannitol and urea clearance in the proximal and 69% and 71% of mannitol and urea clearance in the distal colon, respectively. Hexamethonium, given after atropine, had no further effects on clearance in either segment (fig 5). The clearance ratio of mannitol/urea was not affected by the two nerve blockers. Compared with the effect of atropine plus DCA (fig 3), clearance of mannitol and urea in the distal segment was significantly increased.
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DCA from fig 1 are included. Values are mean (SEM), n = 7. *p < 0.05 compared with saline+DCA values.

Figure 3 Effect of pretreatment with atropine (atr) (intravenously) on the deoxycholic acid (DCA) effect on clearance of mannitol and urea in the proximal (A) and distal (B) colon. Values for the effect on clearance of DCA from fig 1 are included. Values are mean (SEM), n = 7. *p < 0.05 compared with saline+DCA values.

Effect of pretreatment with atropine (atr) (intravenously) on the deoxycholic acid (DCA) effect on clearance of mannitol and urea in the proximal (A) and distal (B) colon. Values for the effect on clearance of DCA from fig 1 are included. Values are mean (SEM), n = 7. *p < 0.05 compared with the previous period; †p < 0.05 compared with saline+DCA values.

Figure 4 Effect of serosal lidocaine (lido) on the deoxycholic acid (DCA) effect on clearance of mannitol and urea in the proximal (A) and distal (B) colon. Values for the effect on clearance of DCA from fig 1 are included. Values are mean (SEM), n = 7. *p < 0.05 compared with the previous period; †p < 0.05 compared with saline+DCA values.

Net fluid transport

In a previous study,15 we showed that DCA slightly reduced net fluid absorption in the proximal colon but had no significant effect on NFT in the distal colon (table 1). Furthermore, hexamethonium per se increased net absorption in the proximal colon and turned net fluid secretion into net fluid absorption in the distal colon. Pretreatment with hexamethonium did not change the DCA effect on NFT (table 1). In the present study, atropine per se did not change NFT in the proximal or in the distal colon (proximal colon: control +72 (8); atropine +74 (7); distal colon: control −20 (5); atropine −13 (5) µl/min/100 cm²; negative values indicate secretion). During atropine blockade (table 1), DCA in the proximal colon significantly reduced fluid absorption from 74 (7) to 41 (9) µl/min/100 cm²). NFT in the distal colon was unaffected (atropine −13 (5), atropine+DCA −13 (9) µl/min/100 cm²). Using the fluid values from the two studies we found no significant relationship between clearances of mannitol and urea and NFT. This was the case for both the proximal and distal segments in the control, DCA, atropine, and hexamethonium groups.

DISCUSSION

The results of the present study showed that DCA markedly increased the plasma clearance of mannitol and urea, and their clearance ratio, in both the proximal and distal colon, implying a marked increase in epithelial permeability. Similar results have been reported in other in vivo studies.6 8–22 Simultaneously, DCA also induced moderate morphological changes. Our own observations17 as well as those of others5–7 22 23 showed that the lesions induced by DCA in concentrations of 3–5 mM are confined to the surface epithelium, without causing disruption of the epithelial lining. This indicates that the increased permeability is primarily localised to the paracellular pathway and tight junctions. The same conclusion has also been made from in vitro observations24 25 and is supported by the observed reversibility of the effects.21

DCA increased the clearance ratio of mannitol/urea to 0.48 (0.05) and 0.65 (0.05) in proximal and distal colon. These changes in ratios cannot be due to changes in mucosal capillary blood flow as the transport of the probes into the lumen are restricted by the epithelial tight junctions and not by the pores in the capillary membrane. The theoretical ratio of the diffusion constants of the two probes in free water is 0.55–0.60. Clearance values in the presence of DCA in the present study were found to be of a similar order of magnitude in the distal colon and somewhat less in the proximal part of the colon. This indicates that the paracellular pathway allows free diffusion in the distal colon and somewhat restricted diffusion in the proximal segment. Taken together, these observations indicate that the permeability of the tight junctions corresponds to cylindrical pores.
with a radius of 35–50 Å and 25–35 Å in the distal and proximal colon, respectively. The value in the distal colon is similar to values seen in the small intestine. In a previous study, we found that 4 mM DCA had no effect on fluid transport in vivo may therefore to inhibition of absorption due to a direct effect on the surface epithelium.

Taken together with previous work, our in vivo results show that 4 mM DCA, a concentration that can be found in some pathophysiological situations in the human colon, inhibits active absorption and increases colonic motility and mucus secretion but does not stimulate active fluid secretion. Thus the prominent flushing and diluting mechanism seen in the small intestine does not seem to be present in the colon.

Pretreatment of animals with atropine and hexamethonium before administration of DCA markedly inhibited clearance of the two probes in both the proximal and distal colon. Inhibition was of the same order of magnitude in both colonic segments. Theoretically, this effect could be explained by a decrease in solvent drag of the probes through the epithelium due to inhibited fluid secretion. However, neither atropine nor hexamethonium affected DCA induced NFT, as discussed above. This strongly indicates that a large part of the increased clearance values of the permeability probes were due to enhanced epithelial permeability elicited by a nervous reflex containing both nicotinic and muscarinic receptors. Furthermore, as hexamethonium per se only induced a small decrease in clearance of the two probes and neither atropine nor lidocaine alone had any effect on clearance, there is no evidence for any nervous "tone" regulating basic epithelial permeability in the rat colon, at least not under our experimental conditions. Moreover, the clearance ratios were not affected by the nerve blockers which suggests that the nerve mechanism changes the number but not the size of the pores in the epithelium. A similar observation has been made in the small intestine but with the difference that atropine was not inhibiting the DCA effect.

As concluded above, there is strong evidence to suggest that the increased permeability is caused by changes in tight junctions. Furthermore, the blood to lumen technique used in this study in the control situation in principal only reflects the permeability of the crypt epithelium. This strongly suggests that the main increase in permeability due to luminal DCA is at the surface epithelium—that is, where the epithelial lesions are located and where no secretory processes are localised. However, as increased permeability seems to be due to both a direct effect of DCA and a nervous mechanism, location of the enhanced permeability to the crypt region is possible.

Applying lidocaine onto the serosal surface of the small intestine only anaesthetises the outer myenteric plexus of the ENS and the external innervation. In rat colon, the longitudinal muscle layer has a similar thickness (Jodal, unpublished observation)—that is, serosally applied lidocaine can be expected to mainly affect the myenteric plexus also in the colon. Accordingly, lidocaine also inhibited the

Table 1 Changes in net fluid transport (ΔNFT) induced by 4 mM deoxycholic acid (DCA) in the proximal and distal colon after pretreatment of animals with saline (control), atropine, or hexamethonium (Hx)

<table>
<thead>
<tr>
<th>ΔNFT</th>
<th>Control-DCA</th>
<th>Atropine-DCA</th>
<th>Hx-DCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal colon</td>
<td>-50 (17) (8)*</td>
<td>-33 (9) (6)*</td>
<td>-35 (17) (6)*</td>
</tr>
<tr>
<td>Distal colon</td>
<td>-45 (27) (8)</td>
<td>3 (8) (6)</td>
<td>-36 (21) (6)</td>
</tr>
</tbody>
</table>

NFT is expressed in µl/[min/100 cm²].
n values are given in parentheses. *p<0.05.
A minus sign indicates net fluid secretion.
The control and hexamethonium values are taken from Sun and colleagues.
DCA induced increase in clearance of both probes in the proximal segment to the same extent as hexamethonium and atropine while the effect in the distal colon was not significant. We therefore propose that the nerve reflex inducing the increased permeability may be differently organised in the two parts of the colon. In the proximal colon, the myenteric plexus plays a crucial role whereas in the distal colon an intact submucosal plexus seems to suffice for the permeability increasing neural mechanism to operate.

Finally, a brief comment on the potential clinical relevance of our key finding: that is, that the colonic permeability response to luminal agents, particularly in the distal colon, may be neurally regulated. Both microscopic colitis and ulcerative colitis have a highly unpredictable clinical course. In microscopic colitis, the disease may for example resolve for unknown reasons, and in ulcerative colitis we do not know what triggers exacerbations. Patients with ulcerative colitis also have a markedly increased mucosal permeability to for example, 35Cr-EDTA. If the mechanism described in the present study also exists in humans, it may well contribute to the variable clinical course of inflammatory bowel disease, including an impact on central nervous effects. If this mechanism increases permeability sufficiently to influence antigen load, it might be a potential target for anti-inflammatory therapy. Interestingly, luminal lidocaine has been reported to have a beneficial effect in distal ulcerative colitis, an effect that may be induced via a nervous influence on epithelial permeability. Thus our data encourage the search for mechanisms in humans.

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