Nitric oxide donating compounds stimulate human colonic ion transport in vitro

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Abstract

Background—Nitric oxide (NO) has been recently implicated as a possible mediator of bowel inflammation and has also been shown to stimulate electrogenic chloride secretion in rat and guinea pig intestine. This study therefore investigated the effect on two NO donors, sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) on human colonic ion transport.

Methods—Changes in short circuit current (Δ SCC) in response to nitric oxide donating compounds were measured in muscle stripped normal human colon mounted in Ussing chambers. The ion species and intracellular mechanisms responsible for Δ SCC evoked by SNP were investigated.

Results—Basolateral SNP caused a progressive rise in Δ SCC over the range 10^{-7} to 10^{-4} M ($ED_{50}=2.5 \times 10^{-5}$ M). SNAP 10^{-4} M also evoked a qualitatively similar Δ SCC compared with SNP 10^{-4} M. Basolateral SNP evoked a greater Δ SCC than apical and this was significantly attenuated by bumetanide 10^{-4} M ($52.9 \pm 10.1\%$) and in chloride free media ($68.3 \pm 7.3\%$). Δ SCC response to SNP was not significantly changed by basolateral 4-acetamido-4'-isothio-cyano-2,2'-disulphonic acid stillbene (SITS 10^{-3} M) an inhibitor of sodium/bicarbonate exchange, or apical amiloride 10^{-5} M an inhibitor of sodium absorption. SNP induced Δ SCC was also significantly reduced by piroxicam (mean (SEM)) 10^{-5} M (57.9 (11.9%)), nordihydroguaretic acid 10^{-4} M (48.0 (12.9%)), tetrodotoxin (TTX 10^{-6} M, 52.3 (9.1%)), and practically abolished by TTX and piroxicam together (96.8 (3.3%)).

Conclusion—NO donors stimulate human colonic ion transport in vitro. For SNP, increased Δ SCC is at least due in part to chloride secretion, and the response seems to be transduced through enteric nerves and by local prostanoid synthesis. This study provides evidence that NO may be another important mediator of ion transport in human colon.

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as a constitutive (calcium dependent unaffected by glucocorticoids) or inducible (calcium independent, induction inhibited by glucocorticoids) enzyme in many tissue types.^{2,3} In recent years NO has also been implicated in regulating an increasing number of physiological and pathophysiological activities in the gastrointestinal tract.⁴ Among its activities in the gastrointestinal tract, NO has been shown to be an inhibitory non-cholinergic non-adrenergic neurotransmitter of intestinal and biliary tract motility^{5–7} and a regulator of mucosal permeability.⁸

It is also becoming apparent that in addition to regulating gastrointestinal motility and permeability, NO may also be an important mediator of intestinal ion transport. Recent animal experiments have shown that NO applied directly,⁹ or via NO donating compounds^{9–11} to experimental animal intestine in vitro, stimulates electrogenic chloride secretion, a mechanism responsible for secretory diarrhoea.¹² Furthermore, the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) attenuates castor oil¹³ and laxative induced¹⁴ diarrhoea in rats in vivo. Recently, inducible nitric oxide synthase (iNOS) has been shown to be increased in animal models of experimental colitis¹⁵ and in active idiopathic ulcerative colitis in humans,^{16,17} suggesting a role for NO in the pathogenesis of these conditions. NO is generated by a number of immunological cell types including mast cells,¹⁸ neutrophils,¹⁹ and macrophages²⁰ all of which have been shown to be present in increased quantities in the lamina propria and the lumen of inflamed bowel.

It is increasingly recognised that species variation is a feature of intestinal secretion in response to secretagogues. One of us has previously demonstrated species variability in response to histamine stimulation of human colonic ion transport in vitro.²¹ In addition, it has also been recently demonstrated that *Clostridium difficile* toxin B may be more important in the pathogenesis of pseudomembranous colitis than toxin A in human colon, which differs from data in experimental animals.²² For these reasons we have used normal colonic mucosa mounted in Ussing chambers to investigate ion transport responses to NO donating compounds and to identify possible mechanisms by which this action occurs.

Methods

Specimens and tissue preparation

Tissues were obtained from patients undergoing colonic resection for carcinoma (n=40)

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Nitric oxide (NO) was originally described as endothelial derived relaxing factor¹ and in recent years has been established to be an important biological mediator in a number of tissue functions.² NO is generated from L-arginine by NO synthase (NOS), which exists

or diverticular disease ($n=3$). Left sided specimens ($n=34$) were taken from the descending or sigmoid colon and right sided specimens ($n=9$) from the caecum and ascending colon. All tissues were macroscopically normal and at least 4 cm away from the edge of the tumour or diseased area. In all cases, the resection margins adjacent to where the tissues were taken from, were confirmed to be histologically normal. Specimens were transferred immediately in Krebs–Henseleit solution (composition; NaCl 118 mmol/l, KCl 4.7 mmol/l, CaCl_2 2.5 mmol/l, MgSO_4 1.2 mmol/l, KH_2PO_4 1.2 mmol/l, NaHCO_3 25 mmol/l, and d-glucose 11.1 mmol/l) at 4°C to the laboratory. Mucosal sheets were stripped of their underlying smooth muscle by blunt dissection and mounted in Ussing chambers. The time from resection to tissues being mounted in Ussing chambers was approximately 20 minutes. In some experiments, the chloride containing salts of the Krebs–Henseleit solution were replaced with sodium gluconate 117 mmol/l, potassium gluconate 4.7 mmol/l, and calcium sulphate dehydrate 2.5 mmol/l. The study was approved by the ethics committee of the University Hospital, Queens Medical Centre, Nottingham.

Ussing chamber studies

Four sheets of mucosa obtained from each patient were mounted simultaneously in Ussing chambers (window area 1.13 cm^2) and bathed on either side with 10 ml of Krebs–Henseleit solution. Tissues were maintained at 37°C , gassed with 95% O_2 /5% CO_2 using an air lift system. The bathing solutions were connected via agar bridges (3MKCl in 3% agar) to calomel electrodes to measure the potential difference between the two tissues. Tissues were voltage clamped to zero potential difference using a DVC-1000 automatic voltage clamp (World Precision Instruments, New Haven, CT) with Ag–AgCl electrodes connected to the agar bridges. Short circuit current (SCC) was continually monitored using an MP 100 data acquisition system (Biopac Systems) linked to the voltage clamp. The preparation was unclamped every five minutes to measure open circuit voltage and changes in resistance were derived from voltage and ΔSCC using Ohm's law.

Tissues were allowed to establish a stable SCC for at least 45 minutes prior to any drug addition. Drugs were added to solutions bathing either the serosal (basolateral) or the mucosal (apical) side of the chamber 15 minutes prior to SNP or SNAP. Changes in short circuit current (ΔSCC) were determined from the difference between basal and maximum SCC deflection after a drug was administered. At the end of each experiment the cholinomimetic agent carbachol (10^{-4}M) was applied to the basolateral side of the preparation to confirm tissue viability. Results (ΔSCC) are expressed in $\mu\text{A}/\text{cm}^2$. All data are presented as mean (SEM) and statistically significant differences between mean values were assessed by means of Student's t test for

paired or unpaired samples as appropriate. Drugs were added basolaterally unless otherwise stated.

Materials

Sodium nitroprusside, piroxicam, bumetanide, carbachol, amiloride, nordihydroguaiaretic acid (NDGA), and N^G -nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma Chemical, Poole, Dorset, UK. S-nitroso-N-acetyl-penicillamine (SNAP) and 4-acetamido-4'-isothio-cyano-2,2'-disulphonic acid stillbene (SITS) were obtained from Calbiochem-Novabiochem, La Jolla, CA.

Results

Basal parameters

In all, 145 separate colonic specimens from 43 patients (34 left sided, and nine right sided) were included for analysis of baseline parameters and responses to NO donating drugs. Table I shows basal SCC, resistance and conductance as well as responses to basolateral carbachol 10^{-4}M . Although basal SCC and voltage appeared somewhat lower in left compared with right colon this did not reach statistical significance (Table I). Tissue conductance and the response to carbachol 10^{-4}M was similar in right and left sided specimens used in these studies. A pharmacological approach was used to identify charge carrying ions and possible mechanisms of action of NO donors. Piroxicam 10^{-5}M , tetrodotoxin 10^{-6}M , and amiloride 10^{-4}M significantly reduced basal SCC and SITS 10^{-3}M significantly increased basal SCC but none of these drugs when administered alone significantly changed ΔSCC response to carbachol compared with controls (10^{-4}M Table II).

SCC responses in human colon to stimulation with NO donors

SNP evoked a concentration dependent increased ΔSCC from 10^{-7} to 10^{-4}M ($\text{ED}_{50}=2.5 \times 10^{-5}\text{M}$, Fig 1). SNP 10^{-4}M exhibited significantly reduced responses to a second application at a similar concentration after 30 minutes; first addition 29.7 (11.9) $\mu\text{A}/\text{cm}^2$, second addition -2.8 (0.7) $\mu\text{A}/\text{cm}^2$ ($p<0.05$, $n=7$). Therefore to avoid problems of tachyphylaxis when constructing a concentration response curve, each piece of data was

TABLE I. Basal parameters for right and left colons

	Right colon ($n=9/34$)	Left colon ($n=34/113$)	p Value
Basal SCC ($\mu\text{A}/\text{cm}^2$)	97.2 (11.9)	71.1 (11.1)	0.26
Voltage (mV)	10.2 (1.6)	7.6 (1.2)	0.14
Conductance (mS/ cm^2)	10.1 (1.0)	11.4 (1.2)	0.74
Response to carbachol 10^{-4}M (ΔSCC $\mu\text{A}/\text{cm}^2$)	74.1 (13.5)	94.8 (8.5)	0.2

Data are expressed as mean (SEM), n =number of patients/specimens from each patient. Although basal SCC and voltage values were lower in left compared with right colons, this did not attain statistical significance (unpaired t test). Mean conductance values and ΔSCC responses to carbachol (10^{-4}M) were similar for right and left colons.

TABLE II Influence of drugs on basal short circuit current and carbachol stimulated changes in short circuit current

Stimulus	Piroxicam (10 ⁻⁵ M, n=7)	TTX (10 ⁻⁶ M, n=6)	Piroxicam 10 ⁻⁵ M and TTX (10 ⁻⁶ M, n=6)	NDGA (10 ⁻⁴ M, n=7)	L-NAME (10 ⁻³ M, n=7)	Amiloride (10 ⁻⁴ M, n=6)	Bumetanide (10 ⁻⁴ M, n=7)	SITS (10 ⁻³ M, n=9)	Bumetanide 10 ⁻⁴ M and SITS (10 ⁻³ M n=6)
None	5.4 (2.1)	6.9 (0.6)	2.6 (1.9)	1.9 (1.7)	1.9 (1.7)	0.8 (2.9)	3.5 (2.5)	1.0 (1.1)	1.4 (1.2)
Drug	-37.1 (8.2)**	-28.3 (7.2)**	-54.0 (12.7)**	-30.2 (9.9)**	4.2 (4.2)	-46.6 (16.1)*	2.4 (8.0)	18.0 (3.5)**	6.5 (8.2)
Carbachol 10 ⁻⁴ M	109.2 (13.0)	109.2 (13.0)	137.4 (22.7)	75.0 (22.7)	91.4 (22.7)	89.8 (20.5)	109.4 (13.1)	103.9 (27.2)	120.0 (19.6)
Drug+ Carbachol 10 ⁻⁴ M	114.9 (21.0)	66.5 (12.8)	43.3 (8.8)†	70.6 (14.8)	114.6 (9.6)	112.3 (16.6)	6.7 (2.8)‡	91.1 (14.1)	17.2 (5.8)‡

Effects of drugs on basal and carbachol stimulated short circuit current in human colon. All drugs were added basolaterally except amiloride, which was added apically. Change in short circuit current (Δ SCC μ A/cm²) was measured over a 15 minute period after drug administration and compared with untreated control tissues over the same period (* $p < 0.05$, ** $p < 0.01$). At the end of each experiment carbachol 10⁻⁴M was added and the maximum Δ SCC was compared in drug treated and untreated tissues († $p < 0.05$, ‡ $p < 0.01$). Comparisons were made using Student's paired t test. Data shown as mean (SEM).

derived from a single stimulation of each tissue by SNP. There was no significant reduction in the response to carbachol after a second shot of SNP; (Δ SCC response to carbachol 10⁻⁴M after SNP 10⁻⁴M was 104.6 (9.4) μ A/cm², n=30 after a single addition and 93.5 (14.3) μ A/cm², n=7; after a second addition, $p = 0.6$). An alternative NO donor SNAP 10⁻⁴M was added and evoked a similar type of response to SNP 10⁻⁴M (Fig 2). For the remainder of the experiments SNP 10⁻⁴M was used as this concentration gave the maximum Δ SCC response.

Sidedness of the response to SNP
In paired experiments, SNP 10⁻⁴M evoked a Δ SCC of 65.5 (24.9) μ A/cm² when added basolaterally, which was significantly greater than the response of 14.1 (2) μ A/cm² observed when SNP 10⁻⁴M was added apically ($p = 0.05$, n=6).

Ionic basis of the response to SNP
Bumetanide 10⁻⁴M, an inhibitor of the Na/K/Cl cotransporter necessary for chloride secretion²³ was added basolaterally 15 minutes prior to SNP. In paired tissues this concentration of bumetanide significantly reduced the Δ SCC response to SNP by 52.9 (10.1)%; (from 66.1 (16.3) to 35.4 (14.8) μ A/cm²,

$p < 0.05$; n=7) and practically abolished the response to carbachol 10⁻⁴M by 89.2 (4.5)% (from 109.4 (13.0) to 6.7 (2.8) μ A/cm², $p < 0.01$; n=6). Basolateral SITS 10⁻³M (an inhibitor of sodium/bicarbonate transport in rat colon²⁴ and renal proximal tubules²⁵) did not significantly affect Δ SCC response to SNP ($p = 0.09$, n=9) when added alone, but in combination with bumetanide 10⁻⁴M reduced Δ SCC significantly greater than with bumetanide alone, (80.0 (6.7)% v 52.9 (10.1)% reduction respectively $p < 0.05$, n=7 Fig 3). Amiloride 10⁻⁵M, an inhibitor of electrogenic sodium absorption in human colon,²⁶ was added apically but did not affect the response to basolateral SNP 10⁻⁴M (SNP

Figure 1: Concentration response curve to basolateral SNP in human colon. SNP evoked a concentration dependent increased Δ SCC in human colon from 10⁻⁷ to 10⁻⁴M. Results are mean (SEM) of six patients. Doses were added in separate tissues from the same patients.

Figure 2: Time course response to SNP(A) and SNAP(B). Mean Δ SCC responses to SNP (n=32) and SNAP (n=8) over time. Values for Δ SCC represent differences in SNP and SNAP stimulated Δ SCC from the baseline to maximum response in tissues described. Note that both SNP and SNAP evoke qualitatively similar increased Δ SCC in human colon.

Figure 3: Investigation of the charge carrying ion(s) responsible for ΔSCC with SNP. SNP (10^{-4}M) was administered to the basolateral side of the tissues 15 minutes after basolateral addition of bumetanide (10^{-4}M), SITS (10^{-3}M), bumetanide (10^{-4}M) and SITS (10^{-3}M), and in chloride free media. ΔSCC values obtained were compared with tissues treated with basolateral SNP (10^{-4}M) alone in normal Krebs solution. The effect of SNP was significantly attenuated by bumetanide, bumetanide and SITS, and in chloride free media.

control; 55.9 (21.8) $\mu\text{A}/\text{cm}^2$ v SNP and amiloride; 59.6 (23.8) $\mu\text{A}/\text{cm}^2$; $p = 0.8$, $n = 6$).

As these inhibitor experiments suggested that electrogenic chloride secretion was the main contributor to SNP induced increased ΔSCC , confirmatory ion substitution studies were performed. In chloride free media, ΔSCC response to SNP was reduced by 68.3 (7.3)% (from 36.2 (13.6) to 7.0 (2.2) $\mu\text{A}/\text{cm}^2$ $p < 0.05$, $n = 7$; Fig 3) compared with paired tissues bathed in normal Krebs solution. ΔSCC response to SNP was restored when the chloride free media was replaced with normal Krebs solution (data not shown).

Mechanism of action of SNP

All antagonists were added basolaterally 15 minutes prior to SNP. Mean peak ΔSCC responses to SNP 10^{-4}M were reduced by the cyclooxygenase inhibitor piroxicam 10^{-5}M by 57.9 (11.9)% (from 75.0 (13.4) to 29.0 (6.9) $\mu\text{A}/\text{cm}^2$, $p < 0.05$; $n = 7$). In addition nordihydroguaiaretic acid 10^{-4}M , which has both cyclooxygenase and lipoxygenase inhibiting properties, significantly reduced ΔSCC response to SNP by 48.0 (12.9)% (from 47.4 (14.6) to 21.2 (7.4) $\mu\text{A}/\text{cm}^2$, $p < 0.05$; $n = 7$).

The neuron blocker tetrodotoxin 10^{-6}M (an inhibitor of fast sodium channels in nerves²⁷) reduced SNP induced ΔSCC by 52.3 (9.1)% (from 75.0 (13.4) to 29.0 (6.9) $\mu\text{A}/\text{cm}^2$, $p < 0.05$; $n = 6$) and the combination of TTX 10^{-6}M and piroxicam 10^{-5}M virtually abolished ΔSCC with SNP 10^{-4}M (96.8 (3.3)% reduction, $p < 0.001$, $n = 6$; Fig 4). The ΔSCC response to carbachol 10^{-4}M was not affected by piroxicam, TTX, or NDGA at the concentrations used but was significantly reduced by the combination of piroxicam 10^{-5}M and TTX 10^{-6}M together (Table II). The iNOS inhibitor L-NAME 10^{-4}M did not change ΔSCC response to SNP 10^{-4}M (SNP alone 56.7 (22.0) $\mu\text{A}/\text{cm}^2$, SNP and L-NAME 52.6 (12.7) $\mu\text{A}/\text{cm}^2$, $p = 0.8$; $n = 7$).

Discussion

There is increasing evidence that NO may control many important functions of the gastrointestinal tract⁴ and another role that has been recently recognised is its ability to regulate intestinal ion transport in animals.⁹⁻¹¹ However the role of NO in regulating human colonic ion transport has not previously been evaluated. Using two different NO donating

Figure 4: Investigation of possible mechanisms responsible for ΔSCC changes with SNP. SNP ($10^{-4}M$) was added basolaterally 15 minutes after basolateral piroxicam ($10^{-5}M$), TTX ($10^{-6}M$), TTX ($10^{-6}M$) and piroxicam ($10^{-5}M$) together, and NDGA ($10^{-4}M$). Piroxicam, TTX, and NDGA all significantly reduced ΔSCC response compared with basolateral SNP ($10^{-4}M$) alone. The combination of TTX and piroxicam together practically abolished the response to SNP.

compounds we have shown that these can also stimulate human colonic ion transport.

Both SNP and SNAP have widely been used as NO donating compounds.⁹⁻¹¹ SNP is a potent vasodilator used in clinical practice and causes vascular relaxation by releasing NO, which acts on guanylate cyclase.²⁸ The mechanism by which SNP releases NO is not fully understood but it is thought to occur both spontaneously,²⁹ and after contact with biological tissues.³⁰⁻³² SNP has also been recently shown to cause increased cyclic-GMP production in isolated human colonic mucosa,³³ which may account for its secretory action. Although most of the biological activity of SNP is thought to be due to NO, it may also have activity independent of NO, which can inhibit mucosal electrolyte transport.³⁴ Therefore SNAP, a nitrosothiol that is structurally unrelated to SNP but which also releases NO,³⁵ was used as an alternative NO donor. In human colon both SNP and SNAP gave a qualitatively similar increased ΔSCC (Fig 2). As repeated basolateral administration of SNP caused tachyphylaxis, a concentration response curve was constructed using separate tissues. The fact that repeated administration of SNP did not effect the subsequent ΔSCC

response to carbachol shows that SNP induced tachyphylaxis is a real phenomenon rather than a decreased sensitivity due to a toxic affect. The ED_{50} for SNP in human colon is $2.5 \times 10^{-5}M$, which is of the same order of magnitude as for rat colon $8 \times 10^{-5}M$.¹⁰

Basal short circuit current in human colon was significantly reduced by apical amiloride indicating that basal ion transport was at least in part accounted for by sodium absorption. Previous studies have shown both basal electrogenic sodium absorption and electro-neutral Na-Cl co-transport in human colon *in vitro*.^{26,36} In this study basal short circuit current was also significantly reduced by piroxicam, NDGA, and tetrodotoxin indicating that continuous synthesis of prostaglandins as well as local enteric nerves contribute to basal electrical tone, which has been previously demonstrated in human colon.²¹

The ionic basis of the increased ΔSCC in response to SNP was investigated both by using pharmacological inhibitors of known ion transporting processes and by ion replacement studies. We have concluded from these studies that electrogenic chloride secretion is the main contributor to the increased ΔSCC as bumetanide and chloride free media inhibited

Δ SCC by 53% and 68% respectively. However, there may also be a contribution from bicarbonate secretion as the combination of SITS and bumetanide reduced Δ SCC greater than bumetanide alone although SITS alone did not significantly reduce Δ SCC to SNP. Amiloride at a concentration known to inhibit electrogenic sodium absorption in human colon²⁶ had no effect on SNP induced Δ SCC increase indicating that electrogenic sodium absorption does not contribute to SNP induced increased Δ SCC. These data are in keeping with the effect of SNP on experimental animal intestine. Bumetanide has been shown to attenuate the effect of SNP in rat^{9,10} and guinea pig intestine¹⁰ in vitro. In addition, ion flux studies in voltage clamped rat colon have demonstrated increased electrogenic chloride secretion and reduced mucosal to serosal sodium and chloride flux with a residual flux thought to be due to bicarbonate secretion.¹⁰ Our studies suggest that SNP evokes similar ion transport events in human colon.

The mean Δ SCC response to SNP was greater when administered to the basolateral side of the preparation compared with apical side in paired tissues. To investigate possible mechanisms further, we used specific inhibitors of eicosanoid synthesis and neuronal transport applied basolaterally prior to the addition of SNP. Tetrodotoxin, an inhibitor of nerve function, and piroxicam and NDGA inhibitors of local prostanoid synthesis, significantly attenuated Δ SCC to SNP, while both tetrodotoxin and piroxicam together virtually abolished it. This suggests that the secretory response to NO requires enteric nerve function and local prostanoid synthesis for SNP induced changes in human colonic ion transport. Other Ussing chamber studies on secretagogue stimulated human colonic ion transport with activated mast cells and phagocytes have also shown that ion transport changes seem to be transduced through nerves and local eicosanoid synthesis.^{37,38}

In summary, these studies show that NO donating compounds stimulate human colonic ion transport. For SNP this increased Δ SCC is for the most part due to electrogenic chloride secretion and possibly bicarbonate secretion and occurs through local prostanoid synthesis and via stimulation of enteric nerves. As macrophages, mast cells, and phagocytes have all been shown to have increased iNOS activity when activated in vitro,¹⁸⁻²⁰ and as inflamed bowel contains a much greater quantity of a range of activated immunocytes, increased production of NO could be a possible mediator of diarrhoea in active inflammatory bowel disease through the stimulation of enteric nerves and by local eicosanoid synthesis.

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