Diminished nitroprusside-induced relaxation of inflamed colonic smooth muscle in mice

J. D. van Bergeijk, H. van Westreenen, P Adhien and F. J. Zijlstra

Department of Pharmacology, Faculty of Medicine and Health Sciences, Erasmus University, PO Box 1738, 3000 DR Rotterdam; Department of Gastroenterology, University Hospital Dijkzigt, Dr Molewaterplein 40, 3015 GD Rotterdam, The Netherlands

CA Corresponding Author
Tel: (+31) 10 408 7550 Fax: (+31) 10 436 6839 Email: zijlstra@farma.fgg.eur.nl

Introduction

The dextran sodium sulphate (DSS) induced colitis in mice is at present a well documented experimental model to study formation of inflammatory mediators and the use of therapeutics in ulcerative colitis (UC). This mild colitis is mainly characterized by infiltration of neutrophils, diarrhoea, rectal blood and loss of body weight. Besides the disease dependent release of lipid mediators and cytokines, enhanced colonic nitric oxide (NO) generation could influence the disease activity in UC and Crohn’s disease. Several investigators have demonstrated increased concentrations of NO in intestinal mucosa of patients with inflammatory bowel disease (IBD). Increased concentrations of NO could not only result in damage of epithelial cells and apoptosis, but also to mucosal vasodilatation, an increased vasopermeability and a decreased colonic motility. Therefore high mucosal levels of endogenous NO may mediate gut motor dysfunction and other clinical features as abdominal pain, malabsorption and granulomatous inflammation in IBD. As a result of downregulatory mechanisms, smooth muscle response to NO is inhibited by high endogenous NO production. Moreover reduction of NO synthase activity could lead to a reduced relaxation.

In this study we investigated the effect of NO from exogenous sodium nitroprusside (SNP) on both normal and inflamed murine colonic smooth muscle preparations.

Methods and Materials

Animals/tissues

The study protocol had been approved by the local Animal Ethical Committee (no. 118–97–01). Colonic smooth muscle preparations were obtained from BALB/c mice (female, 20–22 g, IFFA Credo, France). Colitis was induced by adding 10% (w/v) dextran sodium sulphate (MW > 500 000, Pharmacia, Sweden) to their drinking water, ad libitum for 8 days. Controls received tap water. After overnight fasting mice were killed by cervical dislocation. Immediately thereafter the colon was removed. Total length (approx 90 mm) was divided in proximal, middle and distal colonic segments.

Disease activity score

Upon sacrifice, the removed bowel was examined macroscopically. Signs of inflammation (diarrhoea, blood) and changes in tissue appearance (oedema, thickening) were scored (0–2). Pieces of proximal, middle and distal colon were taken for histological analysis. The sections were fixed in 3.6% buffered formalin, pH 7, and embedded in paraffin wax.
Sections cut at 5μm thickness and stained with haematoxylin and azafloxin were examined under a light microscope using a 400× magnification. The histology score obtained from each section (blinded scored including inflammation 0–3, damage/necrosis 0–3 and regeneration 0–3) ranged from 0 to 9 was combined with the macroscopic score (0–3) and used as the disease activity score (DAS; 0–12).

Organ bath

Whole longitudinal colonic smooth muscle preparations (including mucosa, circular layer and neuronal plexus) were mounted in 10 ml double-jacketed organ baths containing Krebs solution heated to 37°C and continuously gassed with 5% CO₂ in O₂. The contractile responses were measured isotonically using Penny & Giles transducers (pre-load 500 mg). Cumulative contraction-response curves were prepared by adding agonists in small volumes. Preparations were pre-contracted with 300 mM KCl, the optimum concentration found in blood vessel organ bath observations. After washing (three times bath fluid contents every 5 min), contraction was induced by the muscarinic receptor agonist carbachol (Sigma Chemicals) in doses of 10⁻⁸ to 10⁻³ M, reaching stable maximal smooth muscle contraction. Contraction was expressed as mg displacement. Without washing relaxation by SNP (Sigma Chemicals) in doses of 8.4·10⁻⁸ to 8.4·10⁻⁴ M was induced. Relaxation was expressed as percentage fall in contraction. After a second washing regime carbachol induced contraction (10⁻³ M) was antagonized by the β₂-receptor agonist salbutamol in doses of 10⁻⁸ to 10⁻³ M. Bath samples were taken for measurements of NO₂/NO₃ (NOₓ) and cyclic GMP (Biotrak-assay, Amersham, UK). Maximum (EC₅₀) and half-maximum (EC₅₀) contractions and relaxations were calculated after computer programmed plotting of dose–response curve fitting. Results are expressed as mean ± standard error. Statistical analysis included Wilcoxon’s test for unpaired samples (in case of DAS activities) and Student’s t-test (other observations).

Results

Inflammation

After macroscopic and microscopic evaluation we observed a mild to moderate disease activity in the DSS-induced colitis mice (DAS in proximal colon: 6.4 ± 1.57 and in middle colon: 6.3 ± 1.10). Distal colonic preparations tended to show a diminished DAS (4.4 ± 0.79), mainly due to the absence of thickening, oedema, faecal blood and diarrhoea. Histologically inflammation was limited to mucosa and submucosa (neutrophil infiltrations), without involving muscular layers.

Contractility

In normal tissues we observed an increased contraction after carbachol in aboral direction (Fig. 1: distal > middle > proximal colon). In inflammation this carbachol-induced contraction was markedly reduced in middle colonic segments. In the non-inflamed

![FIG. 1. Effect of carbachol induced contraction in mouse longitudinal colonic smooth muscle preparations in controls (left panel) and dextran sodium sulphate (DSS)-induced colitis (right panel). Contraction is expressed as mg displacement of the isotonic transducer. n=8.](image)
proximal colon SNP showed a much higher relaxation (mean $E_{\text{max}}$ 48%) than those observed in middle (mean $E_{\text{max}}$ 27%) and distal (mean $E_{\text{max}}$ 12%) colonic preparations, precontracted by carbachol. Nitroprusside-induced relaxation however was significantly reduced ($\geq 70\%$) in DSS-induced inflamed proximal preparations (mean $E_{\text{max}}$ 15%) and of the same magnitude as the middle (mean $E_{\text{max}}$ 22%) and distal (mean $E_{\text{max}}$ 9%) preparations which tended to decrease (Fig. 2). A similar pattern was observed after induction of salbutamol-evoked relaxations between controls and DSS preparations (Fig. 3), although the difference in relaxations between the proximal segments was less prominent.
The net total NO release after addition of SNP was determined as NO$_2$/NO$_3$. No regional nor control-DSS differences were found (Table 1).

### Cyclic GMP

In the bath fluids cGMP content was measured after SNP-evoked relaxation (Table 2). Levels measured in baths from DSS preparations were lower, although this was only significant in proximal preparations \( (P<0.05, \text{ Student's } t\text{-test}) \).

### Discussion

Normal longitudinal colon smooth muscle contraction induced by carbachol showed an aboral increase. A marked decrease in activity was observed in the middle segment of the inflamed colon. However, no correlation was found between severity of inflammation and disturbed contractility, as carbachol-induced effects in the more inflamed colon were less pronounced. We found an increased SNP-induced relaxation in normal proximal tissue, which was observed in the most inflamed, proximal colonic smooth muscle preparations. Normal and inflamed proximal segments converted SNP into NO, measured as NO$_x$. Cyclic GMP contents however were significantly decreased in the inflamed proximal colon bath fluid. This reduction in cGMP was correlated with the diminished SNP-induced relaxation.

In normal, non-inflamed rat colonic segments, Machara \textit{et al.}\textsuperscript{12} found no differences between NO-evoked release of cGMP in proximal and distal preparations, although relaxation in proximal colon was more prominent than in distal colon. This effect was attributed however to the release of vasoactive intestinal peptide (VIP), a mediator with dilative properties, which only was detected in distal colon.

Our study is the first in which contractility was investigated in inflamed colonic preparations from DSS-mice. Very recently it was shown that in longitudinal strips from trinitrobenzenesulphonic acid (TNBS)-treated ileum in guinea pigs there was a two-fold increase in maximal response induced by carbachol and histamine without modification of the EC$_{50}$ values.\textsuperscript{13} This observation is in contrast with our present findings. This could be due to a different model of inflammation, as TNBS is a direct toxin to mucosal epithelium and DSS-induced inflammation is very likely triggered by changes in intestinal macrophage function and bacterial flora.\textsuperscript{14} In our model smooth muscles were not visibly altered. The marked neutrophil infiltration could contribute to an enhanced production of NO, which in turn will lead to a diminished smooth muscle contraction. Furthermore Martinolle \textit{et al.}\textsuperscript{13} used the ileum from guinea pigs, and literature on NO production in both species and ileum is not available yet. Nitric oxide synthetase activity however was five-fold increased in mucosa taken from patients with ulcerative colitis, but unchanged in those from Crohn's disease.\textsuperscript{15} This indicates that cell-specific generation of inflammatory mediators or local susceptibility of smooth muscles will determine the outcome of NO production and contractility.

In controls the salbutamol induced relaxation was similar in all preparations. This indicates the presence of a normal second messenger system, which only was affected in inflamed proximal segments. After SNP application a significant decrease in cGMP levels

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**Table 1. NO$_x$ release in organ baths (µM) after carbachol (10$^{-3}$ M) and nitroprusside (8.4 10$^{-4}$ M) application (n=4)**

<table>
<thead>
<tr>
<th></th>
<th>Resting</th>
<th>Carbachol</th>
<th>Carbachol + NP</th>
</tr>
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<tbody>
<tr>
<td>Proximal</td>
<td>174 ± 9</td>
<td>184 ± 10</td>
<td>860 ± 39</td>
</tr>
<tr>
<td>Middle</td>
<td>168 ± 12</td>
<td>135 ± 33</td>
<td>838 ± 89</td>
</tr>
<tr>
<td>Distal</td>
<td>171 ± 7</td>
<td>152 ± 17</td>
<td>770 ± 65</td>
</tr>
<tr>
<td>DSS-colitis</td>
<td>171 ± 15</td>
<td>166 ± 13</td>
<td>802 ± 65</td>
</tr>
<tr>
<td></td>
<td>170 ± 17</td>
<td>155 ± 7</td>
<td>712 ± 46</td>
</tr>
<tr>
<td></td>
<td>170 ± 10</td>
<td>159 ± 5</td>
<td>665 ± 55</td>
</tr>
</tbody>
</table>

**Table 2. cGMP organ bath levels (nM) after carbachol (10$^{-3}$ M) and nitroprusside (8.4 10$^{-4}$ M) application (n=4)**

<table>
<thead>
<tr>
<th></th>
<th>Resting</th>
<th>Carbachol</th>
<th>Carbachol + NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td>24 ± 12</td>
<td>157 ± 49</td>
<td>609 ± 219</td>
</tr>
<tr>
<td>Middle</td>
<td>8 ± 7</td>
<td>48 ± 3</td>
<td>98 ± 43</td>
</tr>
<tr>
<td>Distal</td>
<td>19 ± 11</td>
<td>99 ± 17</td>
<td>188 ± 55</td>
</tr>
<tr>
<td>DSS-colitis</td>
<td>4 ± 4</td>
<td>100 ± 33</td>
<td>138 ± 33*</td>
</tr>
<tr>
<td></td>
<td>3 ± 3</td>
<td>32 ± 7</td>
<td>42 ± 14</td>
</tr>
<tr>
<td></td>
<td>9 ± 6</td>
<td>68 ± 18</td>
<td>93 ± 25</td>
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</tbody>
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*\(P < 0.05\) DSS vs. controls.
was found. During chronic inflammation of the colon the ongoing production of NO could diminish guanylate cyclase activity resulting in a decreased relaxation.

It is also possible that carbachol-evoked cGMP release will influence the threshold necessary to obtain relaxation after pre-contraction with carbachol. Recently it was shown that the nerve stimulation-induced muscarinic NO release in the guinea pig likely is mediated by M₁ muscarinic receptor activation. COMPARE WITH OBSERVATIONS IN LUNG DISEASES, negative feedback by M₂ muscarinic receptor activation could be depleted in inflamed colonic tissue. In colitis this mechanism of action needs further investigation.

References

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