Proabsorptive and prosecretory roles for nitric oxide in cholera toxin induced secretion

J L Turvill, F H Mourad, M J G Farthing

Abstract

Background—Cholera toxin causes small intestinal hypersecretion by inducing a coordinated response from enterocytes, enterochromaffin cells, enteric neurones, and the vascular supply. Nitric oxide has been implicated in the function of these separate components.

Aims—To explore the role of nitric oxide in the totality of cholera toxin induced secretion in vivo.

Methods—One group of adult male Wistar rats was treated with the nitric oxide synthase inhibitors N^G-nitro-L-arginine methyl ester (L-NAME; subcutaneously or intraluminally), N^G-methyl-L-arginine (L-NMA), or 7-nitroindazole. A second group of rats was treated with L-arginine (intraperitoneally or intraluminally) or D-arginine. The small intestine was isolated between two cannulae and instilled with 75 µg cholera toxin or saline for two hours. Small intestinal perfusion of a plasma electrolyte solution containing [¹⁴C]-PEG was undertaken to determine net water and electrolyte movement. After the experiment macroscopic and microscopic intestinal appearances were noted and jejunal 5-hydroxytryptamine concentrations were determined.

Results—Both L-arginine and L-NAME induced secretion in the basal state, but only when administered intraluminally. Systemically applied L-NAME caused a dose dependent reduction in cholera toxin induced secretion. This was paralleled by L-NMA but not by 7-nitroindazole or by intraluminally applied L-NAME. Systemically applied L-NAME caused notable cyanosis of the intestine, consistent with mesenteric ischaemia, but no microscopic abnormalities. Systemically applied L-arginine but not D-arginine also reduced cholera toxin induced secretion and inhibited 5-hydroxytryptamine release.

Conclusion—Nitric oxide has a duality of roles in cholera toxin induced secretion, acting both as an absorbagogue and a secretagogue. Its mechanisms of action include the maintenance of mucosal perfusion and enterochromaffin cell stabilisation.

(Gut 1999;44:33-39)

In the past decade the free radical nitric oxide (NO) has emerged both as a ubiquitous signalling molecule, mediating a range of regulatory biological processes, and as a killer molecule, released by activated immune cells. NO is formed on demand by generator cells such as endothelium, neurones, enterocytes, myocytes, mast cells, and leucocytes, and also by intestinal bacteria.¹⁻⁴ Being soluble both in water and lipid, it freely traverses cell membranes and passes into adjacent target cells.⁵ NO is produced as a result of the conversion of L-arginine to L-citrulline by stereospecific nitric oxide synthases (NOS).⁶ There are two major isoforms of NOS, the constitutive (cNOS) and the inducible (iNOS).^{7,8} Constitutive NOS is recognised in both endothelial and neuronal cells, as eNOS and nNOS respectively, and may also be present in epithelial cells. When cNOS containing cells are appropriately stimulated, an increase in cytosolic calcium activates NOS to produce a short burst of NO. The low, pulsed levels of NO production which result are thought to facilitate neurotransmission, maintain vascular perfusion and microvascular patency, maintain mucosal integrity, and inhibit immune cell activation and adhesion within the gastrointestinal tract.⁹⁻¹² Normally concentrations of available L-arginine are sufficient for continuous NO synthesis; however L-citrulline may be recycled back to L-arginine by incorporating one nitrogen atom, thereby increasing the availability of NO substrate.^{13,14} Inducible NOS is formed in activated immune cells, endothelial cells, and myocytes and is implicated in host defence mechanisms. It is not a calmodulin dependent enzyme and is generated only after appropriate stimulation. The activation of iNOS therefore occurs about 24 hours after a stimulatory insult but results in the local production of one, two, or three orders of magnitude more NO than the constitutive isoform generates.¹⁵

The effects of NO on the intestinal epithelium, the local microcirculation, the enteric nervous system, and inflammatory cascades have implicated it as a potential mediator of intestinal water and electrolyte transport and a number of groups have investigated this possibility.¹⁶⁻¹⁸ Their findings to date have been contradictory; this probably relates to the

Abbreviations used in this paper: NO, nitric oxide; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMA, N^G-methyl-L-arginine; cNOS, constitutive nitric oxide synthase; inducible nitric oxide synthase; 5-HT, 5-hydroxytryptamine; PES, plasma electrolyte solution; PEG, polyethylene glycol; HPLC, high performance liquid chromatography; CT, cholera toxin; IQR, interquartile range.

Digestive Diseases Research Centre, St Bartholomew's and the Royal London School of Medicine and Dentistry, Turner Street, London E1 2AD, UK
J L Turvill
F H Mourad
M J G Farthing

Correspondence to:
Dr J L Turvill.

Accepted for publication
22 July 1998

Keywords: cholera toxin; nitric oxide; small intestinal transport; 5-hydroxytryptamine; L-arginine; nitric oxide synthase inhibitors

Table 1 Effect of NOS inhibition (L-NAME, L-NMA and 7-nitroindazole) and the NO precursor L-arginine on basal chloride and sodium movement

	Control	L-NAME (60 mg/kg)	L-NMA (50 mg/kg)	7-nitroindazole (100 mg/kg)	L-arginine (1 g/kg)	D-arginine (1 g/kg)	PES + 20 mmol/l L-arginine	PES + 20 mmol/l L-NAME
Number	15	6	6	6	6	6	6	6
Chloride ($\mu\text{mol}/\text{min}/\text{g}$)	2.5 (1.7/5.7)	4.4 (2.6/6.5)	2.4 (-0.4/2.9)	4.7 (3.3/6.0)	-0.8 (-2.7/5.2)	2.0 (0.6/2.4)	-3.8* (-8.5/0.9)	-5.6* (-9/-4.6)
Sodium ($\mu\text{mol}/\text{min}/\text{g}$)	16.7 (12/19)	16.0 (11/19)	13.4 (10/16)	15.6 (14/17)	14.0 (12/16)	14.8 (7/21)	0.5* (-4/8)	-1.5* (-3/-1)

Data are expressed as median (interquartile ranges) in $\mu\text{mol}/\text{min}/\text{g}$ dry weight.

* $p < 0.004$ versus controls (Kruskal-Wallis).

complexity of actions of NO, the lack of specific NOS isoform inhibitors, and the “dose dependent effects” of NO. Even before the discovery of NO, Hellier and colleagues and Hegarty and colleagues perfused the human jejunum with L-arginine and induced small intestinal secretion. These findings were later confirmed by Rolfe and Levin²¹ in stripped and unstripped rat ileum mounted in Ussing chambers and by Stack and Hawkey²² who found that the NO donor, sodium nitroprusside, induced ion transport in human colon in vitro. By contrast, others have found NO to be proabsorptive. Schirgi-Degen and Beubler found that an intravenous infusion of the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), an L-arginine analogue, induced secretion in ligated rat jejunal loops in vivo.²³ Secretion was reversed by L-arginine, sodium nitroprusside, and indomethacin. Mailman used a different NOS inhibitor, N^G-nitro-L-arginine, to show a reduction in rat ileal fluid absorption.²⁴ Maher *et al* showed, in canine Thiry-Vella fistulae, that L-arginine and the NO donor, S-nitroso-N-acetyl penicillamine, increased and L-NAME decreased fluid absorption.²⁵

In keeping with the complexity of roles for NO in physiological states, Mourad *et al* recently showed that NO has both prosecretory and proabsorptive properties.²⁶ Rat small intestine was perfused in situ with isoosmotic solutions containing either L-arginine or L-NAME. An initial infusion containing L-arginine was

found to induce secretion. This was reversed by low concentrations of L-NAME but when higher concentrations of L-NAME were administered, secretion was re-established.

The role of NO in hypersecretory states is largely unexplored. Cholera toxin is the major enterotoxin produced by *Vibrio cholerae* and is responsible for the life threatening secretory diarrhoea that results from infection with this organism.²⁷ The toxin induces a non-inflammatory, small intestinal hypersecretory state and acts after binding to a ganglioside receptor (GM₁) on the enterocyte brush border membrane.²⁸ The toxin is transported transepithelially to the basolateral membrane where it irreversibly activates a stimulatory G protein, so increasing adenylate cyclase activity. This results in a rise in intracellular concentrations of cyclic AMP and the initiation of a metabolic cascade involving the activation of protein kinases, the phosphorylation of apical chloride channels, and the onset of a chloride flux.^{29–32} In addition cholera toxin is known to induce secretion through the activation of the enteric nervous system, as the application of tetrodotoxin or lignocaine inhibits over 50% of the secretory response in vivo.^{33–35} Furthermore, the secretagogue and neurotransmitter 5-hydroxytryptamine (5-HT) has been implicated as a major factor in the pathogenesis of cholera toxin induced secretion.^{36–38} It is released from enterochromaffin cells after exposure to the toxin and appears to act both on the enteric nervous system and on the enterocyte directly to drive secretion.^{39–41} The extent of 5-HT release has recently been closely correlated with the degree of secretion induced by cholera toxin.⁴²

The objective of this study was to determine the role and mechanism of action of NO in an established in vivo model of maximal cholera toxin induced small intestinal secretion. Studies were performed using non-specific NOS inhibitors (L-NAME and N^G-methyl-L-arginine (L-NMA)) and a selective nNOS inhibitor (7-nitroindazole) to assess the effect of the inhibition of NO synthesis on secretion.^{7 43 44} We also evaluated the effect of the NO precursor, L-arginine and its inactive enantiomer, D-arginine. Because of the results of earlier studies of basal transport, NOS inhibitors and NO precursors were administered both locally, into the small intestinal lumen, and systemically.

Methods

BASIC EXPERIMENTAL DESIGN

Adult male Wistar rats (180–220 g body weight), fasted for 18 hours with free access to water, were anaesthetised with intraperitoneal sodium pentobarbitone (60 mg/kg) and

Figure 1 Influence of systemic L-NAME (60 mg/kg), L-NMA (50 mg/kg), 7-nitroindazole (100 mg/kg), L-arginine (1 g/kg), and D-arginine (1 g/kg) and intraluminal L-NAME and L-arginine (20 mmol/l added to PES) on basal net small intestinal water movement. * $p < 0.0001$ compared with control.

Table 2 Effect of non-specific NOS inhibitors, L-NAME and L-NMA and the nNOS inhibitor, 7-nitroindazole on cholera toxin (CT) induced net small intestinal chloride and sodium movement

	Number	Net Cl ⁻ flux ($\mu\text{mol}/\text{min}/\text{g}$)	Net Na ⁺ flux ($\mu\text{mol}/\text{min}/\text{g}$)
CT	16	-12.3 (-15.3 to -11.3)	-16.9 (-18.0 to -15.2)
+ L-NAME 20 mg/kg	8	-10.0* (-10.7 to -7.2)	-12.7* (-15.0 to -10.9)
+ L-NAME 40 mg/kg	9	-3.5† (-5.3 to -2.5)	-6.4† (-8.2 to -5.0)
+ L-NAME 60 mg/kg	6	-5.9† (-7.9 to -4.8)	-6.9† (-8.6 to -4.7)
+ L-NMA 50 mg/kg	6	-4.7† (-6.3 to -1.7)	-3.9† (-4.4 to -0.4)
+ 7-nitroindazole 50 mg/kg	11	-10.8 (-11.9 to -8.8)	-13.4 (-17.2 to -12.8)
+ 7-nitroindazole 100 mg/kg	6	-10.5 (-12.6 to -8.4)	-15.2 (-16.3 to -14.1)
+ peanut oil 10 mg/kg	6	-11.0 (-11.5 to -9.7)	-16.5 (-17.2 to -15.1)

Data are expressed as median and interquartile ranges.

* $p < 0.002$ versus cholera toxin; † $p < 0.0001$ versus cholera toxin.

maintained by interval injections (15–30 mg/kg) as necessary throughout the experiment. Animals were kept at 37°C using a heat pad. The abdomen was opened through a midline incision and a cannula was inserted into the proximal small intestine 5 cm beyond the duodenojejunal flexure. A distal cannula was similarly inserted into the terminal ileum (1–2 cm proximal to the ileocaecal valve) and both were fixed by ligation. The isolated small intestine was gently flushed with isotonic saline (37°C) and air to clear it of residual contents.⁴⁵ After returning the intestine to the abdominal cavity and closing the abdomen, 75 μg cholera toxin in 6 ml isotonic saline or saline alone was instilled into the small intestine and both cannulae were clamped. After two hours' incubation, the clamps were removed and the intestine perfused at a rate of 0.5 ml/min with plasma electrolyte solution (PES) containing Na⁺ 140, K⁺ 4, Cl⁻ 104, and HCO₃⁻ 40 mmol/l to which 5 g/l polyethylene glycol 4000 (PEG) and 4 $\mu\text{Ci}/\text{l}$ ¹⁴C-PEG had been added. Thirty minutes elapsed to ensure the establishment of a steady state after which three consecutive 10 minute collections of the effluent were obtained from the distal cannula. The samples were stored at -50°C for up to 48 hours prior to analysis of net water and electrolyte movement. At the end of the experiment rats were killed by an overdose of pentobarbitone; the perfused segment was removed, rinsed,

Figure 2 Influence of L-NAME (20, 40, and 60 mg/kg), L-NMA (50 mg/kg), and 7-nitroindazole (50 and 100 mg/kg) on cholera toxin (CT) induced net small intestinal water movement ($\mu\text{l}/\text{min}/\text{g}$ dry intestinal weight). * $p < 0.02$ compared with CT; † $p < 0.02$ compared with CT+L-NAME 20 mg/kg; NS, CT versus CT+7-nitroindazole.

blotted, and weighed and then desiccated in an oven at 80°C for 18 hours to obtain the dry weight.

GROUP 1: INHIBITION OF NITRIC OXIDE SYNTHESIS
Immediately prior to the instillation of cholera toxin or saline, rats were pretreated with one of the following: the non-specific NOS inhibitors, L-NAME (20, 40, or 60 mg/kg in 0.5 ml saline subcutaneously), L-NMA (50 mg/kg in 0.5 ml saline subcutaneously), or the specific nNOS inhibitor, 7-nitroindazole (50 or 100 mg/kg in 10 mg/kg peanut oil intraperitoneally). Vehicle controls (saline alone and peanut oil alone (10 mg/kg intraperitoneally)) were also included. Finally, one group of rats was not pretreated but was perfused with PES containing 20 mmol/l L-NAME.

At the end of the experiment, samples of small intestine from rats that had been pretreated with L-NAME 40 mg/kg, were obtained for histological assessment. Small intestinal tissues were fixed in formalin, embedded in paraffin wax, and stained with haematoxylin and eosin for light microscopy. Histological assessment was performed by a single, blinded histopathologist.

GROUP 2: ADMINISTRATION OF NITRIC OXIDE PRECURSOR

Immediately prior to the instillation of cholera toxin or saline, rats were pretreated with one of the following: L-arginine (0.5 or 1.0 g/kg in 1.5 ml water intraperitoneally), D-arginine (1.0 g/kg in 1.5 ml water intraperitoneally), or L-arginine (1.0 g/kg) plus 7-nitroindazole (50 mg/kg). The experimental design included a saline control and a group of rats which was not pretreated but was perfused with PES containing 20 mmol/l L-arginine.

After this experiment, full thickness samples of small intestine were obtained, by freeze clamping, for determination of 5-HT and tryptophan concentrations by high performance liquid chromatography (HPLC) with fluorimetric detection. After weighing, samples were stored in liquid nitrogen and then prepared by Ultra Turrax homogenisation in 1 ml ice cooled 10% wt/vol perchloric acid containing 2 mmol/l sodium EDTA and centrifugation at 14 000 rpm for 15 minutes. The supernatants were stored at -50°C before same day injection into the HPLC column.⁴⁶ A 100 mm × 4.6 mm internal diameter ODS column was eluted using an LKB 2150 pump at a flow rate of 1 ml/min at ambient temperature.⁴⁷ A JASCO 821FP fluorescence detector with xenon lamp was set at: ex 290 nm, em 335 nm, attenuation 8, gain ×10 and was connected to an integrator. The degassed mobile phase consisted of acidified 100 mmol/l ammonium acetate and methanol. The limit for detection of 5-HT by this technique was determined as 1 fmol/ μl . Results are expressed as pmol/mg dry weight. Tryptophan served as an internal standard.

Samples of small intestine from rats which had been pretreated with L-arginine were obtained for histological assessment as described above.

Table 3 Effect of the addition of L-arginine (20 mmol/l) and L-NAME (20 mmol/l) to the perfusion solution on cholera toxin (CT) induced small intestinal water and electrolyte secretion

	CT	CT+ PES + L-arginine	CT+ PES + L-NAME
Number	16	8	8
Net Cl ⁻ movement	-11.6 (-15.3 to -10.3)	-9.2 (-13.8 to -7.5)	-8.5 (-13.0 to -7.5)
Net Na ⁺ movement	-17.1 (-18.1 to -13.5)	-17.0 (-18.1 to -13.5)	-15.9 (-16.4 to -12.7)

Data are expressed as median (interquartile ranges) in $\mu\text{mol}/\text{min}/\text{g}$ dry weight. CT + PES + L-arginine, NS versus CT; CT + PES + L-NAME, NS versus CT.

Cholera toxin was obtained from the Swiss Serum and Vaccine Institute, Berne. Radiolabelled polyethylene glycol (^{14}C -PEG 4000) was obtained from Amersham International. L-NAME, L-NMA, and 7-nitroindazole were obtained from Sigma chemicals and all other chemicals were supplied by British Drug House.

ANALYSIS OF PERFUSION

^{14}C -PEG concentrations in the effluent were measured in triplicate by liquid scintillation spectroscopy in an LKB Wallac Ultra-beta 1210 scintillation counter. The mean of the net fluid movement of the three consecutive effluent samples was calculated and expressed as $\mu\text{l}/\text{min}/\text{g}$ dry intestinal weight. Positive values denote net absorption and negative values net secretion. Chloride concentration was determined by a Corning 925 chloride analyser; sodium was determined by flame photometry (Instrumentation Laboratories 943). Solute flux is expressed as $\mu\text{mol}/\text{min}/\text{g}$.

Steady state conditions were confirmed by less than a 5% variation in water movement between consecutive 10 minute collections. Values were accepted only if ^{14}C -PEG recovery fell between 95 and 105%.^{45 48}

STATISTICAL ANALYSIS

Results are expressed as median and interquartile ranges. Differences in net fluid and electrolyte movement and differences in 5-HT and tryptophan concentrations were examined using a non-parametric analysis of variance (Kruskal-Wallis test) with intergroup analysis.

Figure 3 Influence of L-arginine 0.5 and 1.0 g/kg and D-arginine 1.0 g/kg on cholera toxin (CT) induced net small intestinal water movement. * $p < 0.002$ compared with control; † $p < 0.0003$ compared with CT+L-arginine 0.5 g/kg; NS, CT versus CT+D-arginine.

Results

GROUP 1: INHIBITION OF NITRIC OXIDE SYNTHESIS
All the control rats absorbed water and electrolytes from the small intestine (median net water movement $127.2 \mu\text{l}/\text{min}/\text{g}$ dry weight, interquartile range 102.3 to 154.9 , $n=15$). Basal absorption of water was not affected by systemic pretreatment with the non-specific NOS inhibitors, L-NAME or L-NMA, or the selective nNOS inhibitor, 7-nitroindazole. Net electrolyte movement paralleled net water movement (fig 1 and table 1). By contrast, the addition of L-NAME 20 mmol/l to the intestinal perfusate caused net small intestinal secretion of water and electrolytes ($-32.6 \mu\text{l}/\text{min}/\text{g}$, interquartile range -26 to -45 , $n=6$; $p < 0.0001$).

Cholera toxin induced net small intestinal secretion of water and electrolytes in all rats ($-82.7 \mu\text{l}/\text{min}/\text{g}$, interquartile range -91.8 to -67.9 , $n=16$; $p < 0.0001$ compared with control). Systemic pretreatment with both L-NAME and L-NMA, but not 7-nitroindazole, notably inhibited cholera toxin induced net small intestinal water secretion (fig 2). There was a dose dependent effect as 40 and 60 mg/kg L-NAME were significantly more antisecretory than 20 mg/kg L-NAME. L-NAME and L-NMA were similarly effective at equivalent doses, suggesting a commonality of action. Net chloride and sodium movement paralleled net water movement (table 2). Cholera toxin induced secretion was not altered by the peanut oil carrier ($-71.7 \mu\text{l}/\text{min}/\text{g}$, interquartile range -78.1 to -66.3 , $n=6$; $p=0.4$).

The addition of 20 mmol/l L-NAME to PES did not modify cholera toxin induced net small intestinal secretion of water and electrolytes (table 3).

The macroscopic appearance of the small intestine in rats pretreated with systemic L-NAME 20, 40, and 60 mg/kg, was notably cyanotic. However the microscopic appearance after L-NAME 40 mg/kg, the minimum dose to cause a maximal antisecretory effect, was normal. In particular there was no evidence of mucosal disruption, intravascular thrombosis, haemorrhage, or inflammatory infiltrate. The small intestinal appearance was consistent, therefore, with reversible mesenteric ischaemia.

GROUP 2: ADMINISTRATION OF NITRIC OXIDE PRECURSOR

Basal absorption of water was not affected by systemic pretreatment with either the NO precursor L-arginine or its enantiomer, D-arginine. Net intestinal electrolyte absorption paralleled net water movement. However, the addition of L-arginine to the intestinal perfusate caused a net small intestinal secretion of water and electrolytes ($-7.3 \mu\text{l}/\text{min}/\text{g}$, interquartile range -9 to 2 , $n=6$; $p < 0.0001$) (fig 1 and table 1). Systemically administered L-arginine dose dependently inhibited cholera toxin induced net small intestinal secretion of water (fig 3). The inactive enantiomer, D-arginine (1.0 g/kg), however, had no effect. Net chloride and sodium movement paralleled net water movement (table 4). 7-Nitroindazole given in

Figure 4 Effect of pretreatment with L-arginine or D-arginine (1 g/kg) on cholera toxin (CT) induced decrease in small intestinal 5-HT concentrations. * $p < 0.0001$ compared with control; † $p < 0.0002$ compared with CT and CT+D-arginine; NS, control versus CT+L-arginine.

combination with 1.0 g/kg L-arginine did not modify the antisecretory effect of L-arginine (water: 1.1 $\mu\text{L}/\text{min}/\text{g}$, interquartile range -7.5 to 18.4; chloride: -4.3 $\mu\text{mol}/\text{min}/\text{g}$, interquartile range -5.4 to -2.0; sodium: -2.4 $\mu\text{mol}/\text{min}/\text{g}$, interquartile range -3.9 to -0.5, $n=6$; $p=0.4$ compared with L-arginine). The addition of 20 mmol/l L-arginine to PES did not modify cholera toxin induced net small intestinal secretion of water and electrolytes ($p > 0.1$) (table 3).

Exposure to cholera toxin ($n=10$) resulted in the depletion of small intestinal 5-HT concentrations when compared with control ($n=11$, $p < 0.0001$). This was prevented by pretreatment with L-arginine but not D-arginine (fig 4). Tryptophan concentrations were used as internal standards and did not significantly differ in the four groups ($p=0.97$). Macroscopic and microscopic small intestinal appearances were unaltered by pretreatment with L-arginine.

Discussion

Few studies have addressed the role of NO precursors and NOS inhibitors, given in isolation, on cholera toxin induced small intestinal water and electrolyte secretion in vivo. The findings of this study show that NO has both proabsorptive and prosecretory effects. Blockade of NO generation by systemic administration of L-NAME or L-NMA dose dependently inhibits cholera toxin induced small intestinal water and electrolyte secretion, without affecting basal transport. We conclude that NO has a permissive role in mediating secretion. Because cholera toxin induces secretion within 30 minutes, before iNOS can be activated, the NO generated to mediate this secretory process is likely to be solely cNOS derived. This implies that low, background concentrations of NO are required for cholera toxin to induce maximal secretion. Similar in vivo findings with NOS inhibitors have been described in *Escherichia coli* heat stable enterotoxin induced secretion and in castor oil, magnesium sulphate, and phenolphthalein induced diarrhoea.^{21 49 50} Reddix *et al* have found that cholera toxin increases NO release from guinea pig ileum mounted in Ussing chambers and that L-NAME inhibits cholera toxin induced increases in short circuit current.⁵¹ Others, using smaller doses of cholera toxin, have reported different in vivo

findings. Qiu *et al* were unable to show any effect on cholera toxin induced secretion using either the NO donor, S-nitroso-N-acetyl-L-cysteine or the nNOS inhibitor, 7-nitroindazole.⁵² Beubler and Schirgi-Degen recently reported that intravenous L-NAME enhanced cholera toxin induced secretion while intravenous L-arginine inhibited it.⁵³ Both authors used the in situ technique of closed small intestinal loops, which has a number of limitations over steady state perfusions. These include alterations in the composition of the intraluminal test solution and the effects of intraluminal distension on intestinal function.

Systemic L-arginine, the NO precursor, but not its inactive enantiomer, D-arginine, also inhibits cholera toxin induced small intestinal water and electrolyte secretion, without affecting basal transport. Beubler and Schirgi-Degen have shown a similar finding.⁵³ While our results at first seem to be contradictory, a similar dichotomy has been described in the role of NO in basal water and electrolyte transport.²⁶ Clearly NO has the potential to act at a number of sites within the host secretory response to cholera toxin. However, the fact that the proabsorptive and prosecretory nitrgic effects do not simply cancel each other out suggests that their mechanisms of action are not directly opposing. We postulate that elevated concentrations of NO activate additional antisecretory pathways. In the absence of iNOS activity, the availability of the NO precursor, L-arginine, is the rate limiting step in intestinal NO generation.^{13 14} By administering an excess of L-arginine, increased NO concentrations result and antisecretory NO mediated pathways are recruited. The failure of 7-nitroindazole to modify the actions of L-arginine suggests that this mechanism is not neurally mediated. The findings of this study suggest that one such L-arginine dependent antisecretory effect is the inhibition of 5-HT release. Intestinal 5-HT, 80% of which is stored in enterochromaffin cells, mediates a substantial proportion of cholera toxin induced secretion. It is released after exposure to the toxin and acts on 5-HT₂ and 5-HT₃ receptors to initiate secretion.^{36 37 39-41} NO is known to inhibit mast cell degranulation and as mast cells and enterochromaffin cells degranulate by a similar calcium dependent mechanism we postulated that NO would also inhibit enterochromaffin cell degranulation.^{4 54} Our finding that the cholera toxin induced depletion of tissue 5-HT concentrations was prevented by L-arginine but not D-arginine supports this hypothesis. We conclude that the elevated concentrations of NO inhibit enterochromaffin cell degranulation and prevent the release of 5-HT which results in the failure of activation of 5-HT dependent secretory pathways.

Possible prosecretory sites of action for NO in cholera toxin induced secretion include the epithelium, the enteric nervous system, the local vascular supply, and immune cells. Confirmation of the findings of Mourad *et al*, that both intraluminal L-arginine and L-NAME stimulate secretion in the basal state supports

Table 4 Effect of L-arginine and D-arginine on cholera toxin (CT) induced net small intestinal chloride and sodium movement

	n	Net Cl ⁻ flux (μmol/min/g)	Net Na ⁺ flux (μmol/min/g)
CT	16	-12.6 (-15.3 to -11.0)	-16.9 (-18.0 to -15.2)
+ L-arginine 0.5 g/kg	11	-10.6* (-11.6 to -6.7)	-10.1† (-12.0 to -8.9)
+ L-arginine 1 g/kg	10	-4.4† (-8.4 to -4.0)	-3.7† (-9.8 to -1.8)
+ D-arginine 1 g/kg	8	-12.5 (-14.0 to -11.3)	-17.5 (-20.0 to -13.5)

Data are expressed as median (interquartile ranges).

*p<0.01 versus cholera toxin; †p<0.0001 versus cholera toxin.

the potential of a direct action of NO on the epithelium.²⁶ The demonstration that systemically applied L-arginine and L-NAME do not affect basal transport reinforces the importance of the local availability of these agents. It is clear however that NO has differing roles in cholera toxin induced secretion compared with the basal state. The same doses of L-arginine and L-NAME, administered via the perfusate, had no effect on cholera toxin induced secretion and we conclude that NO has no direct antisecretory effect on the epithelium in vivo. Next we considered the action of NO on the enteric nervous system. NOS immunoreactivity is present throughout the enteric nervous system.¹⁵ Rolfe and Levin found that *E coli* heat stable enterotoxin induced secretion was mediated by NO acting on the myenteric plexus.²¹ By contrast, Li *et al* studied simultaneous longitudinal muscle contractions and short circuit current mediated by transmural field stimulation and found that neuronally acting NO inhibited muscular contraction but had no effect on the short circuit current.⁵⁵ We found that 7-nitroindazole, at doses known to decrease nNOS activity and modify *Clostridium difficile* toxin A induced secretion, had no effect on cholera toxin induced secretion and so conclude that the inhibition of neuronal NO has no measurable effect in mediating cholera toxin induced secretion in vivo.^{44 52}

Cholera toxin induced secretion is accompanied by intestinal hyperaemia.⁵⁶ Although neither L-arginine nor L-NAME altered small intestinal histological appearances, systemic L-NAME caused sufficient reversible ischaemia to result in intestinal cyanosis. The mucosa is supplied by a countercurrent arteriovenous plexus in the villus core that provides a limited oxygen delivery to the apex of the villus.⁵⁷ As a result, the oxygen supply, mucosal metabolic rate, and the efficiency of ATP production of apical enterocytes are highly sensitive to alterations in vascular perfusion. The efficacy of the countercurrent multiplier and the concentration gradient it generates is entirely dependent on the integrity of villus perfusion. Bohlen and Lash have found that enterocyte respiration can be maintained, despite NOS inhibition, by increasing cellular oxygen extraction.⁵⁸ However it is likely that the reduction in villus blood flow through the countercurrent multiplier, caused by L-NAME and L-NMA, is sufficient to enhance passive absorption of water and electrolytes across the epithelium.

The histological findings also confirm that cholera toxin induces secretion without activating a cellular inflammatory response. While NO is known to modulate free radical generation, combining with superoxide to form the

highly toxic peroxynitrite with activation of immune cells, the role of these agents in cholera toxin induced secretion is at present unclear.

In conclusion, NO exerts both prosecretory and proabsorptive influences in cholera toxin induced secretion in vivo. NO, generated from cNOS, plays a major role in mediating cholera toxin induced secretion. Evidence points to its role in maintaining vascular perfusion. We found no evidence, however, specifically to implicate the intestinal epithelium or the enteric nervous system. Furthermore, an excess of NO release, resulting from the administration of L-arginine, has an additional proabsorptive effect. We confirmed that the prevention of small intestinal 5-HT depletion represents one such antisecretory pathway. As over 80% of small intestinal 5-HT is derived from enterochromaffin cells we conclude that the NO inhibits enterochromaffin cell degranulation. The resulting proabsorptive effect is, therefore, a consequence of the failure of cholera toxin to recruit any 5-HT dependent secretory pathways.

The authors gratefully thank Professor D Perrett and Dr I James of the Department of Metabolism and Endocrinology, St Bartholomew's Hospital, and Dr P Domizio of the Department of Histopathology, St Bartholomew's Hospital, for their assistance and expertise during this study.

- Nichols K, Staines W, Krantis A. Nitric oxide synthase distribution in the rat intestine: a histochemical analysis. *Gastroenterology* 1993;105:1651-61.
- M'Rabet-Touil H, Blachier F, *et al*. Characterization and ontogenesis of nitric oxide synthase activity in pig enterocytes. *FEBS Lett* 1993;331:243-7.
- Brittain T, Blackmore R, Greenwood C. Bacterial nitrite-reducing enzymes. *Eur J Biochem* 1993;209:793-802.
- Kanwar S, Wallace J, Befus D. Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. *Am J Physiol* 1994;266:G222-9.
- Henry Y, Lepoivre M, Drapier J, *et al*. EPR characterisation of molecular targets for NO in mammalian cells and organelles. *FASEB J* 1993;7:1124-34.
- Palmer R, Rees D, Ashton D, *et al*. L-arginine is the physiological precursor for the formation of nitric oxide in endothelium dependent relaxation. *Biochem Biophys Res Commun* 1988;153:1251-6.
- Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992;6:3051-64.
- Lowenstein C, Dinerman J, Snyder S. Nitric oxide, a physiologic messenger. *Ann Intern Med* 1994;120:227-37.
- Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* 1989;ii:997-1000.
- Geiger J, Nolte C, Butt E. Role of cAMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of antagonist-evoked calcium elevation in human platelets. *Proc Natl Acad Sci USA* 1992;89:1031-5.
- Ney P, Schroder H, Schor K. Nitrovasodilator-induced inhibition of LTB₄ release from PMN may be mediated by cGMP. *Eicosanoids* 1990;3:243-5.
- Rand M. NANC transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. *Clin Exp Pharmacol Physiol* 1992;19:147-69.
- Hecker M, Sessa W, Harris H, *et al*. The metabolism of L-arginine and its significance for the biosynthesis of endothelium derived relaxing factors: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc Natl Acad Sci USA* 1990;87:8612-6.
- Drexler H, Zeiher A, Meinzer K, *et al*. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet* 1991;338:1546-50.
- Moncada S, Palmer R, Higgs E. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 1991;43:109-42.
- Salzman AL. Nitric oxide in the gut [review]. *New Horizons* 1995;3:33-45.
- Barry M, Aloisi J, Pickering S, *et al*. Nitric oxide modulates water and electrolyte transport in the ileum. *Ann Surg* 1994;219:382-8.
- Rao R, Riviere P, Pascaud X, *et al*. Tonic regulation of mouse ileal ion transport by nitric oxide. *J Pharmacol Exp Ther* 1994;269:626-31.
- Hellier M, Thirumalai C, Holdsworth C. The effect of amino acids and dipeptides on sodium and water absorption in man. *Gut* 1973;14:41-5.

- 20 Hegarty J, Fairclough P, Clark M, *et al.* Jejunal water and electrolyte secretion by L-arginine in man. *Gut* 1981;22:108–13.
- 21 Rolfe V, Levin R. Enterotoxin *Escherichia coli* STa activates a nitric oxide-dependent myenteric plexus secretory reflex in rat ileum. *J Physiol* 1994;475:531–7.
- 22 Stack W, Hawkey C. Sodium nitroprusside, a nitric oxide donating compound, stimulates human colonic ion transport in vitro. *Gut* 1994;35:S53.
- 23 Schirgi-Degen A, Beubler E. Significance of nitric oxide in the stimulation of intestinal fluid absorption in the rat jejunum in vivo. *Br J Pharmacol* 1995;114:13–18.
- 24 Mailman D. Differential effects of luminal L-arginine and N^G-nitro-L-arginine on blood flow and water fluxes in rat ileum. *Br J Pharmacol* 1994;112:304–10.
- 25 Maher MM, Gontarek JD, Jimenez RE, *et al.* Endogenous nitric oxide promotes ileal absorption. *J Surg Res* 1995;58:687–92.
- 26 Mourad F, O'Donnell L, Andre E, *et al.* L-Arginine, nitric oxide, and intestinal secretion: studies in rat jejunum in vivo. *Gut* 1996;39:539–44.
- 27 Finkelstein R, LoSpalluto J. Production of highly purified cholera toxin and cholera toxin. *J Infect Dis* 1970;121(suppl):S63–72.
- 28 King CA, van Heyningen WE. Deactivation of cholera toxin by a sialidase resistant monosialosylganglioside. *J Infect Dis* 1973;127:639–47.
- 29 Field M. Modes of action of enterotoxin from *Vibrio cholerae* and *Escherichia coli*. *Rev Infect Dis* 1979;1:918–25.
- 30 Chang EB, Rao MC. Intracellular mediators of intestinal electrolyte transport. In: Field M, ed. *Diarrheal diseases*. New York: Elsevier, 1991:49–72.
- 31 Field M, Rao MC, Chang EB. Intestinal electrolyte transport and diarrheal disease I. *N Engl J Med* 1989;321:800–6.
- 32 Field M, Rao MC, Chang EB. Intestinal electrolyte transport and diarrheal disease II. *N Engl J Med* 1989;321:879–83.
- 33 Cassuto J, Jodal M, Tuttle R, *et al.* On the role of intramural nerves in the pathogenesis of choleraic secretion. *Scand J Gastroenterol* 1981;16:377–84.
- 34 Jodal M. Neuronal influence on intestinal transport. *J Intern Med* 1990;228:125–32.
- 35 Lundgren O, Svanik J, Jivegard L. Enteric nervous system, I. Physiology and pathophysiology of the intestinal tract. *Dig Dis Sci* 1989;34:264–83.
- 36 Osaka M, Fujita T, Yanatori Y. On the possible role of intestinal hormones as the diarrhoeagenic messenger in cholera. *Virchows Arch B Cell Pathol* 1975;18:287–96.
- 37 Nilsson O, Cassuto J, Larsson PA. 5-Hydroxytryptamine and cholera secretion: a histochemical and physiological study in cats. *Gut* 1983;24:542–8.
- 38 Bearcroft CP, Perrett D, Farthing MJG. 5-Hydroxytryptamine release into human jejunum by cholera toxin. *Gut* 1996;39:528–31.
- 39 Beubler E, Horina G. 5-HT₂ and 5-HT₃ receptor subtypes mediate cholera toxin-induced intestinal fluid secretion in the rat. *Gastroenterology* 1990;99:83–9.
- 40 Hansen MB, Skadhauge E. Ketanserin and granisetron reduce cholera toxin-induced hypersecretion in pig jejunum. *Scand J Gastroenterol* 1994;29:908–15.
- 41 Mourad FH, O'Donnell LJ, Dias JA, *et al.* Role of 5-hydroxytryptamine type 3 receptors in rat intestinal fluid and electrolyte secretion induced by cholera and *Escherichia coli* enterotoxins. *Gut* 1995;37:340–5.
- 42 Turvill JL, Connor P, Farthing MJG. Enterochromaffin cell 5-HT₃ autoreceptors inhibit cholera toxin induced 5-HT release [abstract]. *Gut* 1997;41(suppl 3):A39.
- 43 Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 1993;329:2002–12.
- 44 Yoshida T, Limmroth V, Irikura K, *et al.* The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. *J Cereb Blood Flow Metab* 1994;12:924–9.
- 45 Rolston DDK, Borodo MM, Kelly MJ, *et al.* Efficacy of oral rehydration solutions in a rat model of secretory diarrhoea. *J Pediatr Gastroenterol Nutr* 1987;6:624–30.
- 46 Barnes JM, Barnes NM, Costall B, *et al.* Reserpine, para-chlorophenylalanine and fenfluramine antagonise cisplatin-induced emesis in the ferret. *Neuropharmacology* 1988;27:783–90.
- 47 Bearcroft CP, Farthing MJG, Perrett D. Determination of 5-hydroxytryptamine, 5-hydroxyindoleacetic acid and tryptophan in plasma and urine by HPLC with fluorimetric detection. *Biomed Chromatogr* 1995;9:23–7.
- 48 Elliot E, Watson A, Walker-Smith J, *et al.* Search for the ideal rehydration solution: studies in a model of secretory diarrhoea. *Gut* 1991;32:1314–20.
- 49 Mascolo N, Izzo A, Barbato F, *et al.* Inhibitors of nitric oxide synthetase prevent castor-oil-induced diarrhoea in the rat. *Br J Pharmacol* 1993;108:861–4.
- 50 Izzo A, Gaginella T, Mascolo N, *et al.* Nitric oxide as a mediator of the laxative action of magnesium sulphate. *Br J Pharmacol* 1994;113:228–32.
- 51 Reddix R, Ceasar S, Niu X. Stimulation of endogenous nitric oxide by cholera toxin in guinea pig ileum [abstract]. *Gastroenterology* 1997;112:A396.
- 52 Qiu B, Pothoulakis C, Castagliuolo I, *et al.* Nitric oxide inhibits rat intestinal secretion by *Clostridium difficile* toxin A but not *Vibrio cholerae* enterotoxin. *Gastroenterology* 1996;111:409–18.
- 53 Beubler E, Schirgi-Degen A. Nitric oxide counteracts 5-hydroxytryptamine- and cholera toxin-induced fluid secretion and enhances the effect of oral rehydration solution. *Eur J Pharmacol* 1997;326:223–8.
- 54 Caplan M, Hedlund E, Hill N. The role of endogenous nitric oxide and platelet-activating factor in hypoxia-induced intestinal injury in rats. *Gastroenterology* 1994;106:346–52.
- 55 Li Y, Weisbrodt N, Lodato R, *et al.* Nitric oxide is involved in muscle relaxation but not in changes in short-circuit current in rat ileum. *Am J Physiol* 1994;266:G554–9.
- 56 Mathan MM, Chandy G, Mathan VI. Ultrastructural changes in the upper small intestinal mucosa in patients with cholera. *Gastroenterology* 1995;109:422–30.
- 57 Jodal M, Lundgren O. Countercurrent mechanisms in the mammalian gastrointestinal tract. *Gastroenterology* 1986;91:225–41.
- 58 Bohlen HG, Lash JM. Intestinal absorption of sodium and nitric oxide-dependent vasodilation interact to dominate resting vascular resistance. *Circ Res* 1996;78:231–7.