Nitric Oxide Produced by the Enterocyte Is Involved in the Cellular Regulation of Ion Transport

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ABSTRACT

The role of nitric oxide (NO) in the intestinal basal ion transport and under conditions of enterotoxin-induced ion secretion is controversial. Namely it is not clear whether NO enhances or counteracts intestinal ion secretion and whether the effects on transport result from a direct interaction with the enterocyte. The cell origin of NO is also unclear. We have tested the hypothesis that NO produced by the enterocyte directly regulates ion transport processes either in basal condition or in response to cholera toxin-induced secretion. Electrical variables reflecting transepithelial ion transport were measured in Caco-2 cell monolayers mounted in Ussing chambers exposed to the NO synthase inhibitor *N* ω -nitro-L-arginine methyl ester, in the presence or absence of cholera toxin. cAMP concentrations were also measured. NO release was determined by nitrite-nitrate concentration. NO synthase activities were assayed by Western blot analysis. *N* ω -nitro-L-arginine methyl ester had a secretory effect, as judged by increased basal short-circuit current and cAMP concentration. It also increased cholera toxin-induced electrical response and cAMP production. Either cholera toxin or the cAMP analog 8-bromo-cAMP induced a rapidly progressive and Ca²⁺-dependent increase in NO concentration, suggesting a homeostatic up-regulation of the constitutive form of NO synthase.

Western blot analysis showed an increase in constitutive NO synthase enzyme isoform. These results indicate that the enterocyte regulates its own ion transport processes, either in basal condition or in the presence of active secretion, through the activation of a constitutive NO synthase-NO pathway, functioning as a braking force of cAMP-induced ion secretion. (*Pediatr Res* 54: 64–68, 2003)

Abbreviations

NO, nitric oxide
NOS, nitric oxide synthase
cNOS, constitutive nitric oxide synthase
iNOS, inducible nitric oxide synthase
NO₂⁻/NO₃⁻, nitrite/nitrate
L-NAME, *N* ω -nitro-L-arginine methyl ester
L-NIL, *N*-iminoethyl-L-lysine
CT, cholera toxin
G, conductance
I_{sc}, short circuit current
PD, transepithelial potential difference
8Br-cAMP, 8-bromo-cAMP

Intestinal water and electrolyte transepithelial transport is under a complex control by several agents including neurotransmitters, hormones, or paracrine agents (1). There is evidence that NO takes part in the intestinal ion transport processes with an effect that involves the enteric nervous system, the suppression of prostaglandin formation, and the opening of K⁺ channels (2, 3).

NO is a gas with a half-life of less than 5 s, rapidly degrading to nitrite and nitrate in the presence of oxygen and

water. NO is produced by a number of cells, including neuronal cells, and released in various organs. Being soluble in both water and lipids, it freely passes into adjacent target cells (2, 4). NO is generated from L-arginine by a family of enzymes, NOS, whose activity can be stereospecifically inhibited by L- but not by D-arginine analogs (5). Two NOS are constantly active in the intestine and are termed cNOS. They include the neuronal NOS (NOS 1) and the endothelial NOS (NOS 3). Both are Ca²⁺/calmodulin-dependent, produce small amounts of NO in short burst, and are involved in several homeostatic processes. A third NOS form, which is Ca²⁺/calmodulin-independent, is activated by various signal molecules, including proinflammatory cytokines. This latter form, termed iNOS (NOS 2), requires at least 2 h to be activated, and released NO can be measured no earlier than 3–4 h after induction. Maximal NO

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production by this mechanism is commonly observed 6–24 h after induction, and peak concentration lasts 24–72 h (2, 6, 7). NO is thus produced by the enterocyte through both the cNOS and the iNOS forms (8, 9).

NO may play a dual role in the intestinal ion transport processes. In physiologic conditions, the basal cNOS activity is implicated in the regulation of the proabsorptive tone in the intestine, but whether this is the result of a direct effect on the enterocyte, or involves a neurologic control of intestinal blood flow, is still not clear (2, 3). On the contrary, an overproduction of NO by iNOS in pathologic conditions, such as inflammatory intestinal diseases, results in net ion secretion (1–12).

NO appears to have some role also in stimulated secretion. In animal models, CT-induced fluid secretion was down-regulated by NO (2). However, others did not detect any modification in CT-induced fluid secretion after administration of NOS inhibitors or NO donors (13). Recently, Turvill *et al.* (14) hypothesized that NO has a dual role in CT-induced secretion, acting either as an absorbagogue or as a secretagogue in the rat perfused small intestine, depending on its concentration. These apparently conflicting findings are probably related to the multiple effects of NO, dose-dependent effects, and different experimental models. In the majority of studies, whole intestinal tissue was used, which contains endothelium, neurons, myocytes, mast cells, and leukocytes, all potential sources of NO. This makes it difficult to track the origin and the effects of NO in the enterocyte. The use of intestinal cell lines to study intestinal electrolyte transport would directly address these issues. Indeed, previous studies have identified both iNOS and cNOS forms in cultures of intestinal epithelium, and Caco-2 cell monolayers have been previously used for studying NO production in response to live bacteria (15).

The aim of this study was to test the hypothesis that NO produced by the enterocyte acts as an intracellular regulator of ion transport both in basal conditions and in response to CT-induced ion secretion. We have used an *in vitro* model, based on Caco-2 cells, previously validated to investigate the regulation of ion transport at the cellular level and NO production (16–18).

METHODS

Cell culture. Caco-2 cells (code BS-TCL87) were obtained from the Istituto Zooprofilattico Sperimentale (Brescia, Italy). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with a high glucose concentration (4.5 g/L) supplemented with 10% FCS, 1% nonessential amino acids, penicillin (50 mU/mL), and streptomycin (50 mg/mL) and were incubated in 5% CO₂-95% air. The medium was changed daily.

Ion transport studies. Cells were grown on uncoated polycarbonate transwell filters as previously described and used for intestinal transport studies at 15 d postconfluence (16). The filter area was 4.9 cm². Each filter was mounted in an Ussing chamber (World Precision Instrument, Sarasota, FL, U.S.A.) as a flat sheet between the mucosal and the serosal compartments. Each compartment contained 10 mL of Ringer's solution with the following composition (in mM): NaCl, 114; KCl, 10; Na₂HPO₄, 1.65; NaH₂PO₄, 0.3; CaCl₂, 1.25; MgCl₂, 1.1; NaHCO₃, 15; glucose,

19. The incubation fluid was gassed with 95% O₂-5% CO₂ and maintained at 37°C by a thermostat-regulated circulating pump. The following electrical variables were monitored by an automatic voltage-clamp (DVC 1000, World Precision Instrument) as previously described (19), before and after the addition of CT or the NOS inhibitor L-NAME: PD, I_{sc} , and tissue ionic G . I_{sc} is expressed as microamperes per square centimeter ($\mu A/cm^2$), G as millisiemens per square centimeter (mS/cm²), and PD as millivolts (mV). Caco-2 cell monolayers were exposed to maximal effective doses of CT (6×10^{-8} M) added to the mucosal side in the presence or absence of a maximal effective concentration of L-NAME (2×10^{-4} M) added to both sides. CT and L-NAME maximal effective concentrations were determined by dose-response experiments previously performed (data not shown). Cell viability was checked at the end of each experiment by measuring the I_{sc} increase after the serosal addition of theophylline (5 mM).

Intracellular cAMP concentration. cAMP concentration in Caco-2 cells was determined by a commercial kit (Biotrak cAMP assay system; Amersham International, Amersham, U.K.), as previously described (20).

NO₂⁻/NO₃⁻ concentration. The combined concentrations of NO₂⁻ and NO₃⁻, the degradation products of NO in the culture medium, were determined by the Griess reaction after nitrate reduction as previously described (21). Total NO₂⁻/NO₃⁻ production is therefore referred to as NO production. Caco-2 cell monolayers were grown on plates and used at 15 d postconfluence. Experiments were also performed in Ca²⁺-free Ringer's solution to investigate whether cNOS, which is the Ca²⁺/calmodulin-dependent NOS form, rather than the iNOS form was involved. The modified Ca²⁺-free Ringer's solution had the following composition (in mM): Na₂HPO₄, 1.65; NaH₂PO₄, 0.3; NaHCO₃, 15; NaCl, 53; KCl, 10; Na₂SO₄, 30.5; MgCl₂, 2.35; glucose, 19; and EDTA, 0.5. In addition, to further investigate the iNOS activity as a possible source of the NO production induced by CT or by 8Br-cAMP, we performed experiments using the specific iNOS inhibitor L-NIL (0.5 μM) (22).

NOS expression. NOS expression was estimated by Western blot analysis. Caco-2 cells were exposed to CT (6×10^{-8} M) or to 8Br-cAMP (0.1 mM) for 1, 6, and 24 h. Cells were then scraped into PBS buffer and lysed in the following buffer (KCl, 60 mM; β -mercaptoethanol, 14 mM; EDTA, 2 mM; HEPES, pH 7.9, 15 mM; sucrose, 0.3 M; aprotinin, 5 $\mu g/mL$; leupeptin, 10 $\mu g/mL$; pepstatin, 2 $\mu g/mL$; phenylmethylsulfonyl fluoride, 0.1 mM) containing 1% Tergitol (Nonidet P-40). Total extracts were centrifuged at $1500 \times g$ for 20 min at 4°C. Protein content was determined by the Bradford method (Bio-Rad Laboratories, Munich, Germany). The supernatant containing the solubilized proteins was boiled for 5 min in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue). Cell protein (50 μg /lane) was added to SDS-PAGE and transferred to a nitrocellulose membrane (BioBlot-NC-Costar; Corning Incorporated, Canada). Blots were blocked with T-TBS buffer (Tris-HCl, pH 8.8, 10 mM; NaCl, 150 mM; Tween 20, 0.05%) containing 3% albumin, and probed for 1 h with affinity-purified anti-human NOS 1 (1:2000), NOS 2 (1:200), or NOS 3 (1:1000) rabbit polyclonal antibodies. Bound antibody was detected with anti-rabbit immunoglobulin

horseradish peroxidase-linked whole antibody and developed by chemiluminescence reaction (Amersham Pharmacia Biotech, U.K.). All incubations and washes were carried out at room temperature with gentle shaking.

γ -Interferon (50,000 U/mL) was used as a positive control in experiments performed using anti-iNOS antibodies, as previously reported (17).

Chemicals. All chemicals were of reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Culture media were from Life Technologies (GIBCO BRL, Milan, Italy). Transwell filters and supports were from Costar (Costar Italia, Milan, Italy). Anti-cNOS and anti-iNOS polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-iNOS polyclonal antibodies were purchased from Transduction Laboratories (ABD Company, Lexington, KY, U.S.A.).

Statistical analysis. All data were expressed as mean $\pm$ SD. Each experiment was run in duplicate and was repeated at least three times. Repeated-measures ANOVA was applied with Bonferroni test for multiple comparisons. The significance was set at 5%.

RESULTS

Intestinal transport studies. The simultaneous addition of L-NAME to both sides of the Caco-2 cell monolayer induced an increase in I_{sc} . The electrical response was slow in the first 35 min, then I_{sc} increased, with a peak 55 min after the addition of the NOS inhibitor. The increase in I_{sc} was totally caused by an effect on PD, as no modifications of G values were recorded. The addition of CT to the luminal side of the Caco-2 cell monolayer induced a similar increase in I_{sc} , without modifications in G . The electrical response was slow in the first 25 min, then I_{sc} increased, with a peak 35 min after toxin addition. Preincubation of Caco-2 cells for 5 min with L-NAME produced a further increase of the CT-induced I_{sc} rise, which was significantly greater than the sum of each individual testing molecule (Fig. 1).

cAMP determination. Intracellular cAMP concentrations were determined after 1 h of incubation with CT in the presence or absence of L-NAME. CT induced a 6-fold increase in the basal cAMP intracellular concentration. The addition of L-NAME produced a significant increase in basal cAMP. It also resulted in a further significant increase in CT-stimulated intracellular cAMP concentration. Again, the effect of CT and L-NAME was synergistic, being greater than the sum of each individual effect (Fig. 2).

NO_2^-/NO_3^- concentration. One hour of incubation with CT induced an increase in NO concentration of approximately 9-fold. The effect was Ca^{2+} -dependent, as the NO increase in response to CT addition was blunted in the absence of Ca^{2+} . In contrast, preincubation with the specific iNOS inhibitor, L-NIL, did not prevent the CT-induced NO increase (Fig. 3). Similar results were obtained after incubation with the cAMP analog, 8Br-cAMP, as shown in Figure 3. 8Br-cAMP was able to induce a dose- and time-dependent NO production by the enterocyte. The maximal effect was obtained at the concentration of 0.1 mM (Fig. 4) and after 30 min of incubation (Fig. 5). In this condition NO production did not show a further increase, indicating a saturation of the effect.

Figure 1. A, time course of the effect on I_{sc} of the CT and L-NAME addition, alone or in combination, to Caco-2 cells mounted in Ussing chambers. Arrows indicate the time of addition of each agent. B, maximal I_{sc} increases after the addition of CT and of L-NAME alone or in combination to Caco-2 cells mounted in Ussing chambers. Data are mean $\pm$ SD of six different observations. * $p < 0.05$ vs control; ** $p < 0.05$ vs L-NAME or CT alone.

Figure 2. Modifications of the intracellular cAMP concentration in Caco-2 cells after 1 h of incubation with CT and L-NAME, alone or in combination. Data are mean $\pm$ SD of six different observations. * $p < 0.05$ vs control; ** $p < 0.05$ vs L-NAME or CT alone.

NOS expression. Because L-NAME is a nonselective inhibitor of NOS (2), experiments were performed to assess the precise identity of NOS involved using Western blot analysis. In basal conditions cNOS (NOS 1) protein expression in Caco-2 cells was negligible, and there was no evidence of the other constitutive (NOS 3) or inducible (NOS 2) forms. Western blot analysis performed after 1 h of incubation with CT revealed stimulation of cNOS (NOS 1) protein expression, as shown by the amplification of a 160-kD band that corresponded to human NOS 1. A similar result was obtained after stimulation with 8Br-cAMP (Fig. 6). Neither CT nor 8Br-cAMP was able to elicit NOS 2 or NOS 3 protein expression with up to 24 h of incubation (data not shown).

DISCUSSION

Fluid and electrolyte secretion into the intestinal lumen is a process that serves several functions. It promotes optimal

Figure 3. Total NO production in Caco-2 cells determined in basal condition and after stimulation with CT or 8Br-cAMP, in standard conditions, in a Ca^{2+} -free medium, and in the presence of the specific iNOS inhibitor, L-NIL. Data are mean $\pm$ SD of six different observations. * $p < 0.05$ vs basal values; ** $p < 0.05$ vs standard condition.

Figure 4. Dose-response relationship of NO production to 8Br-cAMP exposure in Caco-2 cells. Increasing concentrations of 8Br-cAMP were added to Caco-2 cell monolayers, and NO production was determined after 1 h of incubation. Data are mean $\pm$ SD of six different observations.

Figure 5. Time course effect of 8Br-cAMP (0.1 mM) addition on NO production in Caco-2 cells. Data are mean $\pm$ SD of six different observations.

nutrient digestion and absorption, works in concert with mucins to protect the mucosa, and rids the gut of potentially noxious pathogens and toxins. To prevent excessive fluid losses, water and ions fluxes need to be finely tuned through effective braking mechanism (23). The present study provides evidence for the ability of the enterocyte to limit its own ion

Figure 6. cNOS protein expression in Caco-2 cell monolayers after 1 h of incubation with CT and 8-Br-cAMP. The cNOS protein expression is revealed by the appearance of a 160-kD band that corresponds to human NOS 1 (neuronal NOS). Shown is a representative of three experiments.

secretion through a mechanism that involves NO. The increase in I_{sc} elicited by the NOS inhibitor L-NAME suggests that the intracellular NOS concentration produced in the enterocyte is essential to regulate basal transepithelial ion transport. L-NAME inhibition of NO production resulted in an increased cAMP concentration and I_{sc} response, suggesting that in basal conditions NO maintains the intracellular concentration of cAMP at a low level. cAMP could therefore be the target of NO to regulate ion transport in the enterocyte.

The combined addition of CT and of L-NAME resulted in a significant increase in I_{sc} compared with that obtained with CT alone, suggesting that NO is also effective in limiting active secretion. Again, the electrical effects were closely linked to cAMP intracellular concentration. Thus a close relationship exists between the modifications of cAMP and the NO effects on ion transport, both in basal conditions and in the presence of active secretion. This may be related to an inhibitory effect by NO on adenylate cyclase, similar to that described in human monocytes (24).

$\text{NO}_2^-/\text{NO}_3^-$ determination and Western blot experiments contributed to clarifying the mechanisms of NO production and its role in response to cAMP-mediated ion secretion. The increased $\text{NO}_2^-/\text{NO}_3^-$ production in response to CT or to 8Br-cAMP indicates that NO synthesis by the enterocyte is increased in response to stimulated secretion. NO overproduction was associ-

ated with an up-regulation of NOS 1 activity, as suggested by four distinct lines of evidence: 1) the time course of NO production in response to CT or 8Br-cAMP, whose rapid increase is typical of a cNOS rather than iNOS activity (2); 2) the Ca^{2+} dependence of the effect, again typical of cNOS activity because it is known that the activity of iNOS is Ca^{2+} /calmodulin-independent (2); 3) the lack of effect elicited by the specific inhibitor of iNOS (L-NIL) on NO production after stimulation with CT or 8Br-cAMP; and 4) the Western blot gels performed with specific anti-cNOS or anti-iNOS antibody, supporting the selective cNOS activation in response to CT or 8Br-cAMP. It has been suggested that up-regulation of NOS 1 may be a component of the normal cellular stress response (25). Thus expression levels of the low-output enzymes NOS 1 and NOS 3 may be adjusted to meet local demand (25). Overall these findings are consistent with the concept of self-regulation of ion transport by the enterocyte, which operates in basal condition and, homeostatically, in conditions of stimulated secretion. *In vivo* evidence supporting the role of NO in a homeostatic mechanism comes from the increased levels of NO in both plasma and urine of patients with cholera (26).

Our data are consistent with previous results by Beubler and Schirgi-Degen (27) reporting that i.v. administration of L-NAME enhanced CT-induced ion secretion in rat ligated jejunal loops, whereas the NO precursor L-arginine inhibited it. Similarly inhibition of endogenous NO synthesis by equimolar concentrations of L-NAME caused a secretory response of water and electrolytes in animal models (2, 28, 29). However, others have reported that the NO substrate L-arginine induced electrogenic secretion in Caco-2 cells (18). These apparently conflicting results may be explained by dose-related phenomena (2). The concentration of L-arginine may play a crucial role in determining the proabsorptive or secretagogue effects. Wapnir *et al.* (30) have shown that low concentration of L-arginine, added to the oral rehydration solution, stimulates water and electrolyte absorption in the rat jejunum, whereas higher L-arginine concentrations induced a secretory shift of fluid and electrolyte transport. Alternatively, conflicting findings may be related to a species-specific effect of NO (for example, ileum *versus* human enterocytes), or to the nature of the specimens used (*i.e.* whole intestinal tissue, which contains inflammatory, neural, and endocrine-paracrine cells, all potential sources of NO) (31).

The effects of NO in response to CT in Caco-2 cells resemble those observed with other secretagogues including prostaglandin E_2 , *Escherichia coli* heat-stable enterotoxin, and *Clostridium difficile* toxin A (27), and suggest that NO is able to down-regulate fluid secretion elicited by different secretagogues, including those acting through intracellular mechanisms other than cAMP. This may suggest that NO is released in response to cyclic nucleotide concentration.

CONCLUSIONS

In conclusion, this work provided direct evidence that the enterocyte plays a direct role in the homeostatic regulation of its own ion transport processes, through the activation of the cNOS-NO pathway. This network functions as a braking force of ion secretion both in basal and in cAMP-stimulated condi-

tions. The target of this effect is cAMP, which in turn triggers an increase in NOS 1 expression, likely to be interpreted as a homeostatic response.

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