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DIRECT EVIDENCE FOR NITRIC OXIDE STIMULATION OF ELECTROLYTE SECRETION IN THE RAT COLON

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Nitric Oxide (NO) is synthesized in the intestinal tract and may serve as a physiological regulator of intestinal ion transport and/or a pathophysiologic mediator of secretory diarrhea associated with inflammatory mucosal diseases. Indirect approaches, employing inhibitors of nitric oxide synthase or compounds capable of donating NO in solution, have been used to demonstrate the effects on gastrointestinal muscle and the mucosa. To determine directly whether nitric oxide itself is capable of stimulating electrolyte secretion we mounted muscle-stripped rat distal colon in Ussing chambers and monitored short-circuit current (Isc), as an indicator of effects on mucosal ion transport. Comparisons were made to sodium nitroprusside (SNP). NO and SNP stimulated concentration-dependent (0.1 μM to 100 μM) increases in Isc, with NO being more potent than SNP. The EC_{50} for NO was approximately 8 μM compared to a value < 20 μM for SNP. The response to NO was immediate. In contrast, SNP required a mean lag-time of 41 $\pm$ 4 seconds, and a significantly longer time was required for SNP to reach its maximum effect. The response to both of these agonists was blocked by bumetanide, indicating that they were stimulating a chloride ion secretory response. The cyclooxygenase inhibitor piroxicam, the neurotoxin tetrodotoxin and the inhibitor of guanylate cyclase, methylene blue, all inhibited the response to both agonists. These studies demonstrate that NO itself can stimulate chloride secretion by the rat colonic mucosa through a prostaglandin-dependent, and partially neural mechanism that may involve guanylate cyclase.

KEY WORDS: Nitric Oxide, sodium nitroprusside, rat colon, chloride secretion.

INTRODUCTION

Nitric oxide radical (NO) is believed to be a mediator of neural, cardiovascular, immune and gastrointestinal function[1,2]. NO is biosynthesized from L-arginine through the actions (under different conditions) of three or more isoforms of NO synthase[3], at least one of which is present in the myenteric plexus of the intestine[4]. The existence of the synthase enzyme within the enteric nervous system, in close proximity to the epithelium, suggests the possibility that NO could be a physiologic regulator of intestinal ion transport. Furthermore, NO is generated by neutrophils,[5] macrophages[6] and mast cells,[7] implicating it as a potential pathophysiologic mediator of the secretory diarrhea associated with inflammatory bowel disease (IBD). Additional support for the involvement of NO in intestinal mucosal function comes from indirect evidence. The NO synthase inhibitor N^G-nitro-L-arginine

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methyl ester (L-NAME) antagonizes the fluid accumulation due to castor oil[8] and TNBS-induced colitis,[9] and sodium nitroprusside (SNP) stimulates chloride secretion,[10,11] measured as an increase in short-circuit current (Isc).

There are potential problems associated with these indirect approaches. The NO synthase inhibitors may have multiple or non-specific effects,[12,14] requiring additional control experiments. SNP is not entirely suitable because, along with NO it generates thiocyanate and cyanide,[15] the latter of which is capable of inhibiting electrolyte transport *in vitro*[16]. Other NO donors such as S-nitroso-N-acetylpenicillamine (SNAP) or 3-morpholinosydnonimine N-ethylcarbamide (SIN-1) also are not entirely appropriate to use. Along with NO, SIN-1 produces peroxynitrite, $OH^{\cdot}$ and superoxide,[17] the latter of which (in the presence of superoxide dismutase) yields H_2O_2, a known colonic secretagogue.[18,19] In the case of SNAP, hydrolysis by tissue enzymes might release penicillamine which, as a reducing agent,[15] could have effects of its own on epithelial transport. And, evidence that NO is released from donor molecules in sufficient amounts to produce the observed responses[1,2,10,11] is difficult to obtain due to its rapid breakdown as NO is formed in solution.

Over fifteen years ago nitric oxide gas was used to activate guanylate cyclase,[20] and more recently to demonstrate direct effects on tissues *in vitro*.[21,22] In the present study, we show that NO itself is a stimulant of Isc (chloride secretion) in the rat colon.

METHODS

Preparation of Intestinal Tissues

Male Sprague-Dawley rats (200–300 g, Charles River Breeding Laboratories, Wilmington, MA) were maintained on a standard laboratory diet and allowed free access to food and water before they were killed by cervical dislocation. The distal colon was excised and placed immediately in oxygenated modified Krebs-Ringer buffer (KRB) solution of the following composition (millimolar): NaCl 120.2, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2 and $NaHCO_3$ 25, glucose 11.1. The tissue was carefully stripped of its underlying longitudinal and circular muscle layers by blunt dissection. The resulting preparation was shown histologically to consist of the mucosa and submucosa.[23] Adjacent tissues (paired) were mounted as flat sheets on pins between two lucite half chambers (World Precision Instruments, Inc., New Haven, CT) having an area of 0.64 cm^2 and bathed on both sides by 10 ml of buffer solution, circulated by gas lift and maintained at 37°C by water jacketed reservoirs. The solution was gassed continuously with 5% CO_2 in O_2 and maintained at pH 7.4.

Electrical Measurements

Electrical measurements were monitored with an automatic voltage clamp (TR100-F, JWT engineering, Overland Park, KS). Direct connecting voltage and current passing electrodes (World Precision Instruments, Inc. New Haven, CT) were utilized to measure transepithelial potential difference and Isc. The Isc was continuously recorded on a Gould model 2800S recorder (Gould Inc., Cleveland, OH). Tissues were equilibrated under short-circuited conditions until the current had stabilized (usually 30–45 min). All responses shown in figures 1,3,4 are the maximum achieved (Δmax Isc), regardless of the time required.

Preparation of NO solution

Stock solutions of NO were prepared immediately before use, essentially by the method of Shikano *et al.*[21] One hundred ml of KRB solution was placed in a rubber-stoppered glass flask. The flask was evacuated under vacuum for 30 min at room temperature then flushed on ice (0°C) with O_2-free argon gas for 20 min. Nitric oxide gas (Matheson, 99.99%) was bubbled through the solution for 1 min and it also saturated the atmosphere in the flask. After vortexing, and twenty minutes longer on ice, the undissolved NO was removed by flushing the flask with argon gas.

Preparing the solutions in the above manner and assuming saturation, the maximal concentration of NO was 3.3 mM.[21] Thus, when 0.3 ml of buffer saturated with NO was added to the serosal side (10 ml capacity) of the Ussing chamber (after removal of the same volume), this represented an NO concentration upper limit of 0.11 nM. The initial concentrations of NO were calculated on this basis. The half-life of NO in physiologic buffer at 37°C is 30.4 ± 2.2 sec,[22] a long enough time to stimulate and maintain the Isc response (because the circulation time in the Ussing chamber is only a few seconds and maximal responses occurred in <3 min). Nevertheless, because of expected breakdown of NO after addition to the tissue, the concentrations referred to in the figures and the table may be slightly over-estimated.

Effects of Drugs

Bumetanide, piroxicam, TTX and methylene blue were added to the serosal side of the Ussing chamber 15 min before addition of the NO or SNP. Piroxicam and bumetanide were dissolved in dimethyl sulfoxide (final concentration 0.1%). Addition of the solvent itself did not affect Isc. All other drugs were dissolved in deionized water or KRB. NO and SNP did not alter the pH of the serosal solution. Carbachol (100 mM) was added at the end of each experiment to confirm tissue viability.

Chemicals

Bumetanide, methylene blue, piroxicam, tetrodotoxin and sodium nitroprusside were purchased from Sigma Chemical Co. (St. Louis, MO). Nitric oxide (99.99%) was obtained from Matheson Gas. All chemicals used for the Krebs-Ringer electrolyte solution were of the purest grade available from Sigma Chemical Co. or J.T. Baker (Phillipsburg, NJ).

Data analysis

Data are expressed as means ± standard error for maximal changes in Isc ($\mu A/cm^2$). Dunnett's or Student's *t*-test were used (as indicated in the table and figure legends) to determine statistical significance of a particular comparison.

RESULTS

NO and SNP evoked concentration-dependent (0.1 μM to 100 μM) increases in the Isc response in the rat colon; above 10 μM NO produced a greater effect and reached a higher maximum than SNP (Figure 1). The EC_{50} value for NO was 8 μM. The EC_{50} for SNP would be >20 μM (because a maximum was not clearly reached). The

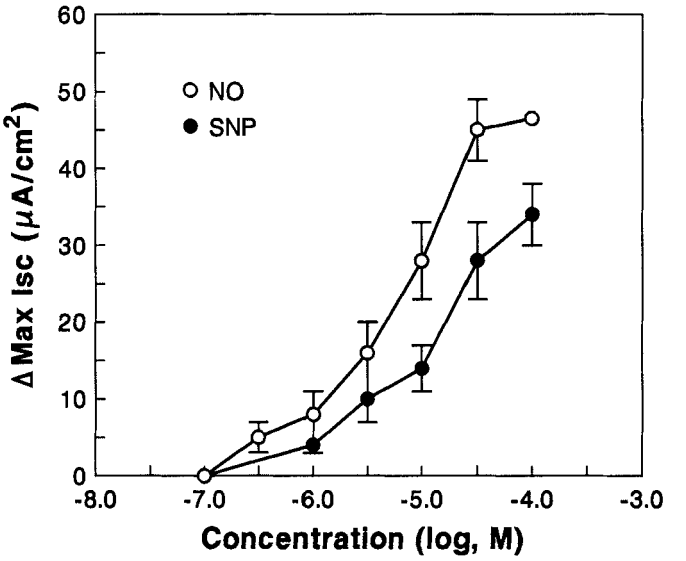

Figure 1 Concentration-response curve to serosal NO and SNP in rat colon. Each point is the mean ± SE for 3 to 5 tissues, except for NO at 10^{-4}M, which is the mean of two separate experiments.

responses shown in Figure 1 were recorded as the maximum achieved, independent of the time required. However, at all concentrations there was a delay in the initiation of the SNP response, in contrast to an immediate response observed upon addition of NO.

The time lag to initiation of the response to 50 μM SNP (Figure 2) and time required to reach the maximum was significantly greater for SNP compared to NO (Table 1). The lag-time decreased, but not significantly so, as the concentration of SNP was increased from 1.0 μM to 50 μM (Figure 3). The rate of decay of the response (approximately 10 min) was essentially the same for NO and SNP. Carbachol, a muscarinic agonist that stimulates epithelial cells to actively secrete chloride ions,[24] produced a rapid typical response in tissues previously exposed to NO or SNP (Figure 2), indicating normal tissue viability and responsiveness.

The NO-evoked Isc response was inhibited by the $Na^+/K^+/Cl^-$ co-transport inhibitor bumetanide,[25] the cyclooxygenase inhibitor piroxicam,[26] the axonal conduction blocker tetrodotoxin (TTX)[2] and methylene blue, an inhibitor of soluble guanylate cyclase[28] (Figure 4). The response to an equimolar concentration of SNP (50 μM) was inhibited to the same degree as the NO response.

DISCUSSION

There is increasing evidence, accumulated indirectly through the use of L-arginine or NO synthase inhibitors, that NO is a regulator of intestinal function.[2,4,8,9,29-31] The results of the present paper support this idea and go one step further by demonstrating an effect of NO itself on colonic mucosal secretion. SNP is widely used as an NO donating molecule but there are problems, such as its potential intrinsic effects

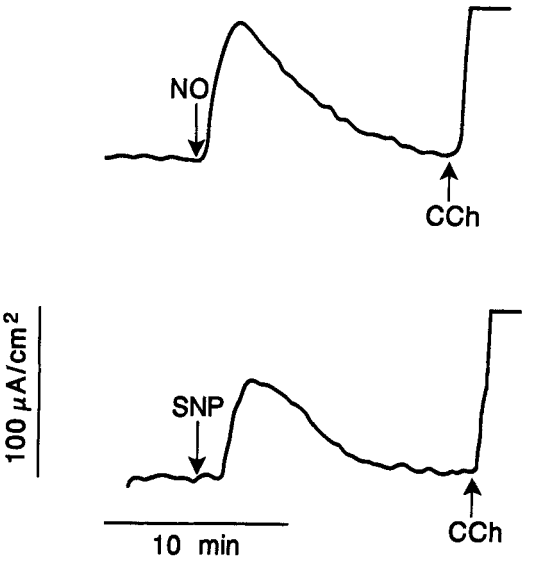

Figure 2 Typical tracing of NO- and SNP-induced Isc increases in rat colon. Each agonist (50 μM) was added to the serosal of the Ussing chamber at the time indicated by the arrows. The muscarinic agonist carbachol (CCh) was added (100 μM) after NO or SNP, to confirm tissue viability. Note the immediate response to NO as opposed to the lag-time (on average 41 ± 4 seconds, see Table 1) for SNP.

TABLE 1
Comparative Effects of NO and SNP on Isc Across Rat Colon

Agonist	Time (Sec) to Start of Response	Time (Sec) to Achieve ΔMax Isc	ΔMax Isc ($\mu A/cm^2$)	n
NO	3 ± 1	131 ± 7	47 ± 6	5
SNP	41 ± 4**	170 ± 9*	29 ± 4	3

The initial concentration of both the agonists was 50 μM. Values are means ± SE.
*$p < 0.05$, **$p < 0.001$ compared to NO (Student's t-test).

and the release of cyanide,[15] associated with its use. Just as NO may not be identical to endothelium-derived relaxing factor,[21,23] SNP cannot be assumed to be identical to NO in its actions, as SNP has effects that appear to be independent of NO.[33,34]

SNP elicits fluid secretion in the mouse intestine[35] and stimulates Isc and chloride secretion in the guinea pig ileum[10] and rat colon,[11] respectively. Our results with bumetanide, and TTX (Figure 4) confirm that SNP stimulates chloride ion secretion by the rat colon, partly by activation of intrinsic nerves,[11] and show that NO itself produced this same profile of response. Importantly, NO acted faster and was more potent than SNP.

That prostaglandins contribute to the NO and SNP response is suggested from the inhibition by the cyclooxygenase inhibitor piroxicam (Figure 4). Other investigators have reported a prostaglandin component to the effect of NO on canine colonic muscle.[36] Prostaglandins are stimulants of colonic secretion[37] and may participate in the

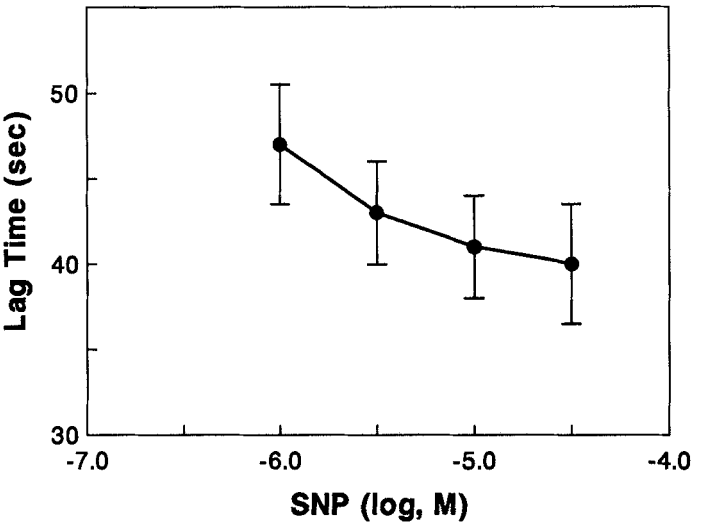

Figure 3 Illustration of the relationship between lag-time and onset of the Isc response in rat colon. SNP was added to the serosal chamber. Each point is the mean ± SE for three to five animals.

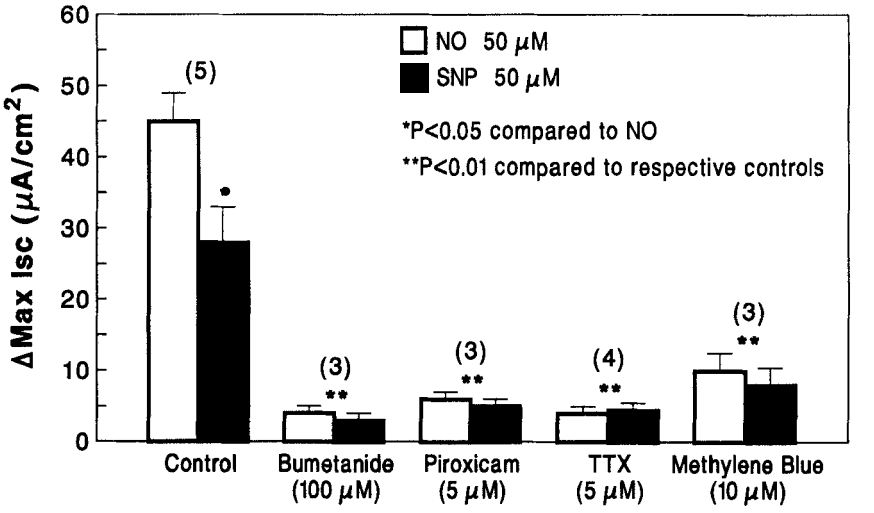

Figure 4 Effects of bumetanide, piroxicam, TTX and methylene blue on the Isc response to NO and SNP in rat colon. NO and SNP inhibitors were added to the serosal side of tissue; inhibitors were added 15 min before NO or SNP. The numbers of tissues (animals) are shown in parentheses. Means ± SE are shown. SNP produced a response significantly (Student's paired *t*-test) less than NO, and all drug treatments significantly (Dunnett's *t*-test) depressed the control response.

NO response through a direct action on the epithelial cells or indirectly via activation of neural pathways. The reactive oxygen metabolites H_2O_2 and NH_2Cl stimulate colonic secretion through a prostaglandin related neural mechanism.[18,19] The identity of the neurotransmitter responsible is unknown. It is known, however, that NH_2Cl releases acetylcholine (a colonic secretagogue)[38] from colonic submucosal nerves.[39] Another endogenous stimulant of colonic secretion, vasoactive intestinal peptide (VIP), is claimed to be released by NO through a presynaptic action on myenteric neurons.[30] Ricinoleic acid (the active component in the laxative castor oil), reported to act through NO and prostaglandins,[40] releases VIP from the intestinal mucosa.[41]

The effects of NO and SNP were both inhibited by methylene blue, an inhibitor of guanylate cyclase activation.[28] Stimulation of guanylate cyclase and cGMP formation in intestinal epithelial cells leads to chloride secretion.[42] Therefore, although we did not measure cGMP production, our results with methylene blue suggest that NO or its decomposition product(s) promote the secretory response in part through an effect on (neural or epithelial cell) guanylate cyclase. SNP stimulates cGMP production[43] and methylene blue inhibits SNP-induced electrolyte transport in the rat colon, as reported previously,[11] and in the present study.

Compared to NO, the lag-time (41 ± 4 sec) to initiate the SNP response was significantly greater (by 10-fold), and a longer period of time was required to reach the maximal ΔIsc (see Figure 2, Table 1). These data, along with our finding of a concentration-related decrease in lag-time to SNP, suggest that time is required for SNP to generate a product that evokes the response.

SNP generates CN^-, a known inhibitor of epithelial electrolyte transport[16]. It is possible that CN^- contributes to a blunting of the SNP response compared to NO at all concentrations. The concentration of CN^- generated would likely be small enough ($< 100\,\mu M$) to compromise the metabolic capacity of the cells responsible (indirectly or directly) for the secretion without completely stopping cellular respiration.[16] However, the rate of decay of the response (~10 min.) was similar for both agonists, indicating that the effect of NO, like SNP, was readily reversible, and the tissue responded robustly to CCh stimulation, validating its viability.

NO decomposes spontaneous in the presence of molecular oxygen to yield nitrogen oxides, nitrite and nitrate.[44,45] The latter two anions were previously demonstrated to have no effect on Isc in rat colon,[11] making it unlikely that they contribute to the NO or SNP response. However, it is possible that other nitrogen oxides, especially N_2O_3 are involved in the action of NO, especially in the pathophysiologic secretory response to mucosal inflammation (*vide infra*). SNP cannot be assumed to be free of direct effects on ion transport, as it has been cautioned that SNP has a strong NO^+ character, which may be responsible for its biological effects.[45]

Based upon our data, a scheme can be proposed for the possible interactions among NO (or N_2O_3 and other NO metabolites), neurons and prostaglandins to bring about Cl^- secretion (Figure 5). Physiologically, NO might be released by non-adrenergic non-cholinergic enteric nerves; pathophysiologically, granulocytes would contribute NO.[46] Fibroblasts are believed to be a major source of prostaglandins generated by mucosal stimulation of Isc by H_2O_2[18]. NO might evoke release of prostaglandins as H_2O_2 does, and this would be blocked by piroxicam. The prostaglandins, through stimulation of epithelial cell receptors linked to adenylate cyclase,[47] would promote Cl^- secretion into the intestinal lumen. A second pathway contributing to the response could involve stimulating cholinergic neurons or neurons containing VIP, which can stimulate muscarinic receptors linked to guanylate cyclase

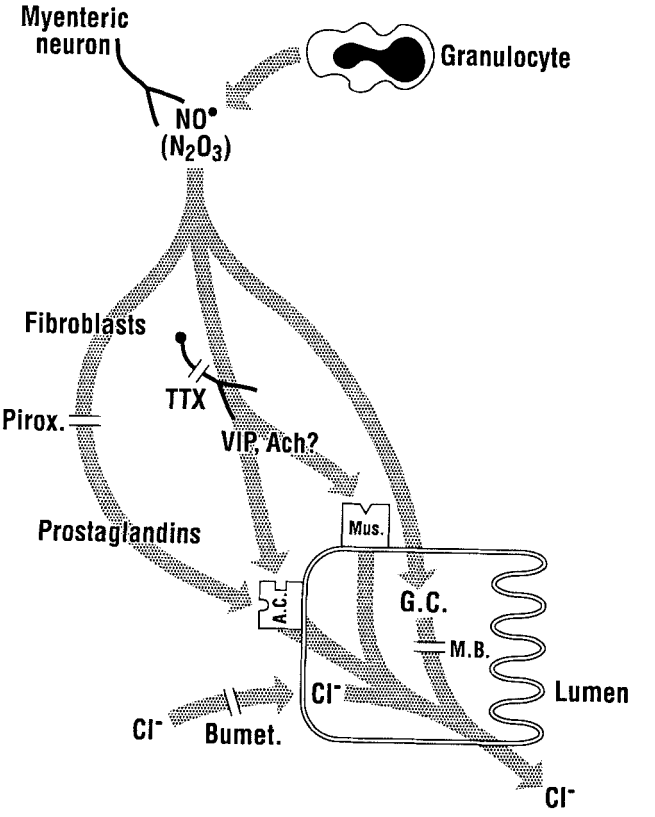

Figure 5 Scheme for the mechanisms potentially responsible for NO evoked Cl^- secretion by rat colon. Physiologically, NO (N_2O_3 or nitrogen oxides other than NO_2 or NO_3) may arise from myenteric neurons; in inflammatory mucosal disease the source could be granulocytes. Stimulation by NO of fibroblasts (or other mesenchymal elements) may generate prostaglandins, which are known to evoke Cl^- secretion through an adenylate cyclase (A.C.) coupled mechanism. This would be blocked by the cyclooxygenase inhibitor piroxicam (Pirox.). NO might also release vasoactive intestinal peptide (VIP) or acetylcholine (Ach) from submucosal neurons, with subsequent activation by these known secretagogues of epithelial cell A.C. and muscarinic receptors (Mus.), linked to Cl^- secretory mechanisms. An alternative pathway could be through direct stimulation of cytosolic guanylate cyclase (G.C.), which is inhibited by methylene (M.B.). Bumetanide (Bumet.) is an inhibitor of $Na^+/K^+/Cl^-$ Co-transport responsible for Cl^- accumulation within the epithelial cell. Bumet. will block the effects of NO because it prevents Cl^- from entering the cell, therefore the anion cannot be secreted into the lumen.

or receptors coupled to adenylate cyclase, respectively.[47] This response would be blocked by TTX. Finally, NO might directly stimulate guanylate cyclase; this response would be blocked by methylene blue.

The significance of NO-induced colonic secretion must be considered from at least two perspectives. One is as a physiologic regulator of colonic function. By adding electrolytes (and thus water) to the feces, transit of material through the colon and defecation will be facilitated. Another, potentially more important effect of NO relates to the pathophysiology and therapy of IBD. Roediger et al,[48] detected significantly elevated levels of nitrite in 78% of patients with acute ulcerative colitis,

along with abnormal colonic electrolyte transport. More recently, L-NAME was shown to prevent the luminal fluid accumulation in TNBS-induced colitis in guinea pigs,[9] implicating NO as a mediator of the pathophysiology in this animal model of IBD. Inflammatory granulocytes can provide a source of NO.[46] In the inflamed mucosa NO may react with superoxide and other oxygen metabolites to yield peroxynitrite,[5,17] nitrite and nitrosamines,[46] resulting in the release of neurotransmitters,[39] cell injury, and a direct influence on epithelial function.

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References

1. S. Moncada, R.M.J. Palmer and E.A. Higgs (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews*, **43**, 109–142.
2. M.E. Stark and J.H. Szurszewski (1992) Role of nitric oxide in gastrointestinal and hepatic function and disease. *Gastroenterology*, **103**, 1928–1949.
3. H.H.H.W. Schmidt, J.S. Pollock, M. Nakane, U. Förstermann and F. Murad (1992) Ca^{2+}/calmodulin-regulated nitric oxide synthases. *Cell Calcium*, **13**, 427–434.
4. P. Kostka, E. Jang, E.G. Watson, J.L. Stewart and E.E. Daniel (1993) Nitric oxide synthase in the autonomic nervous system of canine ileum. *Journal of Pharmacolog and Experimental Therapeutics*, **264**, 234–239.
5. T.B. McCall, N.K. Boughton-Smith, R.M.J. Palmer, B.J.R. Whittle and S. Moncada (1989) Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochemical Journal*, **261**, 293–296.
6. K.-E. Kröncke, V.K-. Bachofen, B. Berchick, V. Burkart and H. Kolb (1991) Activated macrophages kill pancreatic syngeneic islet cells via arginine-dependent nitric oxide generation. *Biochemical Biophysical Research Communications*, **175**, 752–758.
7. E.Y. Bissonnette, C.W. Hogaboam, J.L. Wallace and A.D. Befus (1991) Potentiation of tumor necrosis factor-α-mediated cytotoxicity of mast cells by their production of nitric oxide. *Journal of Immunology*, **147**, 3060–3065.
8. N. Mascolo, A.A. Izzo, B. Barbato and F. Capasso (1993) Inhibitors of nitric oxide synthetase prevent castor-oil-induced diarrhoea in the rat. *British Journal of Pharmacology*, **108**, 861–864.
9. M.J.S. Miller, H. Sadowska-Krowicka, S. Chotinaruemol, J.L. Kakkis and D.A. Clark (1993) Amelioration of chronic ileitis by nitric oxide synthase inhibition. *Journal of Pharmacology and Experimental Therapeutics*, **264**, 11–16.
10. W.K. MacNaughton, A. Calarco and A. Krantis (1992) Nitric oxide (NO) donating compounds stimulate electrolyte transport in the guinea pig intestine. *Gastroenterology*, **102**, A233.
11. K.T. Wilson, Y. Xie, M. Musch and E. Chang (1993) Sodium nitroprusside stimulates anion secretion and inhibits NaCl absorption in rat colon. *Journal of Pharmacology and Experimental Therapeutics*, (In Press).
12. C. Nathan (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB Journal*. **6**, 3051–3064.
13. D.L. Granger, J.B. Hibbs Jr., J.R. Perfect and D.T. Durack (1990) Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *Journal of Clinical investigation*, **85**, 264–273.
14. G. Werner-Felmayer, E.R. Werner, D. Fuchs, A. Hausen, G. Reibnegger and H. Wachter (1990) Tetrahydrobiopterin-dependent formation of nitrite and nitrate in murine fibroblasts. *Journal of Experimental Medicine*, **172**, 1599–1607.
15. J.N. Bates, M.T. Baker, R. Guerra and D.G. Harrison (1991) Nitric oxide generation from nitroprusside by vascular tissue: evidence that reduction of the nitroprusside anion and cyanide loss are required. *Biochemical Pharmacology*, **42** (Suppl), s157–165.
16. M.J. Stutts, T. Gatzy and R.C. Boucher (1988) Effects of metabolic inhibition on ion transport by dog bronchial epithelium. *Journal of Applied Physiology*, **64**, 253–258.
17. N. Hogg, V.M. Darley-Usmar, M.T. Wilson and S. Moncado (1992) Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochemical Journal*, **281**, 419–424.

18. S.S. Karayalcin, C.W. Sturbaum, J.T. Wachsman, J-.H. Cha and D.W. Powell (1990) Hydrogen peroxide stimulates rat colonic prostaglandin production and alters electrolyte transport. *Journal of Clinical Investigation*, **86**, 60–68.
19. H. Tamai, J.F. Kachur, D.A. Baron, M.B. Grisham and T.S. Gaginella (1991) Monochloramine, a neutrophil-derived oxidant, stimulates rat colonic secretion. *Journal of Pharmacology and Experimental Therapeutics*, **257**, 887–894.
20. F. Murad, C. Mittal, W.P. Arnold, S. Katsuki and H. Kimura (1978) *Advances in Cyclic Nucleotide Research*, Vol. 9. (eds. W.J. George and L.J. Ignarro). Raven Press, New York, p. 145.
21. K. Shikano, C.J. Long, E.H. Chilstein and B.A. Berkowitz (1988) Comparative pharmacology of endothelium-derived relaxing factor and nitric oxide. *Journal of Pharmacology and Experimental Therapeutics*, **247**, 873–881.
22. R.M.J. Palmer, A.G. Ferrige and S. Moncada (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* (Lond), **327**, 524–526.
23. Z-.C. Wu and T.S. Gaginella (1981) Functional properties of noradrenergic nervous system in rat colonic mucosa: uptake of [^{3}H]norepinephrine. *American Journal of Physiology*, **241**, G137–G142.
24. K. Dharmsathaphorn and S.J. Pandol (1986) Mechanism of chloride secretion induced by carbachol in a colonic epithelial cell line. *Journal of Clinical Investigation*, **77**, 348–354.
25. K. Dharmsathaphorn, K.G. Mandel, H. Masui and J.A. McRoberts (1985) Vasoactive intestinal polypeptide-induced chloride secretion by a colonic epithelial cell line: direct participation of a basolaterally localized Na^+, K^+, Cl^- cotransport system. *Journal of Clinical Investigation*, **75**, 462–471.
26. R.M. Burch, W.C. Wise and P.V. Halushka (1983) Prostaglandin-independent inhibition of calcium transport by nonsteroidal anti-inflammatory drugs: differential effects of carboxylic acids and piroxicam. *Journal of Pharmacology and Experimental Therapeutics*, **227**, 84–91.
27. T. Narahashi (1972) Mechanism of action of tetrodotoxin and saxitoxin on excitable membranes. *Federation Proceedings*, **31**, 1117–1123.
28. C.A. Gruetter, P.J. Kadowitz and L.J. Ignarro (1981) Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerin, sodium nitrite, and amyl nitrite. *Canadian Journal of Physiology and Pharmacology*, **59**, 150–165.
29. K.M. Sanders and S.M. Ward (1992) Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *American Journal of Physiology*, **262**, G379–G392.
30. J.R. Grider, K.S. Murthy, J.-G. Jin and G.M. Makhlouf (1992) Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *American Journal Physiology*, **262**, G774–G778.
31. S.J. Konturek, J. Bilski, P.K. Konturek, M. Cieszkowski and W. Pawlik (1993) Role of endogenous nitric oxide in the control of canine pancreatic secretion and blood flow. *Gastroenterology*, **104**, 896–902.
32. P.R. Myers, R.J. Jr. Minor, R. Jr. Guerra, J.N. Bates and D.G. Harrison (1990) Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature* (Lond), **345**, 161–163.
33. L. Kiedrowski, E. Costa and J.T. Wroblewski (1992) Sodium nitroprusside inhibits N-methyl-D-aspartate-evoked calcium influx via a nitric oxide- and cGMP-independent mechanism. *Molecular Pharmacology*, **41**, 779–784.
34. L. Kiedrowski, H. Manev, E. Costa and J.T. Wroblewski (1991) Inhibition of glutamate-induced cell death by sodium nitroprusside is not mediated by nitric oxide. *Neuropharmacology*, **30**, 1241–1243.
35. D.D. Thomas and F.C. Knoop (1982) The effect of calcium and prostaglandin inhibitors on the intestinal fluid response to heat-stable enterotoxin of Escherichia coli. *Journal of Infectious Diseases*, **145**, 141–147.
36. S.M. Ward, H.H. Dalziel, K.D. Thornbury, D.P. Westfall, D.P. and K.M. Sanders. (1992) Nonadrenergic, noncholinergic inhibition and rebound excitation in canine colon depend on nitric oxide. *American Journal of Physiology*, **262**, G237–G243.
37. T.S. Gaginella (1990) Eicosanoid-mediated intestinal secretion. In *Secretory Diarrhea* (eds E. Lebenthal and M. Duffey), Raven Press, New York, pp. 15–30.
38. P.E.T. Isaacs, C.L. Corbett, A.K. Riley, P.C. Hawker and L.A. Turnberg (1976) *In vitro* behavior of human intestinal mucosa. The influence of acetylcholine on ion transport. *Journal of Clinical Investigation*, **58**, 535–542.
39. T.S. Gaginella, M.B. Grisham, D.B. Thomas, R. Walsh and C. Moummi (1992) Oxidant-evoked release of acetylcholine from enteric neurons of the rat colon. *Journal of Pharmacology and Experimental Therapeutics*, **263**, 1068–1073.
40. E. Beubler and J. Juan (1979) Effect of ricinoleic acid and other laxatives on net water flux and prostaglandin E release by the rat colon. *Journal of Pharmacy Pharmacology*, **31**, 681–685.

41. T. Frye, B. Kerzner, T.M. O'Dorisio and T.S. Gaginella (1979) Ricinoleic acid mediated vasoactive intestinal polypeptide release in rat jejunum. *Clinical Research*, **27**, 717.
42. M. Field, L.H. Graf, W.J. Laird and P.L. Smith (1978) Heat-stable enterotoxin of Escherichia coli: *in vitro* effects of guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. *Proceedings of the National Academy of Sciences*, USA, **75**, 2800–2804.
43. K.T. Wilson, A.B. Vaandrager, J. de Vente, M.W. Musch, H.R. de Jonge and E.B. Chang (1993) Second messenger pathways involved in sodium nitroprusside-stimulated colonic electrolyte transport: localization of cyclic nucleotide and PGE2 production to the subepithelium. *Gastroenterology*, **104**, A290
44. M.A. Marletta (1988) Mammalian synthesis of nitrite, nitrate, and N-nitrosating agents. *Chemical Research and Toxicology*, **1**, 249–257.
45. J.S. Stamler, D.J. Singel and J. Loscalzo (1992) Biochemistry of nitric oxide and its redox-activated forms. *Science*, **258**, 1898–1902.
46. Grisham, K. Ware, H.E. Jr. Gilleland, L.B. Gilleland, C.L. Abell and T. Yamada (1992) Neutrophil-mediated nitrosamine formation: role of nitric oxide in rats. *Gastroenterology*, **103**, 1260–1266.
47. T.S. Gaginella (1990) Neurochemical receptor pharmacology of intestinal secretion. In *Secretory Diarrhea* (eds E. Lebenthal and M. Duffey), Raven Press, New York, pp. 163–178.
48. W.E.W. Roediger, M.J. Lawson, S.H. Nance and B.C. Radcliffe (1986) Detectable colonic nitrite levels in inflammatory bowel disease – mucosal or bacterial malfunction? *Digestion*, **35**, 199–204.

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