Relaxation of distal colonic circular smooth muscle by nitric oxide derived from human leucocytes

S J Middleton, M Shorthouse, J O Hunter

Abstract
The role of nitric oxide (NO) as a mediator of colonic circular smooth muscle relaxation by human leucocytes was investigated. Granulocytes and mononuclear cells were obtained by gradient centrifugation of venous blood from healthy volunteers. Both cell types relaxed precontracted distal colonic circular smooth muscle in a concentration dependent manner. Muscle relaxation was inhibited by preincubation of cells with NG-monomethyl-L-arginine (100 μM) but not by preincubation with Nω-monomethyl-D-arginine (100 μM). Muscle relaxation by cells was reduced by 200 nM oxyhaemoglobin and 10 μM methylene blue but was increased by 60 units/ml superoxide dismutase. Non-viable cells did not produce muscle relaxation. Activation of mononuclear cells by incubation with 100 nM FMet-Leu-Phe increased muscle relaxation, whereas activation of granulocytes did not. Granulocytes and mononuclear cells relax precontracted distal colonic circular smooth muscle in vitro by the release of NO that may contribute to motility disorders of the gut associated with inflammation.

(Gut 1993; 34: 814–817)

PREPARATION OF TISSUE
Male Wistar rats weighing 250–400 g were killed and strips of colonic circular smooth muscle were attached to isotonic transducers (Harvard, Kent, England) in 2 ml organ baths and perfused by oxygenated (95% O2, 5% CO2) Krebs Henseleit solution, composition: (mM/l) NaCl 118; KCl 4-69; MgSO4 1-13; CaCl2 2-56; NaHCO3 25; NaHPO4 1-15; glucose 5-5. This had a pH of 7-4 to 7-6 at 37°C. Muscle strips were mounted with the longitudinal axis parallel to the direction of the circular muscle bundles. Temperature was regulated and pH monitored intermittently. Muscle strips were maintained under a tension of 3 g, which produced near optimal contraction and experiments were commenced after a stabilisation period of two hours, which was found necessary in preliminary studies to ensure consistent muscle performance. Muscle strips were precontracted by 10 μM acetylcholine and the mean amplitude of steady state contractions was measured for two minutes before and after the addition of leucocytes.

CELL PREPARATION
Venous blood from healthy human volunteers aged between 18 and 70 was collected with EDTA or glass shot beads (Scientific Furnishings, Macclesfield, England) to remove platelets
by defibrination. Blood (5 ml) was layered above an 8 ml bilayer of hypaque 1017 and 1119 in equal volumes and centrifuged at 700 g for 25 minutes. Mononuclear cells (macrophages and lymphocytes with or without platelets) and granulocytes (neutrophils, basophils, and eosinophils) were aspirated from two distinct layers. Leucocytes were washed twice in 10 ml Krebs Henseleit solution (37°C) containing 100 nM/1 indomethacin (KHI) to inhibit prostaglandin synthesis, centrifuged at 200 g for 10 minutes, and resuspended in 5 ml KHI. Leucocyte suspensions were accepted if red cell corpuscles were <5% of total and viability was >95% as judged by a trypsin blue exclusion test (Sigma Chemicals Ltd, England). This test was used to ensure a 100% death rate of cells frozen in liquid nitrogen. Cell suspensions were centrifuged at 200 g for 10 minutes and the pellet of cells added to the organ bath in the bathing fluid of which was used to resuspend them for transfer. Experimental controls consisted of a similar procedure without the presence of cells.

**TABLE 1** Substances with known effects on the NO-eGMP pathway affected relaxation of precontracted distal colonic circular smooth muscle by granulocytes

<table>
<thead>
<tr>
<th>Cells 10^8</th>
<th>Mean muscle relaxation (SEM) (%)</th>
<th>No of samples (pairs)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable</td>
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<tr>
<td>Non-viable</td>
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<tr>
<td>Control</td>
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<tr>
<td>Oxyhaemoglobin</td>
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<tr>
<td>N^2-monomethyl-D-arginine</td>
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<tr>
<td>N^2-monomethyl-L-arginine</td>
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<td>Control</td>
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<tr>
<td>Methylene blue</td>
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<td>Control</td>
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<td></td>
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<tr>
<td>Superoxide dismutase</td>
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</tbody>
</table>

Mean relaxations are compared with controls by Student's t test for paired data.

**RESULTS**

**EFFECT OF LEUCOCYTES ON PRECONTRACTED MUSCLE**

Granulocytes and mononuclear cells produced concentration dependent relaxations of circular smooth muscle precontracted with 10 µM/l acetylcholine (Figs 1 and 2). Non-viable leucocytes did not relax muscle. Removal of platelets did not alter relaxation of precontracted muscle by 1×10^8 mononuclear cells which was 22 (7%) with platelets and 26 (10%) without (p<0.1, n=7 pairs). Therefore platelets were not removed from mononuclear cell suspensions in the subsequent experiments as this process reduced cell yield. Substances known to affect the NO-eGMP pathway affected muscle relaxation by leucocytes (Tables I and II). Addition of 200 nM oxyhaemoglobin and 10 µM methylene blue to the organ bath, one and 10 minutes before cells respectively, reduced muscle relaxation. Incubation of cells for 45 minutes with 100 µM N^2-monomethyl-L-arginine reduced muscle relaxation, but 100 µM N^2-monomethyl-D-arginine had no effect. Superoxide dismutase (60 units/ml) added one minute before leucocytes produced an increase in muscle relaxation. Tetrodotoxin (100 nM) did not affect muscle relaxation by leucocytes (n=5, not shown). Leucocytes were activated by incubation for one hour with F-Mer-Leu-Phe 100 nM. Activated mononuclear cells (5×10^8/l) caused a mean muscle relaxation of 43-6 (15%) compared with 8-3 (4%) by paired non-activated cells (n=10 pairs, p<0.05). Activation of granulocytes did not increase muscle relaxation (mean relaxation by activated granulocytes 21-3 (10%) compared with 18-4 (6%) by non-activated cells (n=12 pairs, p=0.7)).
We have shown that granulocytes and mononuclear cells relax colonic circular smooth muscle strips contracted by acetylcholine. The mediator of muscle relaxation is unlikely to be a prostaglandin as leukocytes were incubated with 100 nM indomethacin. Leucotrienes produce contraction of this tissue and thromboxane has no effect. Muscle relaxation was increased by superoxide dismutase and reduced by oxyhaemoglobin and preincubation with methylene blue. Incubation of effector phagocytes with N^6-monomethyl-L-arginine reduced muscle relaxation, whereas incubation with N^6-monomethyl-D-arginine had no effect. Only viable mononuclear cells and granulocytes caused muscle relaxation, suggesting that the relaxing factor is not stored by these cells. Muscle relaxation was not affected by tetrodotoxin and therefore unlikely to be mediated by neural elements. These findings strongly support the suggestion that effector phagocytes relax circular smooth muscle by the release of NO.

Our results are in agreement with those of others who found that activated and non-activated macrophages and granulocytes relax vascular smooth muscle by release of NO that is synthesized from L-arginine by a stereospecific enzyme, NO synthase. Release of NO is increased by activation of macrophages, but not granulocytes, possibly because of the simultaneous increase in production of superoxide anions that react with NO. Leucocytes forming part of the inflammatory infiltrate of ulcerative colitis and at other sites of inflammation in the gastrointestinal tract may produce smooth muscle relaxation via release of NO. Diffusion of NO through the submucosa might be facilitated by the formation of a stabilising adduct with a carrier molecule such as cysteine, or a thiol containing protein such as albumin. Formation of these S-nitrosothiol compounds has been shown to increase the biological half life of NO in physiological solutions from three to five seconds to about 40 minutes. Pacemaker cells are located on the submucosal surface of the circular muscle. These cells not only produce electrical slow wave pacemaker activity responsible for the spontaneous mechanical activity of circular smooth muscle but also form a regenerative surface that propagates this activity. Damage to these cells reduces electrical pacemaker activity and impedes its propagation. NO or its adduct may inhibit this pacemaker activity or the response of myocytes to it, thus reducing spontaneous mechanical activity and causing a reduction in smooth muscle tone. In severe inflammation where the muscularis propria is infiltrated by leucocytes, profound dilatation may occur such as that seen in toxic megacolon. It has recently been shown that NO relaxes the human internal anal sphincter. This may contribute to the urgency to stool often associated with ulcerative colitis if inflammatory cells release NO in sufficient amounts to affect sphincter function.

Smooth muscle relaxation by NO is mediated by raising intracellular cyclic GMP concentrations, which inhibits the release of calcium from intracellular stores and may produce mechanical changes without alterations in membrane potential. This may explain the electromechanical dissociation found by Snape et al as the cause of reduced colonic reflex in patients with ulcerative colitis.

In conclusion, we have shown that human granulocytes and mononuclear cells generate NO in quantities sufficient to relax distal colonic smooth muscle. This may contribute to disorders of motility associated with inflammation.

We are indebted to Professor A W Cutberr for advice, encouragement, and use of facilities, and to his staff at the Department of Pharmacology, Cambridge University, particularly Mr T Redmond and Mr B Gilson for their invaluable technical assistance. We also thank Miss A Lee for preparation of the manuscript.

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Gut 1993 34: 814-817
doi: 10.1136/gut.34.6.814

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