Nitric oxide stimulates cyclic guanosine monophosphate production and electrogenic secretion in Caco-2 colonocytes

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ABSTRACT

Nitric oxide stimulates intestinal ion transport via the activation of enteric nerves, but it is not known whether it regulates intestinal transport function by acting on the epithelium directly. The aim of this study was to determine the influence of nitric oxide on epithelial electrogenic ion secretion, measured as the short-circuit current ($I_{sc}$), using the human colonic carcinoma cell line Caco-2. The cellular mechanisms were examined by measuring epithelial cGMP production, and nitrite release was monitored as an index of nitric oxide synthesized. The nitric oxide substrate L-arginine methyl ester increased nitrite release, electrogenic secretion and cell cGMP production. Pretreatment with L-NAME (N$^x$-nitro-L-arginine methyl ester, 1 mM), but not the D-isomer, significantly reduced the electrogenic secretion and cGMP production evoked by L-arginine methyl ester, implicating nitric oxide synthase involvement. Pretreatment with cystamine, but not Methylene Blue, significantly reduced the maximum $I_{sc}$ and the cGMP release induced by L-arginine methyl ester and the nitric oxide donor sodium nitroprusside, implicating the involvement of particulate guanylate cyclase. In conclusion, nitric oxide stimulates electrogenic ion secretion and cGMP production in intestinal epithelial cells by activating particulate guanylate cyclase. The direct action of nitric oxide on the intestinal epithelium may be important in the regulation of intestinal transport function in health and in inflammatory bowel disease.

INTRODUCTION

Nitric oxide (NO) is an important regulator of intestinal ion transport function, but it is not clear whether it acts on the epithelium directly to regulate ion transport. The exogenous application of NO-donating compounds and NO substrates such as sodium nitroprusside and L-arginine respectively induces electrogenic Cl$^-$ secretion in vitro in human colon [1], rat ileum [2], rat colon [3] and guinea pig small and large intestine [4]. In mutant mice deficient in the NO synthase cofactor tetrahydrobiopterin, ileal ion transport induced by L-arginine is impaired [5], demonstrating the importance of NO synthase cofactors as possible regulatory targets of intestinal transport. Studies in rats in vivo have found that NO may have contrasting actions on intestinal fluid secretion, but these results may be influenced by the site of administration of NO synthase inhibitors. Intraperitoneal administration of the NO synthase inhibitor N$^x$-nitro-L-arginine methyl ester (L-NAME) reduces fluid secretion in rat ileum induced by luminal Escherichia coli heat-stable enterotoxin [2], castor oil [6],

Key words: Caco-2 colonocytes, cyclic guanosine monophosphate, intestinal secretion, nitric oxide.
Abbreviations: $I_{sc}$, short-circuit current; t-NAME, N$^x$-nitro-L-arginine methyl ester; NO, nitric oxide.
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senna and cascara [7], but l-NAME applied intravenously enhances the fluid secretion stimulated by cholera toxin in the rat [8].

This contrasting influence of NO on intestinal transport in vivo may be due to the multiplicity of NO synthase isotypes which are localized in a variety of sites within the gut. Three main isotypes of NO synthase have been identified in mammalian cells and include a constitutive neuronal isotype (Type 1), an inducible isotype localized in inflammatory cells and epithelial cells (Type 2) and a constitutive isotype located in blood vessel endothelium (Type 3) [9]. Although the distribution of NO synthase isotypes in the gut has not been fully characterized, studies have identified both inducible and constitutive forms in cultures of colonic epithelium [10], and the inducible form in cultured colonocytes, suggesting that the epithelium may be an important source of NO in the gut [11]. Indeed, in inflammatory bowel disease the levels of NO within the gut mucosa are significantly increased [12]. Miller et al. [13] found that the administration of l-NAME reduced luminal fluid accumulation in guinea pigs with intestinal inflammation, suggesting that NO may contribute to the pathogenesis of diarrhoea in inflammatory bowel disease.

Since the direct influence of NO on the epithelium is not known and the cellular mechanisms of action are unclear, we examined the effects of the NO donor sodium nitroprusside and the NO substrate l-arginine methyl ester on electrogenic secretion and intracellular cGMP production in the human colonic carcinoma cell line Caco-2. The involvement of guanylate cyclase was examined using cystamine and Methylene Blue.

**METHODS**

**Tissue culture**

Caco-2 colonocytes (passage 80–101) were grown in 75 cm² tissue-culture-treated flasks containing Dulbecco’s Modified Eagle’s Medium supplemented with 10 mM l-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, 0.1% minimum essential medium non-essential amino acids and 10% fetal bovine serum. Cultures were maintained in a humidified incubator with 5% CO₂ at 37 °C and media was replaced every 2−3 days. At confluency, cells were trypsinized (0.25% Trypsin/EDTA) and then seeded at a density of 1 × 10⁴ cells/cm² into (a) 1 cm² diameter trans-wells (Costar U.K.) for the measurement of electrogenic transport, or (b) 24-well multi-well plates for cGMP measurement.

**Measurement of electrogenic secretion**

Confluent monolayers were placed into Ussing-type chambers that were adapted for the accommodation of trans-wells (World Precision Instruments). Cells were incubated at 37 °C in Krebs bicarbonate saline containing the following concentrations of ions: 143.0 mmol/l Na⁺, 125.7 mmol/l Cl⁻, 24.9 mmol/l HCO₃⁻, 5.9 mmol/l K⁺, 2.5 mmol/l Ca²⁺, 1.2 mmol/l H₂PO₄⁻, 1.2 mmol/l SO₄²⁻ and 1.2 mmol/l Mg²⁺. The apical bathing solution contained 10 mM glucose for metabolic requirements and the basolateral bathing solution contained 10 mM mannitol as an osmotic balance. The incubation fluid was circulated through the chambers by gassing with 95% O₂ and 5% CO₂. The short-circuit current (Isc, µA/cm²) and potential difference (mV) across the monolayer were monitored by an automatic voltage-clamp (DVC 1000, World Precision Instruments), and the epithelial resistance (Rₑ, Ω cm²) was calculated according to Ohm’s Law. The maximum change in Isc (ΔIsc = maximum Isc − basal Isc) was calculated as an index of electrogenic ion secretion.

**Measurement of nitrite release**

The release of nitrite into the apical and basolateral bathing solution was measured as an indication of the NO synthesized. The concentration of nitrite was measured by the Greiss reaction (1 ml sample plus 0.5 ml of 1.0% sulphanilamide in 0.1 mol/l HCl plus 0.5 ml of 0.1% naphthylethylaminediamine in H₂O). The absorbance was measured at 540 nm by a spectrophotometer (Cecil CE2040) and concentrations of nitrite calibrated from NaNO₂ curves (in Krebs bicarbonate saline plus either 10 mmol/l mannitol or 10 mmol/l glucose).

**cGMP production**

Caco-2 cells were grown to confluence in 24-well multi-well plates and then incubated short-term in a sterile container in a water bath at 37 °C, and gassed with humidified 5% CO₂ and 95% O₂. cGMP was measured by rinsing the cells in Krebs bicarbonate saline and exposing them to agonists or antagonists which were added to the wells. After incubation, the cells were removed by pipette into Eppendorf tubes, snap frozen in liquid N₂ and stored at −40 °C before measurement. For the measurement of cGMP, cells were centrifuged at 14000 g for 2 min in 0.1 mmol/l perchloric acid, and the cyclic nucleotide content of the supernatant measured by scintillation proximity assay (14C-labelled cGMP, Amersham U.K.), and expressed per cm² of cells (with approximately 7 × 10⁵/cm² at confluency).

**Drugs**

All chemicals were obtained from Sigma Co., U.K.

**Statistics**

The values for Isc, and concentrations of cGMP or nitrite, are presented as means ± S.E.M. with the number of experiments given in parentheses. Statistical significance...
Nitric oxide and colonocyte secretion was tested using Student’s unpaired \( t \)-test with Bonferroni’s procedure applied where comparisons were made between multiple groups. A \( P \) value < 0.05 was considered significant.

**RESULTS**

**Basal electrical activity, nitrite release and cGMP content of Caco-2 cells**

Before measurement of electrogenic secretion, the Caco-2 cells were acclimatized in the Ussing chambers for 10 min to obtain a stable baseline. The basal values in confluent Caco-2 colonocytes in 23 experiments were \( I_{sc} \), 6.5±2.2 \( \mu \)A/cm\(^2\); potential difference, 1.7±0.2 mV; \( R_t \), 240.2±25.1 \( \Omega \)cm\(^2\). Basal nitrite release into the apical and basolateral bathing solutions was not significantly different [apical, 0.065±0.005 \( \mu \)mol/l; basolateral, 0.073±0.007 \( \mu \)mol/l (\( n = 23 \), \( P > 0.05 \)), and the basal cGMP content of Caco-2 cells was 6.85±1.72 fmol/cm\(^2\) (\( n = 8 \)).

**Effects of sodium nitroprusside, L-arginine and L-arginine methyl ester on electrogenic secretion**

The effects of adding sodium nitroprusside, L-arginine and L-arginine methyl ester to the apical and basolateral bathing solutions on the electrogenic secretion across Caco-2 cells were measured. The results obtained are shown in Figure 1. The L-arginine methyl ester induced changes in \( I_{sc} \) which were significantly greater (\( P < 0.05 \)) than those induced by sodium nitroprusside and L-arginine (L-arginine methyl ester, 23.0±3.9 \( \mu \)A/cm\(^2\); sodium nitroprusside, 6.5±2.3 \( \mu \)A/cm\(^2\); L-arginine, 5.5±1.2 \( \mu \)A/cm\(^2\); \( n = 6 \)).

The \( 
\triangle \) \( I_{sc,max} \) induced by L-arginine methyl ester was significantly inhibited (\( P < 0.01 \)) by adding bumetanide (0.1 mmol/l) to the incubation solution for 15 min in comparison to controls, suggesting that the secretory \( I_{sc} \) is mediated by Cl\(^-\) ions [control, 16.3±1.9 \( \mu \)A/cm\(^2\); bumetanide, 6.8±1.4 \( \mu \)A/cm\(^2\) (\( n = 6 \)].

**Effects of L-NAME and D-NAME on L-arginine methyl ester-induced electrogenic secretion and nitrite release**

The effects of L-NAME and D-NAME (1 \( \mu \)mol/l and 1 mmol/l), placed in the incubation solutions 10 min before the addition of L-arginine methyl ester (1 mmol/l), are shown in Figure 2. L-NAME (1 mmol/l) significantly reduced the electrogenic response to L-arginine methyl ester by 76% compared with D-NAME controls (\( n = 6 \), \( P < 0.006 \)). Pretreatment with 1 \( \mu \)mol/l L-NAME did not significantly alter the electrogenic responses compared with D-NAME controls (\( n = 6 \), \( P > 0.05 \)). The release of nitrite into the basolateral bathing solution evoked by L-arginine methyl ester was reduced signifi-
Figure 4 Effects of sodium nitroprusside and L-arginine methyl ester on cell cGMP levels as a function of (A) concentration (1 μmol/l–10 mmol/l, n = 8) and (B) time (1–15 min, n = 8).

Values are given as means ± S.E.M.

Effects of L-arginine methyl ester and sodium nitroprusside on cGMP production

The effects of L-arginine methyl ester and sodium nitroprusside on cGMP production are shown in Figure 4. Both L-arginine methyl ester and sodium nitroprusside stimulated cGMP production in a concentration- and time-dependent manner. Concentrations of 1 mmol/l L-arginine methyl ester and 10 mmol/l sodium nitroprusside stimulated the greatest accumulation of cGMP in Caco-2 cells [L-arginine methyl ester, 15.8 ± 2.0 fmol/cm²; sodium nitroprusside, 30.0 ± 4.2 fmol/cm² (n = 8); see Figure 4A]. The production of cGMP stimulated by 1 mmol/l L-arginine methyl ester was maximal at 10 min, whereas the production of cGMP stimulated by sodium nitroprusside was maximal within 2 min (Figure 4B).

Effects of antagonists on the cGMP production induced by L-arginine methyl ester and sodium nitroprusside

The effects of 1 μmol/l and 1 mmol/l cystamine, Methylene Blue, D- and L-NAME on cell cGMP production induced by L-arginine methyl ester and sodium nitroprusside are shown in Table 1. Cystamine significantly reduced the cGMP production induced by L-arginine methyl ester and sodium nitroprusside.
methyl ester \((n = 8, P < 0.005)\) and sodium nitroprusside \((n = 8, P < 0.05)\) compared with controls, whereas Methylene Blue was ineffective in comparison to controls.

Pretreatment with \(\text{L-NAME (1 mmol/l)}\) significantly reduced the cGMP production evoked by \(\text{L-arginine methyl ester (n = 8, P < 0.05)}\) in comparison to \(\text{D-NAME controls (n = 8, P > 0.05)}\).

**DISCUSSION**

The regulation of intestinal electrogenic transport by NO appears to be mediated partly by enteric nerves [2], but it is not clear whether NO also acts on the intestinal epithelium directly. We have demonstrated that the addition of the NO donor sodium nitroprusside, and NO substrates \(\text{L-arginine and L-arginine methyl ester})\, to Caco-2 cells stimulated a concentration-dependent increase in electrogenic ion secretion, measured as the \(I_{sc}\). \(\text{L-Arginine methyl ester induced greater changes in } I_{sc}\) than either \(\text{L-arginine or sodium nitroprusside which may reflect the increased lipophilic nature of the methylated compound. Sodium nitroprusside induced only small changes in electrogenic ion secretion despite stimulating the greatest changes in cGMP production. The small electrogenic response may have been due to the sodium nitroprusside spontaneously producing NO between the head of the fluid circulation system and the cells in the Ussing chambers which was a distance of 15 cm, and thus was not being delivered to the target cells.}

The experiments with \(\text{L-arginine methyl ester support the speculation that intestinal epithelial cells may be an important source of NO. The addition of L-arginine methyl ester produced a rise in the } I_{sc}\text{ that was accompanied by nitrite release into the incubation solution, confirming that NO was produced. The electrogenic responses and nitrite release evoked by L-arginine methyl ester were reduced by L-NAME, indicating the involvement of NO synthases. The NO synthase(s) present in Caco-2 cells remain to be identified but are likely to include both constitutive and inducible isotypes [10]. In inflammatory bowel diseases, the activation of NO synthases by pro-inflammatory cytokines may contribute to the overproduction of NO and result in tissue damage [11].}

Our studies show that \(\text{L-arginine methyl ester increased the } I_{sc}\text{ across Caco-2 cells which was reduced by cystamine, an inhibitor of particulate guanylate cyclase [14,15]. The cystamine had no impact on the nitrite released with the inference that NO production precedes the activation of guanylate cyclase. The involvement of particulate guanylate cyclase was further confirmed by measuring intracellular cGMP levels; cystamine significantly inhibited the cGMP production evoked by both sodium nitroprusside and L-arginine methyl ester. The involvement of particulate guanylate cyclase was further confirmed by the fact that Methylene Blue, an inhibitor of soluble guanylate cyclase [16,17], had no effect on the cGMP production induced by either L-arginine methyl ester or sodium nitroprusside. These data suggest that intestinal epithelial cells synthesize NO which acts on particulate guanylate cyclase in order to raise intracellular cGMP levels. This may be an important mechanism by which NO regulates epithelial electrogenic transport. Membrane-bound guanylate cyclases have been identified on Caco-2 cells [18] and may provide a target for the NO in our experiments. It would be of interest to characterize more fully the interactions between NO and particulate guanylate cyclase in these cells. NO activates soluble guanylate cyclase by binding to endogenous haem component [19] but the mechanism of activation of particulate guanylate cyclase is not known.

We have shown that NO activates intestinal epithelial electrogenic secretion by acting directly on cells and raising intracellular cGMP levels. Previous studies demonstrated that NO stimulated cGMP-dependent electrogenic transport in guinea pig intestine [4] and rat colon [3], but the site of action of NO was unclear. Some studies suggest that NO stimulates electrogenic secretion via the activation of enteric nerves. Stack et al. [1] found that NO-induced electrogenic secretion in human colon \textit{in vitro} was partly mediated by neural mechanisms. Rolfe and Levin [2] demonstrated that electrogenic secretion induced by \textit{Escherichia coli} heat-stable enterotoxin was mediated through a local NO-dependent neural pathway routed through the myenteric plexus. NO synthases have been located in the myenteric plexus of the rat [20]. Therefore, the NO released from the nerves may act on the enterocytes directly to induce secretion, or indirectly via the release of neurotransmitters such as vasoactive intestinal peptide which is co-localized with NO [20]. Our present study confirms that NO may act directly on the intestinal epithelium to induce electrogenic secretion.

The activation of electrogenic secretion in intestinal epithelial cells by NO may be important in pathophysiological processes underlying diarrhoea in inflammatory bowel disease. In colon from patients with ulcerative colitis and Crohn’s disease the levels of inducible NO synthase were elevated [12,21]. Plasma nitrite levels were significantly elevated in patients with inflammatory diarrhoea [22]. In guinea pigs with ileitis the administration of L-NAME reduced the luminal fluid accumulation that accompanied the inflammation, implying that NO synthase and NO are influencing intestinal secretion [13]. In addition to evoking secretion, the NO may contribute to tissue damage in inflammatory bowel disease. The overproduction of NO evokes cell damage [23] and compromises epithelial barrier function [24]. The subsequent increase in intestinal epithelial permeability may exacerbate diarrhoea and increase the...
passage of potentially toxic macromolecules into the host.

In conclusion, intestinal epithelial cells provide a potential source of NO. The NO stimulates epithelial electrogenic transport by the activation of particulate guanylate cyclase and elevation of intracellular cGMP. This direct stimulation of epithelial transport by NO may play a role in the regulation of intestinal ion transport function in health and in inflammatory bowel disease where the overproduction of NO is a regular feature.

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REFERENCES


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