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Regulation of electrolyte transport by nitric oxide in the mouse cecum

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Abstract

The effect and role of nitric oxide (NO) in the regulation of ion transport in the mouse cecum were investigated. L-arginine, used to increase NO production, increased short-circuit current (I_{sc}), a measure of ion transport, in a concentration-dependent manner with a maximal increase of 193.8 ± 65.5 $\mu A/cm^2$. This increase was not changed in Cl^-- or HCO_3^--free buffers, but was significantly decreased in Na^+-free buffer. Using immunohistochemistry, the constitutive form of nitric oxide synthase was found not to be different in the inflamed cecum. The inducible form of the enzyme, however, which was absent in the cecum of normal mice, was present in high levels in the cecum of the colitic mouse. These results suggest that NO causes an increase in Na^+ absorption. The increased levels of inducible NO synthase in the inflamed cecum suggest a role for NO in the pathophysiology of inflammatory bowel disease.

Keywords: Cecum; Ion transport; Short-circuit current; Nitric oxide (NO); Inflammatory bowel disease

1. Introduction

NO is an endogenous intercellular messenger, which mediates a variety of biological activities in different tissues including smooth muscle relaxation, platelet inhibition, neurotransmission, and immune regulation. It is synthesized from L-arginine by the action of the enzyme NO synthase, which exists in distinct isoforms, constitutive NO synthase isoforms, and inducible NO synthase isoform. The constitutive form of the enzyme is activated in response to a stimulant and only results in the release of small amounts of NO (pmol) for short periods of time. The inducible form when activated, however, results in the release of large amounts of NO for longer periods of time. The readily diffusible NO transduces a signal via its action on guanylate cyclase to increase the production of cGMP, which, in turn, mediates further signal transduction pathways.

NO is generated by neutrophils, macrophages and mast cells implicating a role for NO in the pathophysiology of inflammatory bowel disease. Immunohistochemical staining of NO synthase revealed the presence of the inducible enzyme in the trinitrobenzene sulfonic acid-treated inflamed ileum of guinea pigs and the colon of colitic rhesus monkeys in the epithelial and lamina propria cells, while it is only present in myenteric plexus in control ileum (Ribbons et al., 1995).

Recently, it has been suggested that nitric oxide (NO) may be included in the list of inflammatory mediators (Boughton-Smith et al., 1993). However, under normal conditions, NO has been shown to serve an important role in the maintenance of tissue and vasculature integrity, neurotransmission, and motility in the gastrointestinal tract (Stark and Szurszewski, 1992). Under physiological conditions, the constitutive NO synthase predominates, and it is important in the function of many cells, including smooth muscle, platelets, neutrophils and epithelial cells. In the small intestine, NO has been shown to be important in the preservation of normal intestinal permeability (Kubes, 1992).

Under pathological states, however, the synthesis of micromolar quantities of NO during inflammation, due to the induction of inducible NO synthase, has been found to be deleterious to the cells through the formation of nitric oxide reactive products (Beckman et al., 1990). Nitric oxide formation may contribute to the gastrointestinal immunopathology during chronic inflammatory events. Active ulcerative colitis is associated with increased levels of

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inducible NO synthase activity (Boughton-Smith et al., 1993). It is possible that inhibition of NO production may reverse the tissue injury and other clinical symptoms associated with inflammation. Blockade of NO production by N^G-nitro-L-arginine-methyl ester (L-NAME) has been shown to have protective effects in animal models of colitis (Miller et al., 1993; Hogaboam et al., 1995).

In this study, the effect and role of NO on ion transport were studied in normal mouse cecum. A mouse model of colitis, the C3H/HeJBir substrain (Sundberg et al., 1994) has been shown to develop a spontaneous, heritable form of idiopathic inflammation. The substrain was used to localize the NO enzymes, both constitutive NO synthase and inducible NO synthase, and compare their distribution to that of the normal cecum.

2. Materials and methods

2.1. Materials

Healthy female C3H/HeJ mice and inbred C3H/HeJBir (colitic mice) were obtained from Jackson Laboratory (Bar Harbor, Maine) and were maintained on a standard diet with free access to water. Mice ($n = 8$) of the substrain C3H/HeJBir, which tested positive for inflammation by occult blood test, were sent to us directly from Jackson Laboratory. All the mice tested had inflammation (of varying degree) in the cecum as was reported recently (Sundberg et al., 1994; Tripodi et al., 1996).

L-arginine, sodium nitroprusside, bovine serum albumin, tetrodotoxin, atropine, N^G-nitro-L-arginine-methyl ester (L-NAME) and acetazolamide were purchased from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit immunoglobulin (IgG) was from Jackson ImmunoResearch Labs (West Grove, PA). Inducible NO synthase (monoclonal immunoglobulin-G (IgG) mouse anti-macrophage NO synthase) and mouse constitutive NO synthase (brain NO synthase, monoclonal IgG mouse brain NO synthase) were purchased from Transduction Laboratories (Lexington, KY).

2.2. Transport studies

The animals were used between 8 and 12 weeks of age. Mice were killed by exposure to 100% CO_2, and the cecum was immediately removed and washed in Krebs–HCO_3 buffer. The cecum was cut open along the mesenteric border and full thickness cecal tissue was mounted as a flat sheet between two Lucite modified Ussing chambers having an area of 0.13 cm^2 and oxygenated and maintained at 37°C. Short-circuit current (I_{sc}) which has been shown to be equivalent to the electrical sum of all ion transport processes occurring simultaneously, was determined. An automatic voltage clamp (WPI, Sarasota, FL), corrected for fluid resistance between the potential difference sensing bridges, provided continuous short-circuiting of the tissue.

Unless specified, the bathing solution consisted of Krebs–HCO_3 composed of (in mM): KCl 4.8, $CaCl_2$ 2.5, NaCl 118.1, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, Glucose 11, pH 7.4 after gassing with 95% O_2–5% CO_2. Experiments with chloride-free or sodium-free buffers were performed by substituting Cl with gluconate or substitute Na^+ with choline. The Cl-free buffer composed of (in mM): Na-gluconate 118, K_2SO_4 4.7, $CaSO_4$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, glucose 11.1, and $NaHCO_3$ 25, pH 7.4 after gassing with 95% O_2–5% CO_2. The Na^+-free buffer composed of (in mM): choline–Cl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.1, choline–HCO_3 25, pH 7.4 after gassing with 95% O_2–5% CO_2. The bicarbonate-free buffer composed of (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.1, and HEPES 25, pH 7.4. The bicarbonate-free buffer was always used in the presence of 1 mM acetazolamide to inhibit endogenous bicarbonate production.

The cecum was divided into 2 pieces and the tissues paired and used if their conductance did not differ by more than 25%. Immediately after being mounted, the tissues exhibited an initial decrease in I_{sc} which stabilized in around 30 min. All I_{sc} and PD values reported were therefore measured after the initial 40 min when the tissue had reached a stable I_{sc}. Unless specified, all drugs were added to the serosal side of the cecal tissue.

Statistical analyses were performed with Student's *t*-tests for paired and unpaired data (Segel, 1976). Unless specified, all results are reported as mean ± S.E.

2.3. Immunohistochemical staining

Sections from normal and colitic mouse cecum were frozen and then sectioned. They were then rehydrated and fixed with acetone for 20 min at room temperature. The slides were rinsed with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, and then incubated with 5% donkey serum in PBS containing 0.1% bovine serum albumin for 30 min. The slides were then washed twice in the PBS buffer in the presence of 0.1% bovine serum albumin for 5 min and were incubated with the antibodies to either mouse-inducible NO synthase (1:25 dilution, monoclonal immunoglobulin-G (IgG) mouse anti-macrophage NO synthase purchased from Transduction Laboratories, Lexington, KY) or mouse constitutive NO synthase (1:25, brain NO synthase, monoclonal IgG mouse brain NO synthase purchased from Transduction Laboratories, Lexington, KY) for 60 min at room temperature. The slides were then washed 3 times with PBS/bovine serum albumin buffer for 5 min each washing, and later incubated with a fluorescein isothiocyanate (FITC)-labelled second antibody (1:50 dilution, Donkey anti-mouse IgGγ2a FITC) for 60 min at room temperature. The slides were then washed 3 times for 5 min each in PBS/bovine serum

albumin buffer. Localization of the NO synthase in question was evaluated using fluorescence microscopy. Isotype controls (first antibody, purified mouse IgG2a) were also performed to test for non-specific labeling.

3. Results

3.1. Effects of NO on basal short-circuit current (I_{sc})

The basal I_{sc} in the normal mouse cecum was found to be 82.6 ± 5.8 $\mu A/cm^2$ ($n = 35$) and was completely dependent on Na^+ absorption (Tripodi et al., 1996). To test the effects of NO on basal I_{sc}, NO was generated through the addition of its precursors, L-arginine or sodium nitroprusside. Serosal addition of different concentrations of L-arginine or sodium nitroprusside caused an increase in NO levels as measured by an ISO-NO meter (WPI, Sarasota, Fl). These increases in NO levels correlated with increases in I_{sc} (Figs. 1 and 2), suggesting that NO either increases anion secretion or cation absorption. L-arginine was found to increase I_{sc} in a concentration-dependent manner with an EC_{50} of around 80 mM. The peak increase in I_{sc} was 193.8 ± 65.5 $\mu A/cm^2$ at around 300 mM L-arginine ($n = 4$, Fig. 3A) and was reached in 5 min after the addition of L-arginine. At this concentration of L-arginine, the concentration of NO produced was 44.3 ± 17.9 nM (Fig. 1). The addition of L-arginine had no effect on the conductance of the tissue ($G = 4.6 \pm 0.6$ mS/cm^2 before the addition of L-arginine vs. $G = 4.7 \pm 0.7$ mS/cm^2 after the addition of L-arginine). Mucosal addition of L-arginine, as well as serosal addition of D-arginine did not have any effect on basal I_{sc} (data not shown).

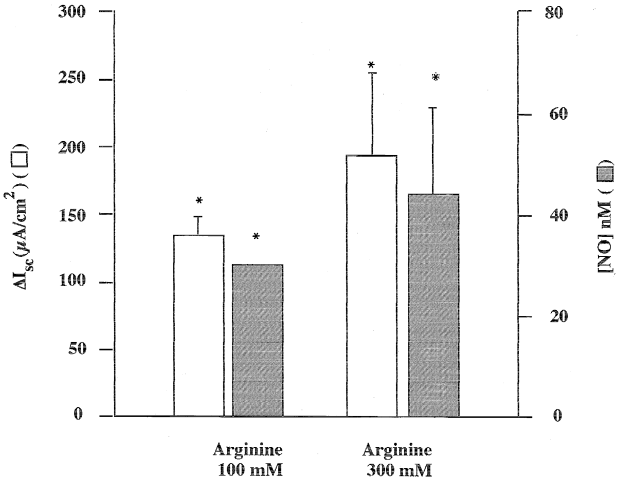

Fig. 1. The effect of two concentrations of L-arginine on short-circuit current and on the levels of NO ($n = 4$ for each concentration). L-arginine was added to the serosal side of the cecal tissue and the maximum increase in I_{sc} recorded. At the same time and the same concentrations of L-arginine, NO levels were measured in the chambers using the ISO-NO meter (WPI). $* P < 0.05$; P value represents the comparison of the effect of L-arginine on I_{sc} as compared to control untreated tissue.

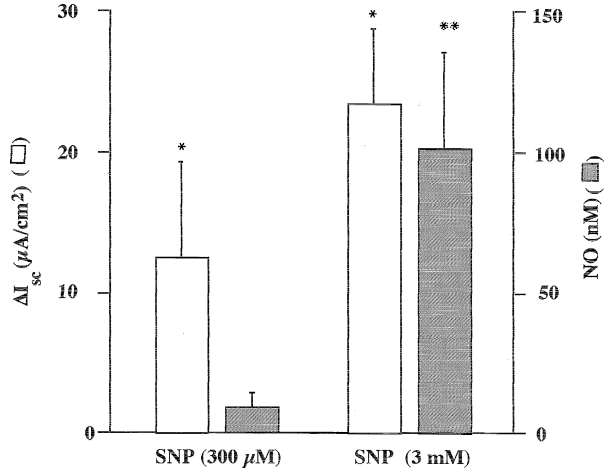

Fig. 2. The effect of two concentrations of sodium nitroprusside on short-circuit current and on the levels of NO ($n = 4$ for each concentration). Sodium nitroprusside was added to the serosal side of the cecal tissue and the maximum increase in I_{sc} recorded. At the same time and the same concentrations of sodium nitroprusside, NO levels were measured in the chambers using the ISO-NO meter (WPI). $** P < 0.005$; P value represents the comparison of the effect of sodium nitroprusside on I_{sc} as compared to control untreated tissue.

Sodium nitroprusside, which generates NO directly and not through the enzymatic pathway, was also used as mentioned before. It also caused an increase in I_{sc} and in NO levels (Fig. 2). However, high concentrations of sodium nitroprusside that produced high concentrations of NO were toxic to the tissue. Hence, L-arginine was used for the rest of the experiments.

To test the effects of NO synthase inhibitors on basal I_{sc} and on L-arginine-induced increase in I_{sc}, the cecal tissue was pretreated with different concentrations of L-NAME prior to the addition of L-arginine. L-NAME had no effect on basal I_{sc} (data not shown) but was able to inhibit L-arginine-induced increase in I_{sc} with an EC_{50} around 4 mM and a maximal effect observed around 10 mM (Fig. 3B). These data suggest that the observed effects of L-arginine on I_{sc} are due to the production of NO only.

3.2. Effects of L-arginine in the presence of neuronal blockers

To determine the mechanism by which L-arginine causes its effects on electrolyte transport, the cecal tissue was treated with either tetrodotoxin, a neuronal Na^+ channel inhibitor that prevents neural input to the epithelial cells, or atropine, a cholinergic inhibitor. L-arginine (100 mM) was added to the serosal side of the cecum after pretreating the tissue with either of the inhibitors, and the peak change in I_{sc} measured and compared to the L-arginine-induced peak change in I_{sc} in control untreated paired cecal tissues. The addition of tetrodotoxin (10 μM) increased the L-

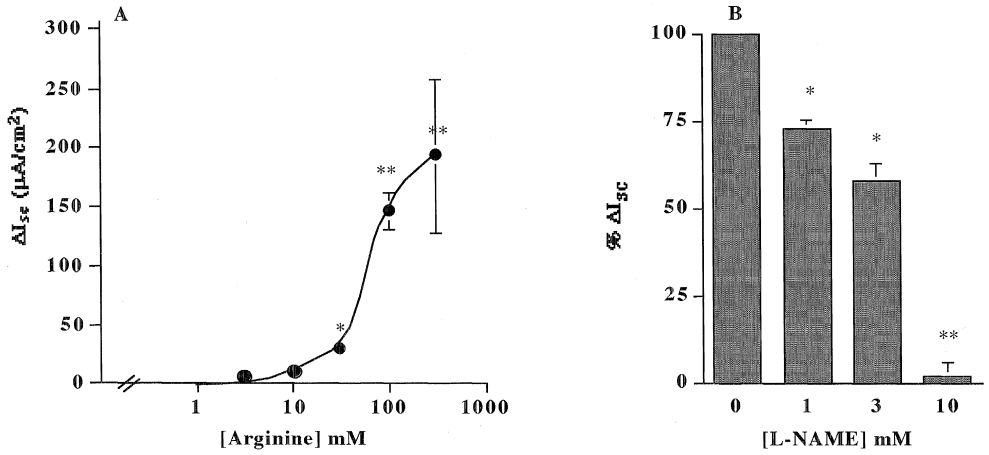

Fig. 3. (A) Concentration-dependent response of the effect of L-arginine on mouse cecum ($n = 4–8$). L-Arginine was added to the serosal surface of the tissue, and the data based on the maximum increase in short-circuit current is shown for each concentration tested. A separate piece of unstripped cecum was used for each determination. (B) Concentration-dependent inhibition of L-arginine's induced increase in short-circuit current by L-NAME. L-NAME was added to the tissue prior to the addition of L-arginine ($n = 4$). The data represents the maximum increase in short-circuit current in the presence of L-NAME and L-arginine. $* P < 0.05$; $** P < 0.005$; P value represents the comparison of the effect of a specific concentration of L-arginine on I_{sc} as compared to control untreated tissue.

arginine-induced increase in I_{sc} significantly (101.9 ± 29.0 $\mu A/cm^2$ in tissue treated with L-arginine alone vs. 157.7 ± 22.3 $\mu A/cm^2$ in tissue pretreated with tetrodotoxin before L-arginine addition, $p < 0.05$, $n = 4$). Atropine (10 μM), however, had no effect on the increase in I_{sc} caused by L-arginine (74.0 ± 40.7 $\mu A/cm^2$ in tissues treated with L-arginine alone vs. 65.4 ± 14.6 $\mu A/cm^2$ in paired tissues pretreated with atropine before L-arginine addition, $n = 4$, NS, Fig. 4). These data suggest that tetrodotoxin is inhibiting the release of a neuronal mediator that antagonizes L-arginine's effects; therefore, suggesting that L-arginine's effects could be mediated in part through non-cholinergic nerves.

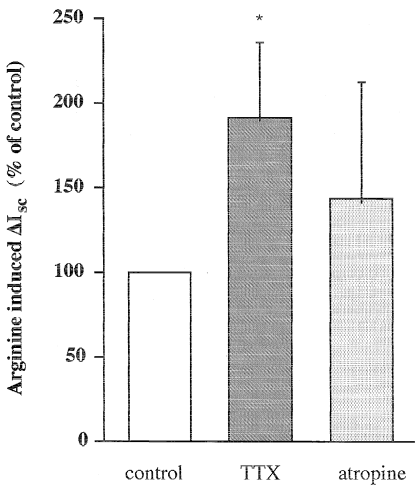

Fig. 4. The effect of L-arginine (100 mM) on short-circuit current alone, in the presence of tetrodotoxin, or in the presence of atropine. The data (mean ± S.E., $n = 4$, $* P < 0.05$. P value represents the comparison of the effect of L-arginine on I_{sc} in normal buffer vs. the experimental treatment) are presented as percentage change in I_{sc} caused by L-arginine.

3.3. The nature of the ion involved in L-arginine action

To determine the nature of the ion(s) whose transport is affected by L-arginine treatment, ion substitution studies were performed. Chloride or sodium ions were substituted for gluconate and choline, respectively, to determine if either was involved in the action of L-arginine. Neither chloride ion replacement by gluconate, or omission of HCO_3^- in the bathing buffer had any effect on L-arginine-induced increase in I_{sc} (Fig. 5). Replacement of Na^+ ions

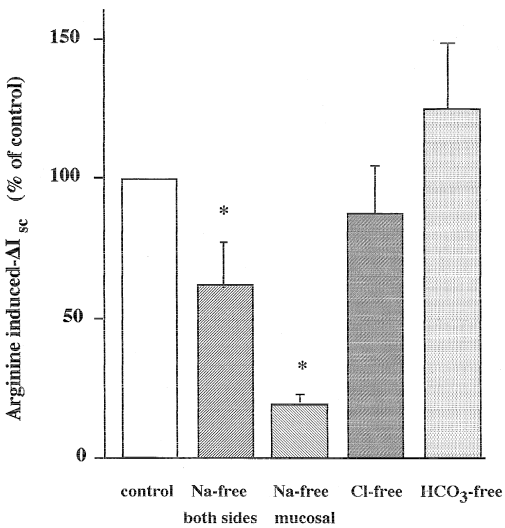

Fig. 5. The effect of L-arginine (100 mM) on short-circuit current in normal buffer, in Na^+-free buffer substituted on both sides of the tissue, Na^+-free buffer only on the apical side, Cl^--free buffer, and in HCO_3^--free buffer. The data (mean ± S.E., $n = 4$, $* P < 0.05$. P value represents the comparison of the effect of L-arginine on I_{sc} in normal buffer vs. the experimental treatment) are presented as percentage change in I_{sc} caused by L-arginine.

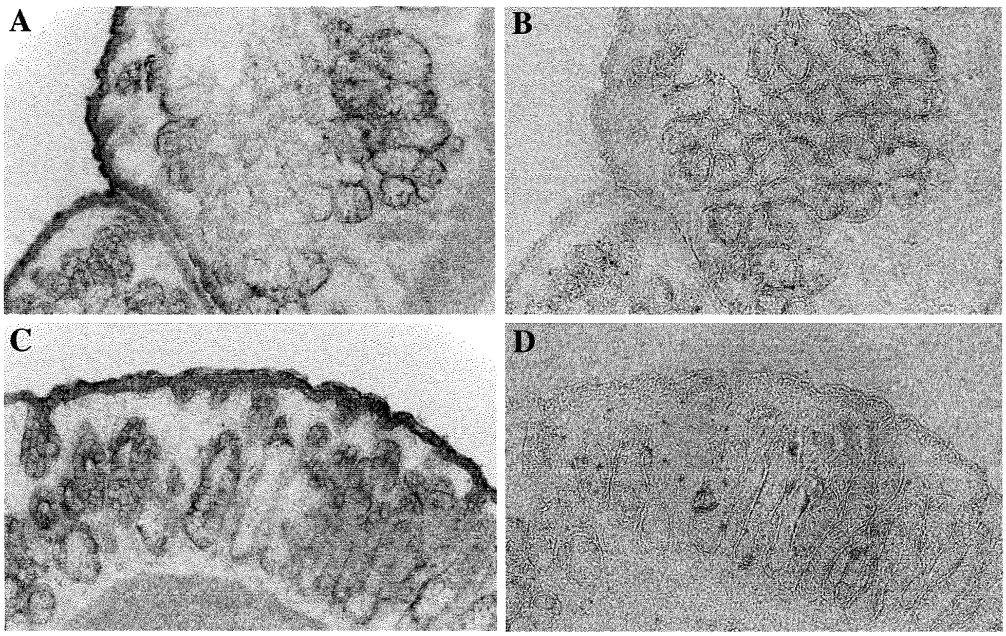

Fig. 6. Immunohistochemical staining showing (A) the presence of constitutive NO synthase in a section of normal epithelium from mouse cecum. (B) a phase contrast of the same section as in A. (C) Immunohistochemical staining showing the distribution of inducible NO synthase in the epithelial layer of the colitic mouse cecum. (D) a phase contrast of the same section as in C. No inducible NO synthase staining was apparent in the control mouse cecal epithelial layer. s: surface layer of the epithelial cells; l: lamina propria; and c: crypt glands.

by choline in the bathing buffer, however, caused a significant decrease in L-arginine-induced increase in I_{sc}. The increase in I_{sc} caused by L-arginine in normal buffer was 144.2 ± 40.0 $\mu A/cm^2$, and in Na^+-free buffer, it was 69.2 ± 5.4 $\mu A/cm^2$ ($n = 4$, $p < 0.05$, equivalent to 52% inhibition, Fig. 5). Similar findings were obtained when Na^+ was replaced by gluconate on the mucosal side only [the increase in I_{sc} caused by L-arginine in normal buffer was 84.6 ± 15.4 $\mu A/cm^2$, and in Na^+-free buffer, it was 15.4 ± 0.0 $\mu A/cm^2$ ($n = 3$, $p < 0.005$, equivalent to 82% inhibition)]. These data suggest that the major effect of L-arginine is through increasing sodium ion absorption to cause the observed increase in I_{sc}.

3.4. Immunohistochemical staining

The distribution of the enzyme nitric oxide synthase was examined using immunohistochemical staining in normal and in inflamed cecum. Both enzymes were studied, the constitutive and the inducible forms. Constitutive NO synthase was present in the lamina propria and in the nerve and muscle layer of the control cecum, while it was absent from the epithelial layer (Fig. 6A,B). In the inflamed cecum, the distribution of constitutive NO synthase was not different (data not shown), suggesting that constitutive NO synthase is not regulated by inflammatory mediators. However, inducible NO synthase, which was absent in the normal cecum (data not shown), was highly induced in the inflamed cecum with distribution that encompasses the whole mucosal layer, as well as the lamina propria, nerves and muscle layers (Fig. 6C,D), suggesting a major role for NO in inflammation.

4. Discussion

Nitric oxide was identified as an intercellular messenger first in blood vessels and later in peripheral and central nervous system. There is increasing awareness that NO contributes to the pathophysiology of chronic inflammatory diseases with inducible NO synthase proposed to play a proinflammatory role. Under basal or physiological conditions, NO is formed by constitutively expressed isoforms of the enzyme NO synthase (Nathan, 1992). Constitutive NO synthase release small amounts of NO in response to increased intracellular Ca^{2+}; in turn, released NO increases intracellular cGMP in the target cells, thereby inducing vascular or intestinal smooth muscle relaxation or modifying neurotransmission. Inducible NO synthase, on the other hand, releases great amounts of NO, which serves a cytostatic or cytotoxic mediator against tumors or invading microorganisms (Nathan, 1992). High levels of NO could promote oxidative reactions and the formation of reactive nitrogen intermediates which involve second-order kinetics rather than just activation of guanylate cyclase, as in the case with constitutive NO synthase. Elevated levels of NO have been found in gut inflammation in animals and humans (Miller et al., 1993, 1995; Ribbons et al., 1995; Middleton et al., 1993).

In this study, we report the effects of NO, through the addition of L-arginine, on the regulation of ion transport in

the mouse cecum. L-arginine increased I_{sc}, a measure of ion movement across the epithelial layer. We attributed this change to increased Na^+ absorption through the non-amiloride sensitive apical Na^+ channel which we have reported recently (Tripodi et al., 1996). NO's effect on I_{sc} was not affected by the removal of Cl^- or HCO_3^- ions, suggesting that NO only regulates Na^+ ion movement with no effect on anion secretion in the cecum. Similar findings were reported in rat jejunum recently (Schirgi-Degen and Beubler, 1995, 1996) where NO has been shown to maintain a proabsorptive tone in the intestine and counteract secretagogue-induced fluid secretion. In the latter study, NO has been shown to activate basolateral K^+ channels, an effect that has been shown to correlate with increased NaCl absorption in the rabbit ileum (Homaidan and Broutman, 1994). Stimulation of guanylate cyclase does not seem to be involved in this action of NO. In rat ileum, however, NO has been found to have a role as a neurotransmitter that mediates relaxation of ileal smooth muscle, but did not mediate changes in epithelial I_{sc}. L-NNA (N^{ω}-nitro-L-arginine), an inhibitor of NO synthase, inhibited L-arginine induced relaxation but failed to change I_{sc} (Li et al., 1994). Through activation of soluble guanylate cyclase and subsequent elevation of guanosine 3′-5′-cyclic monophosphate, NO relaxes vascular smooth muscle. In the intestine, an increase in cGMP due to the activation of guanylate cyclase has been shown to increase chloride secretion (Currie et al., 1992). The fact that in the present study, NO did not have any effect on anion secretion in the mouse cecum, suggests, as in the case of rat jejunum, that the activation of guanylate cyclase is not involved in the action of NO.

The role of NO in the regulation of transmural ion transport in mouse ileum has been previously studied, and the effect of NO was studied by evaluating changes in I_{sc} and potential difference (Rao et al., 1994). NO was found to cause a tonic net pro-absorptive, antisecretory effect on basal I_{sc} in mouse ileum as was observed in our study. In mouse ileum, inhibitors of NO synthase have been found to increase basal I_{sc} and potential difference in the presence of adrenergic and cholinergic inhibitors, effects that were reversed by the addition of L-arginine (Rao et al., 1994). In the mouse cecum, however, we found no effect of NO synthase inhibitor L-NAME on basal I_{sc}, but L-NAME completely abolished the increase in I_{sc} caused by L-arginine treatment. Moreover, we found that tetrodotoxin treatment of the tissue potentiated the effect of L-arginine on I_{sc}. This could be explained by tetrodotoxin inhibiting the release of secretory neurotransmitters, making NO more effective in turning on absorptive pathways.

Nitric oxide is produced in tissues by NO synthase with the liberation of equimolar amounts of citrulline. Citrulline levels were found to be significantly higher in rectal biopsy specimens from patients with active ulcerative colitis than in normals, suggesting that the mucosal NO biosynthesis is increased in active disease and that NO may play a pathogenic role in ulcerative colitis (Middleton et al., 1993). The distribution of both enzymes constitutive NO synthase and inducible NO synthase were therefore studied in normal, as well as in inflamed cecum. The distribution of constitutive NO synthase, which was present in the myenteric plexus and the muscle layer in the control cecum, was not changed in the diseased state. However, inducible NO synthase, which was, absent in normal cecum, was highly present in the cecum of colitic mice. In the inflamed cecum, positive epithelial staining was very extensive and intense, encompassing the complete epithelial layer. The fact that inducible NO synthase was present in the epithelial layer in the inflamed cecum, therefore suggests that NO may play a role in the pathophysiology of inflammatory bowel disease.

5. Conclusion

NO by the addition of L-arginine has been shown to increase I_{sc} in normal mouse cecum. This increase in I_{sc} was correlated with an increase in Na^+ absorption. Inducible NO synthase was found to be induced in the epithelial cells during inflammation, suggesting a role for NO in inflammatory bowel disease, which could be to compensate for the reduced absorption and increased secretion in the diseased state due to the release of inflammatory mediators (Homaidan et al., 1995).

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