Histochem Cell Biol (1996) 105:33–41

ORIGINAL PAPER

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Expression of nitric oxide synthase in mucosal cells of the canine colon

Accepted: 13 June 1995

Abstract The expression of nitric oxide synthase (NOS) in the mucosa of the canine colon was investigated with in situ hybridzation, immunohistochemistry (using isoform specific antibodies), western analysis, and NADPH diaphorase (NADPH-d) histochemistry. In situ hybridization using a common probe for known isoforms of NOS showed that NOS mRNA was strongly expressed in mucosal cells. A gradient in the degree of hybridization was noted from the base of the crypts to the luminal surface. This gradient was also apparent using an endothelial NOS (eNOS)-specific probe. Neural NOS-like immunoreactivity (nNOS-LI) was observed in columnar epithelial cells, and the same population of cells was stained with NADPH-d. Endothelial NOS-like immunoreactivity (eNOS-LI) was also found in mucosal cells; however, this eNOS-LI was confined to mucous cells. These cells were not stained with NADPH-d. The existence of eNOS in mucosal cells was confirmed by in situ hybridization using the probe which specifically hybridized with mRNA of eNOS and by western blots which demonstrated the expression of a 135-kDa protein in mucosal homogenates. The differential expression of NOS isoforms and the gradient in expression along the length of the crypts suggest complex roles for NO in the development of colonic epithelial cells and in secretion and transport functions of the colonic mucosa.

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Introduction

Formation of nitric oxide (NO) occurs via the action of nitric oxide synthase (NOS), which converts L-arginine to NO and L-citrulline. Several isoforms of NOS have been cloned and their sequences are known. These include two constitutive isoforms; neural NOS (nNOS) and endothelial NOS (eNOS), and inducible NOS (iNOS), which is expressed in a number of cells in response to cytokines. Although there are substantial regions of homology in the genes for the known isoforms of NOS, there are also dissimilar regions that cause the products to be immunologically distinguishable (Lamas et al. 1992; Lowenstein et al. 1992; Lyons et al. 1992; Marsden et al. 1992; Michel and Lamas 1992; Sessa et al. 1993; Ogura et al. 1993). Specific antibodies and RNA probes for in situ hybridization studies for each isoform make it possible to determine the cell types that express the various isoforms within a complex tissue (Ceccatelli and Eriksson 1993; Conrad et al. 1993; Liu and Barajas 1993; Myatt et al. 1993; Persson et al. 1993).

Recently, studies have demonstrated that NOS isoforms are widely distributed in a number of cells and tissues, such as the anterior pituitary (Ceccatelli et al. 1993), pancreatic B cells (Schmidt et al. 1992b), hepatocytes (Geller et al. 1993), and tumor cell lines (Sherman et al. 1993). The wide distribution of NOS suggests the involvement of NO in a variety of physiological activities. Others have found NADPH diaphorase staining (a commonly used histochemical marker for NOS) in mucosal tissues of the gastrointestinal tract (Schmidt et al. 1992a; Liu et al. 1995), but the cellular distribution of NOS isoforms in mucosal tissues is unknown. In the present study we characterized the distribution of NOS isoforms in the mucosa of the canine colon using immunohistochemistry and in situ hybridization techniques.

Materials and methods

Tissue preparation

Eleven dogs of either sex were used for this investigation. The animals were killed with an overdose of pentobarbital sodium (100 mg/kg), segments of the proximal colon, ileum, and the body of the stomach were removed and rapidly perfused with Krebs Ringer bicarbonate solution via the terminal mesenteric arteries. This was followed by perfusion with a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After the initial fixation, the segments were cut into small pieces, and these were immersed in the same fixative solution for 1–4 h at 4° C and washed with 0.1 M phosphate buffer. Tissues were embedded in Tissue-Tek OCT compound (Miles) and frozen in liquid nitrogen. Sections (10 μm thick) were cut with a cryostat and placed on glass slides.

Protein preparation of western blotting

Mucosal tissues, harvested by scraping colonic segments from five dogs, were frozen in liquid nitrogen. Pooled samples of mucosa were thawed and sonicated for 30 s in ice-cold buffer A (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, and 10 μM tetrahydrobiopeterin) in the presence of protease inhibitors (i.e. 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 3 μM pepstatin A, and 2 μM aprotinin). The tissue was further processed with a glass/Teflon pestle homogenizer for about 25 cycles. The homogenate was centrifuged at 100000 *g* for 60 min. The supernatant was removed and referred to as the soluble fraction, while the pellet was resuspended in buffer A and referred to as the particulate fraction. Protein content was determined by a Bradfor assay (BioRad) utilizing bovine serum albumin as the standard.

Crude enzyme preparations were separated on 7.5% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). The proteins were then blotted onto nitrocellulose (Hyland-ECL) by dry electroblotting (Millipore) for 60 min. The blots were allowed to air dry 1 h or more and then blocked overnight at 4° C with 6% fat-free dried milk in Tris-saline buffer (40 mM Tris-HCl, pH 7.6 and 300 mM NaCl). The blots were incubated with the primary antibody for 2 h at room temperature. The ascites fraction from the hybridoma H32 was utilized in this study at a dilution of 1:2000 in 2% fat-free dried milk in Tris-saline buffer (see Pollock et al. 1991). The blots were washed four times in Tris-saline buffer and incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobin antibody (1:3000, Amersham) for 1 h at room temperature. Finally, the blots were washed four additional times and the specific proteins were detected by enhanced chemiluminescence (ECL, Amersham).

In situ hybridization

Two types of probes were used for in situ hybridization studies. The sequence of one contained a high degree of homology with the three types of NOS (i.e. nNOS, eNOS, and iNOS), and therefore this probe should hybridize with all types of NOS mRNA. The sequence of the second probe was specific for eNOS and did not hybridize with nNOS or iNOS. Sense probes and sections pretreated with RNase (100 μg/ml) were used as controls.

cDNA encoding the rat brain isoform of NOS (i.e. nNOS; Bredt et al. 1991) was provided by Adele Snowman (Johns Hopkins University). A 229-bp *Bam*HI fragment from the full-length cDNA was subcloned into pGEM3Z and was used for riboprobe synthesis. This fragment corresponds to nucleotides 1601–1830 following the rat brain NOS numbering system, encoding amino acids 534–610 (Bredt et al. 1991). This region is within the putative L-arginine binding domain (Sessa 1994), and it is highly conserved in all known isoforms of NOS (Lyons et al. 1992, Xie et al. 1992).

cDNA encoding bovine endothelial NOS (i.e. eNOS; Sessa et al. 1992) was provided by William Sessa (Yale University). A 403-bp *Eco*RI/*Sal*I fragment representing the first 134 amino acids was subcloned into pGEM3Z and used for riboprobe synthesis. This region is specific for eNOS and has no homology to other known isoforms of NOS.

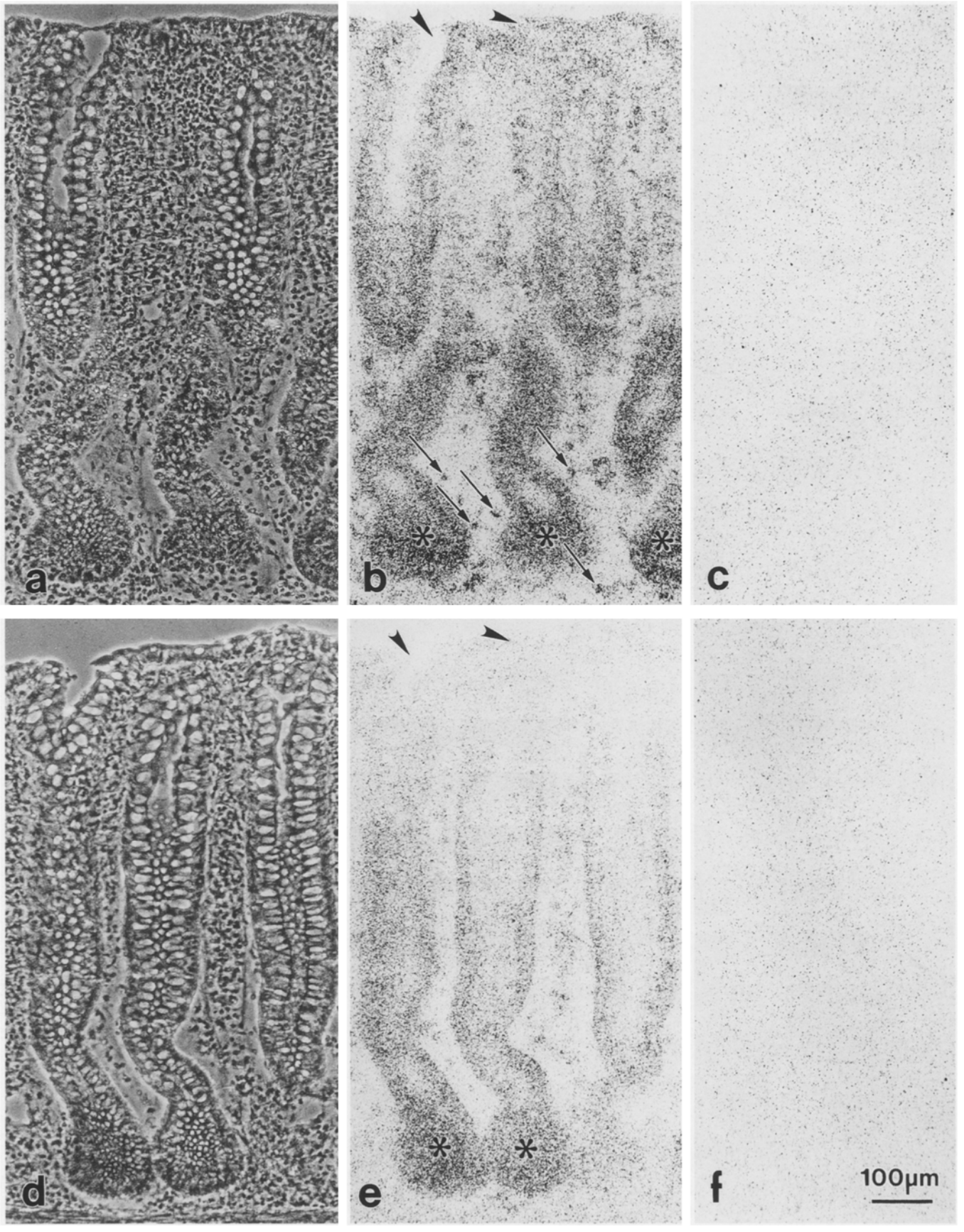
Sense and antisense probes were synthesized from the NOS subclones using [^{35}S]UTP and the Promega riboprobe synthesis system. Specific activity of the probes were all approximately 10^6 cpm/μg. Tissue sections were incubated with proteinase K (0.5 μg/ml) at 37° C for 15 min, postfixed in 4% paraformaldehyde for 10 min, and treated with 0.25% acetic anhydride in 0.1 M triethalolamine (pH 8.0) for 10 min. The sections were allowed to incubate overnight at 42° C in a solution containing 50% (vol/vol) formamide, 0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1X Denhardt's solution, 500 μg/ml yeast tRNA, 10% dextran sulfate, 100 mM dithiothreitol, and 2–5×10^6 cpm/slide of RNA probes. After the incubation the slides were washed in 4X SSC and incubated with 20 μg/ml RNase (Sigma) 37° C for 30 min. They were washed again with 2X SSC, 0.1X SSC at 50° C and were dehydrated with ethanol. For radioautographs, slides were dipped in NTB-2 emulsion (Kodak), exposed at 4° C for 1–3 weeks, and developed in D-19 (Kodak). Some sections were counterstained with buffered hematoxylin (Zymed) for 5 min after development. All sections were mounted with crystal mount (Fisher).

Double staining with NOS immunohistochemistry and NADPH-diaphorase histochemistry

For immunohistochemistry of anti-NOS antibodies, sections (7 μm thick) were treated with 5% normal horse serum for 30 min and incubated with anti-eNOS (1:400 monoclonal antibody; Pollock et al. 1993), or anti-NOS (1:100 monoclonal antibody, Transduction, Lexington, Ky.; McKee et al. 1994), at 4° C overnight. The nNOS monoclonal antibody was raised against a 22.3-kDa protein fragment corresponding to amino acids 1095–1289 of human nNOS. Sections were washed by phosphate-buffered saline (PBS) and were incubated with FITC-conjugated mouse IgG second antibodies for 1 h. Specificity was checked by omitting the primary antibodies or incubation with antibody pre-incubated with excess purified eNOS. Pre-absorption abolished immunoreactivity in sections treated with anti-eNOS antibody, but immunoreactivity was unaffected in sections incubated with anti-nNOS antibody. To avoid non-specific absorption of antibodies to mucus, we also tested the specificity of binding by conducting primary and secondary incubations in the presence of high concentrations of magnesium (7.2 m*M*). This control had no effect on immunoreactivity. To check the sub-cellular distribution of eNOS-like immunoreactivity, some sections were examined with a confocal microscope (MRC-500, BioRad).

After immunohistochemical preparation and observation, sections were incubated with the solution containing 0.25 mg/ml nitro blue tetrazolium (Sigma) and 1 mg/ml β-nicotinamide adenine di-

Fig. 1a–f In situ hybridization of NOS mRNAs in mucosa. **a** Phase-contrast image of section shown in **b**. **b** NOS mRNAs, detected with a probe common for all isoforms of NOS, were expressed in mucosal cells and in cells within the lamina propria which are likely to be macrophages (*arrows*). Hybridization at the base of the crypts (*asterisks*) was greater than at the luminal surface (*arrowheads*). **c** In a serial section hybridization did not occur with a "sense" probe, used as a control. **d** Phase-contrast image of the section shown in **e**. **e** Cells in the mucosa were labelled with an antisense probe specific for eNOS. Though signals were less intense than those using the common NOS probe (**b**), there was a tendency for the base of the crypts (*asterisks*) to show greater hybridization than the luminal surface (*arrowheads*). Cells in the lamina propria did not appear to express mRNA of eNOS. **f** In a serial section, hybridization did not occur with a "sense" probe used as a control. *Scale bar* applies to all panels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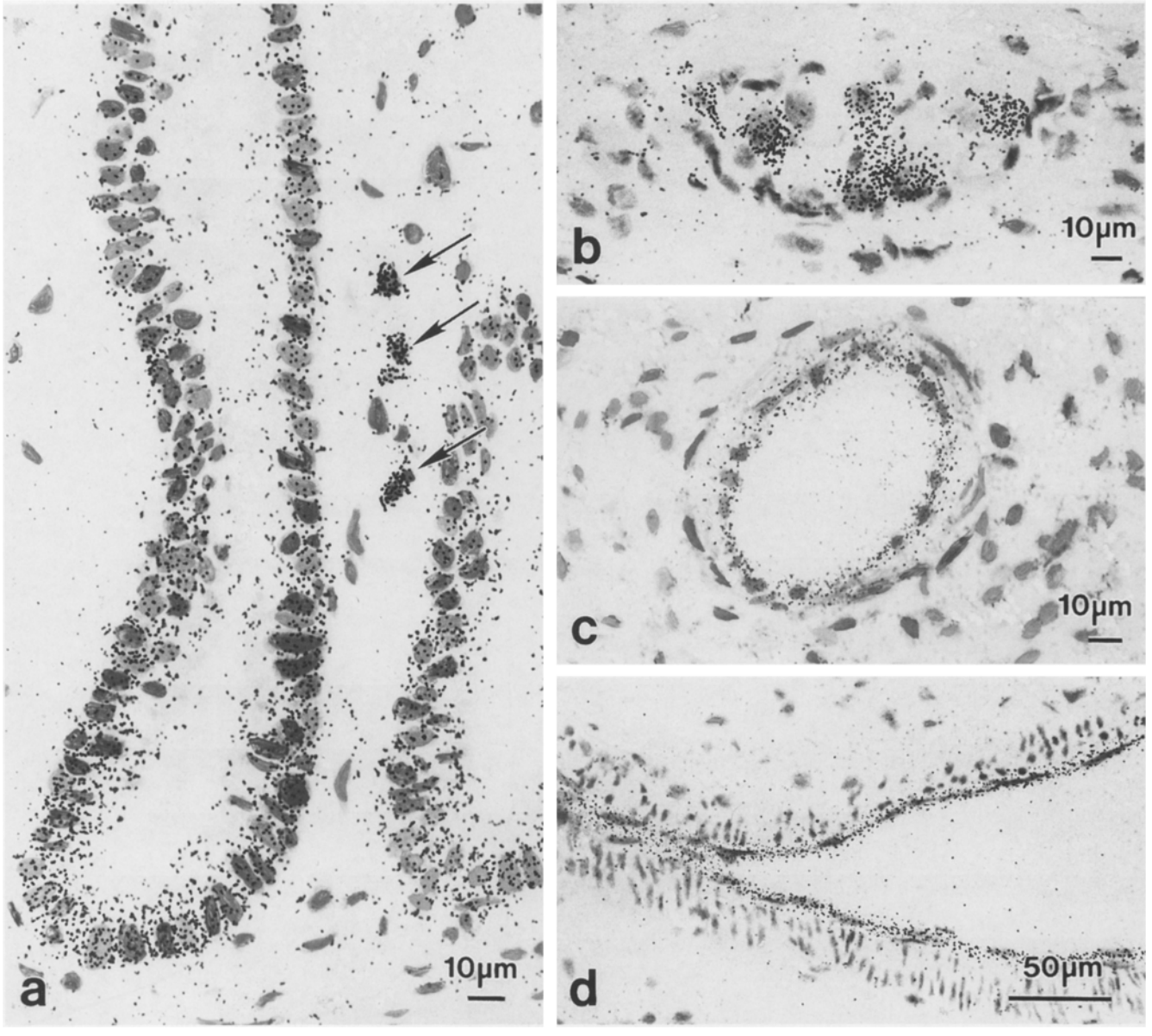
nucleotide phosphate (NADPH; Sigma) in PBS at 37° C for 7 min, rinsed with the same buffer and mounted with Aqua-Mount (Lerner). Controls were processed without NADPH.

Results

In situ hybridization

Both NOS RNA probes hybridized with mRNAs of mucosal cells; however, the distribution of label differed. The common NOS probe, designed to hybridize to all isoforms of NOS, strongly labeled cells in the crypts (Fig. 1a, b). Cells at the base of the crypts were more intensely labeled than cells near the luminal surface. Silver particles were also observed in cells of the lamina propria, suggesting labeling of macrophages (Figs. 1b, 2a). The common NOS probe showed strong signals in some enteric neurons and endothelial cells of blood vessels (Fig. 2b, c). The eNOS specific probe also labeled mucosal cells (Fig. 1d, e); however, the labeling was much weaker than with the common NOS probe. A similar gradient in labeling was noted with the eNOS probe, in which cells along the walls and bases of the crypts were more intensely labeled than cells near the luminal surface. The eNOS probe also hybridized with mRNA in the endothelial cells of blood vessels (Fig. 2d).

Fig. 2a–d In situ hybridization of NOS mRNA in canine colon. The section was stained with hematoxylin. **a** Cells at the base of crypts and within the lamina propria (*arrows*) were labeled strongly with the common NOS probe. Labeled cells in the lamina propria are likely to be macrophages by virtue of their distribution and nuclear properties. **b** Some neurons in a submucosal ganglion (*G*) were also labeled with the common NOS mRNA, confirming previous observations suggesting expression of nNOS by neurons in submucosal ganglia (Ward et al. 1992). **c** Endothelial cells of the arteriole were labeled with the common NOS probe. **d** The probe specific for eNOS also labeled endothelial cells of blood vessels



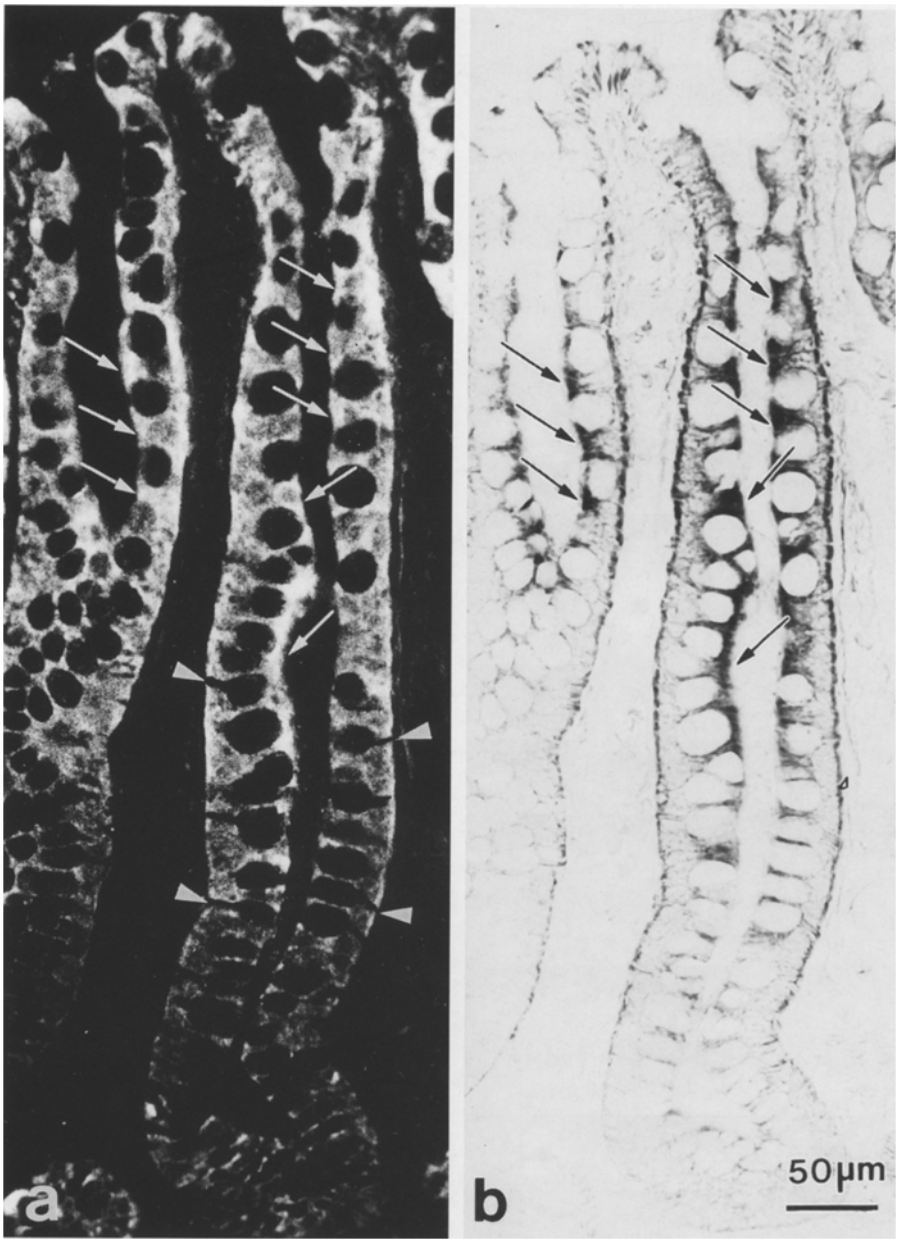


Fig. 3a, b Expression of nNOS-like immunoreactivity and NADPH-d in mucosal cells. Sections were first labeled with anti-nNOS antibodies and then re-processed for NADPH-d. **a** Columnar epithelial cells containing nNOS-LI (some indicated by *arrows*). Mucous cells (goblet cells) were immunonegative (*arrowheads*). **b** Columnar cells were co-labeled with NADPH-d. *Arrows* indicate the same cells as in **a**, which have nNOS-LI and NADPH-d staining. *Scale bar* applies to both panels

NOS immunohistochemistry and NADPH-d histochemistry

Columnar cells in the mucosa contained nNOS-like immunoreactivity (nNOS-LI) and goblet cells were immunonegative (Fig. 3a). Staining with NADPH diaphorase (NADPH-d) showed a similar distribution, with dark staining near the apical and basolateral aspects of the columnar cells (Fig. 3b). The highest density of nNOS-LI was in the mid-region of the crypts, but cells close to the apical surface were also labeled. Very little labeling was observed in cells near the base of the crypts.

Mucosal tissues were also labeled with antibodies specific for eNOS (Pollock et al. 1993). The distribution of cells that expressed eNOS-LI differed from that of cells with nNOS-LI. Mucous cells (goblet cells) were labeled with the eNOS throughout the length of the crypts, but immunoreactivity was most intense at the base of the crypts (Fig. 4a). In goblet cells the immunoreactivity appeared to be restricted to the region of mucous granules, and did not extend into the cytoplasm. The eNOS-LI appeared to be localized to the surface of mucous granules as visualized by confocal microscopy (Fig. 5). The same pattern of immunoreactivity was found in mucous cells of the ileum and gastric gland (data not shown). Co-labeling with NADPH-d showed that diaphorase did not label the same population of cells identified with eNOS-LI (Fig. 4b), suggesting, as previously noted, that NADPH-d is not a marker of eNOS in canine gastrointestinal tissues (Xue et al. 1994). We observed no eNOS-LI within the lamina propria (Fig. 4a), but blood vessels within submucosa and tunica muscularis of the canine

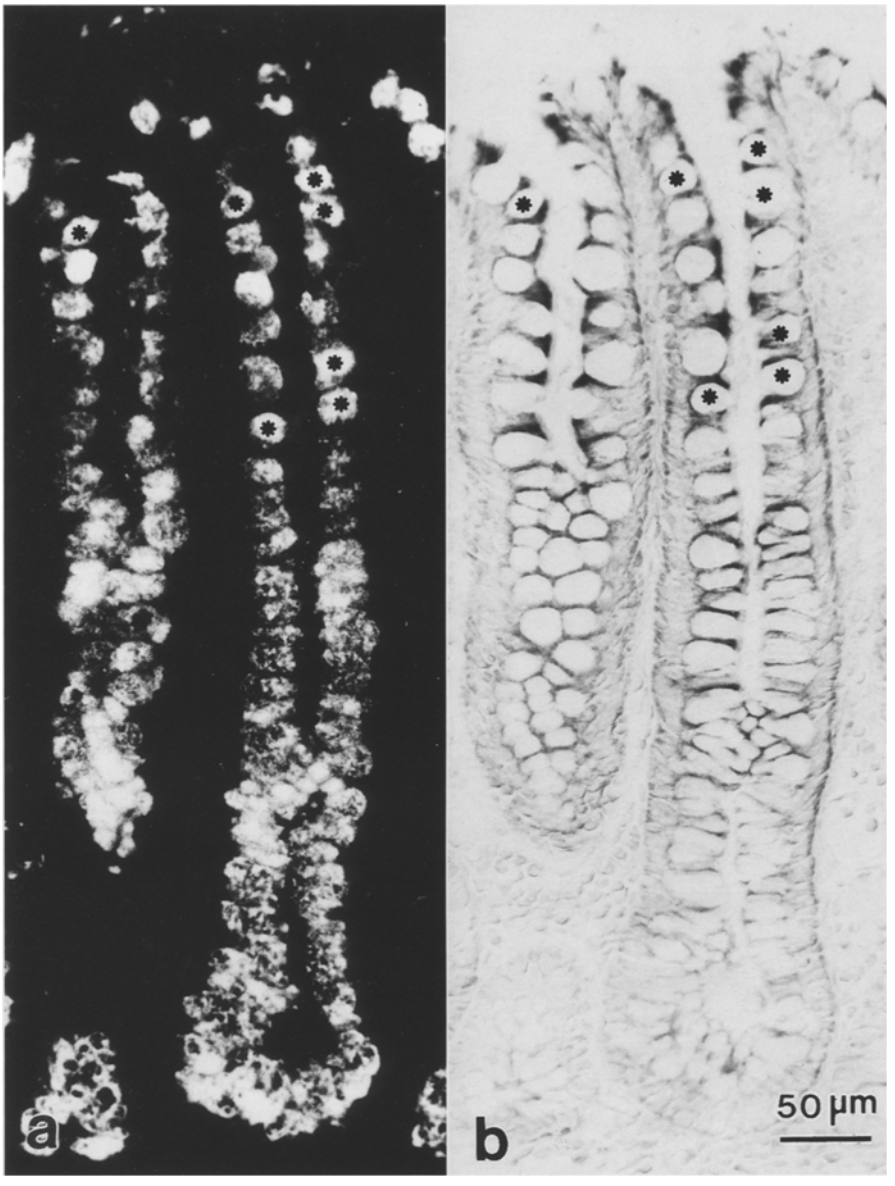


Fig. 4 Expression of eNOS-like immunoreactivity and NADPH-d. **a** Mucous cells contained eNOS-LI (some cells indicated by *asterisks*). Labeling was restricted to the region around mucous granules. Labeled mucous cells were densely distributed in the lower half of the crypts. Labeling was more sparse at the luminal surface of crypts. **b** Mucous cells were not stained with NADPH-d. *Asterisks* in **b** denote the same mucous cells indicated in **a**. Scale bar applies to both panels.

colon were strongly labeled with this antibody (data not shown), as previously reported (Xue et al. 1994).

The expression of eNOS was also confirmed by western blots of homogenates of mucosal scrapings. The eNOS antibody labeled a protein in mucosal homogenates of approximately 135 kDa (Fig. 6). The antibody also labeled a protein of the same size from the particulate fraction of bovine endothelial cells that was used as a control (see Pollock et al. 1991). The specificity of the nNOS antibody was also checked in western blots from homogenates of canine cerebellum, colonic tissues dissected from the region of the myenteric plexus, and mucosal scrapings. Single bands at 160 kDa were obtained from the cerebellum and colonic muscle strips, but

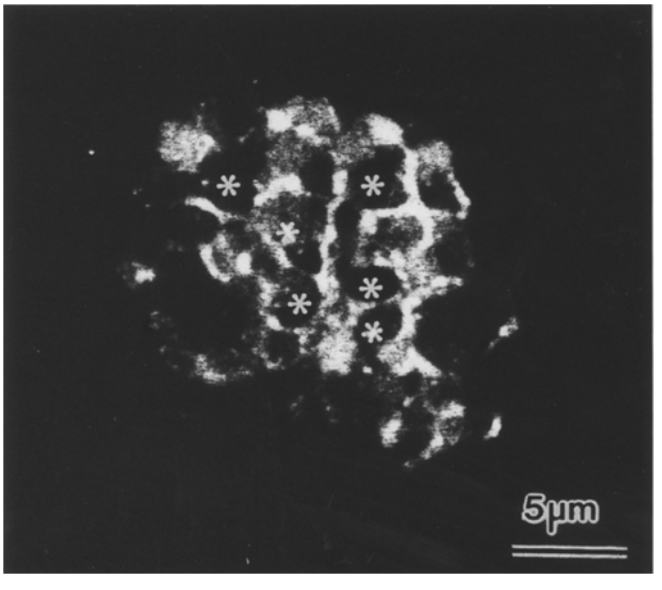


Fig. 5 Confocal image of mucous cell labeled with anti-eNOS antibody. eNOS-LI was observed at the outer perimeter of mucous granules (*asterisks*). The image was formed digitally from an average of six scans

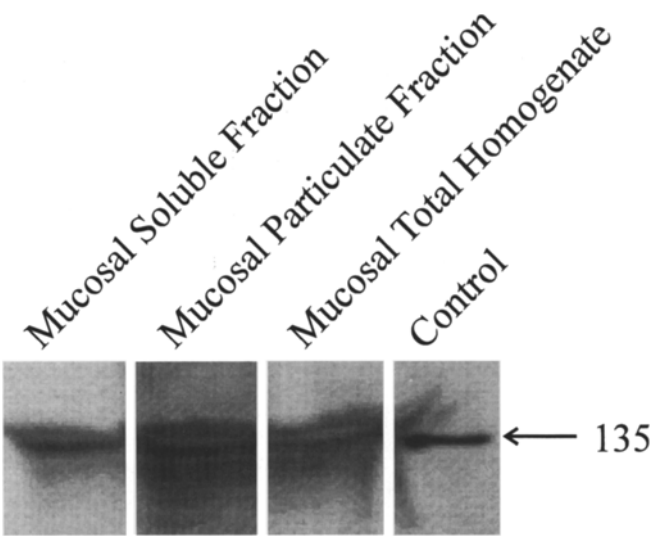


Fig. 6 Western blots of protein extracts from mucosal scrapings. *Lane 1* was loaded with 100 μg from the soluble protein fraction, *lane 2* with 100 μg from the particulate fraction, and *lane 3* with 250 μg of the total homogenate. *Lane 4* was loaded with 10 μg of the particulate fraction of bovine endothelial cells. The latter was used as a control because it is known to contain eNOS, a 135-kDa protein (see Pollock et al. 1991)

nNOS protein levels in unpurified mucosal homogenates were below the level of resolution even when up to 75 mg of protein was loaded onto gels (data not shown).

Discussion

We used four methods to demonstrate the expression of NOS isoforms in cells within the mucosa of the canine colon. Western blots from homogenates of mucosal scrapings demonstrated that the immunoreactivity of the dominant isoform corresponded to a band at 135 kDa. This agrees with the molecular weight of eNOS (Pollack et al. 1991). In situ hybridization studies showed that NOS mRNAs were not uniformly distributed along the walls of the crypts. Cells near the base of the crypts hybridized extensively to the general probe for NOS, and weaker hybridization was noted toward the luminal surface. A similar pattern was observed using the eNOS-specific RNA probe, and the gradient also appeared to be manifest in protein expression because it was apparent in immunohistochemical studies using the eNOS-specific antibody. Furthermore, nNOS-like immunoreactivity (nNOS-LI) was also apparent in cells within the mucosa. nNOS-LI occurred in a different population of cells than eNOS-LI. nNOS-LI was concentrated in the mid-portion of crypts in columnar epithelial cells, and it co-localized with NADPH-d activity, as previously demonstrated in central and peripheral neurons (Dawson et al. 1991; Hope et al. 1991). eNOS-LI was observed in mucous cells, and this activity did not co-localize with NADPH-d activity, as observed in other cell types of the dog (Xue et al. 1994).

The common NOS probes used in the in situ hybridization studies were directed toward a region of homology in the sequences of the known isoforms of NOS (see Materials and methods). Thus, hybridization with this probe could be interpreted as expression of eNOS, nNOS, or iNOS. Macrophages, neurons, and the luminal aspect of blood vessels were also labeled with the common NOS probe. A lower level of hybridization was observed when the eNOS-specific probe was used, but a similar gradient in expression in the mucosa (i.e. crypt to luminal surface). Macrophages and neurons were not labeled with the eNOS-specific probe. The difference in hybridization in the mucosa could have been due to inherent differences in affinity of the two RNA probes, or the intense labeling with the common NOS probe could have been due to the expression of isoforms other than eNOS in the crypt region (e.g. iNOS). The fact that the pattern of eNOS-LI was similar to the gradient of labeling with in situ hybridization tends to suggest that differences in hybridization efficiency might explain the differences in the density of labeling between Fig. 1b and Fig. 1e.

The gradient in NOS expression, as demonstrated by in situ hybridization and immunohistochemical techniques, was a particularly striking finding of the present study. Since proliferation of the colonic epithelium occurs at the base of the crypts (Tsubouchi and Leblond 1979; Morita et al. 1994), our results tend to suggest that NO may be more important in immature cells, and it may play a role in the development of epithelial cells. As cells mature and migrate toward the luminal surface, the level of mRNA and eNOS expression tends to decrease. During this migration, however, cells that have differentiated into columnar epithelial cells begin to express nNOS. The developmental significance and dynamics of NOS expression will require further studies.

Immunohistochemistry using antibodies directed at nNOS and NADPH-d histochemistry suggested that nNOS is expressed in columnar epithelial cells. Others have found that epithelial cells of the gastric and intestinal mucosa, kidney, and lung also express nNOS-LI and/or NADPH-d activity (Schmidt et al. 1992a; Liu et al. 1995), suggesting that nNOS may be commonly expressed in epithelia. The observation of eNOS expression in intestinal mucosal cells is novel, but others have recently reported expression of this isoform in cultured kidney tubular epithelial cells (LLC-PK1 cells; Tracey et al. 1994). The eNOS-LI in goblet cells was distributed around the outer perimeter of mucous granules. The amino acid sequence of eNOS includes an N-myristylation site, possibly explaining the association of eNOS with membranes (Pollock et al. 1991; Sessa et al. 1992); therefore, eNOS may be bound to the membrane of mucous granules. We were unable to determine the reason why goblet cells were not labeled with NADPH-d, but it is possible that fixation destroys the diaphorase activity of membrane-bound NOS (Larsson et al. 1993). Thus, as previously demonstrated, NADPH-d was a poor histochemical marker for formaldehyde-fixed cells containing eNOS-LI (Xue et al. 1994).

At present the functional significance of expression of two NOS isoforms in discrete sub-populations of cells

and the sub-cellular localization of eNOS-LI at the surface of mucous granules is unknown. Several studies have shown that NO and the second messenger pathways activated by NO affect epithelial function. In rat stomach, for example NO and guanosine 3′,5′-cyclic monophosphate (cGMP) stimulate mucous secretion and increase mucous gel thickness (Brown et al. 1992a, b, 1993). The mucous cell fraction isolated from the rat gastric mucosa showed NOS activity measured by conversion of [^{3}H]-arginine to [^{3}H]-citrulline. These data lead to the assumption that the function of the membrane binding eNOS surrounding mucous granules might be to effect mucus secretion. NO donors have also been shown to increase the short circuit current in rat colons mounted in Ussing chambers (Tamai and Gaginella 1993; Wilson et al. 1993), and this effect was attributed to enhanced anion secretion on the basis of radiotracer flux studies and blockade by bumetanide. Wilson and co-workers (1993) also found that NO donors inhibited neutral Na^+ and Cl^- absorption. Kubes and co-workers showed that NO modulates epithelial permeability in the feline small intestine (Kubes 1992: Payne and Kubes 1993) and that close arterial infusion of NOS inhibitors led to an increase of mucosal epithelial permeability as measured by clearance of rhodamine-dextran from the interstitium to the lumen. It is possible that NO produced by nNOS in columnar epithelial cells is involved in the regulation of mucosal membrane permeability. The precise mechanism of the effects of NO are unknown, but some studies have suggested that the mucosal effects of NO are mediated via nerves, prostaglandin synthesis and/or a direct effect involving the synthesis of cGMP (e.g. Tamai and Gaginella 1993; Wilson et al. 1993). Further functional studies will be necessary to determine the physiological significance of NO produced by constitutive NOS isoforms in mucosal tissues.

In conclusion, we have demonstrated the existence of at least two isoforms of NOS in mucosal cells of the canine colon. The two isoforms were expressed in discrete populations of cells, and there appears to be differential expression of NOS as cells migrate from the base of the crypts to the luminal surface. Although NO appears to contribute to the regulation of epithelial transport, at present we know very little about the role of constitutive NOS enzymes in normal mucosal development and function.

Acknowledgements This project was supported by a Program Project Grant, DK41315. The authors are grateful to Ms. N. Horowitz for harvesting mucosal samples for western blots.

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Note added in proof During review of this manuscript an abstract was presented at the American Gastroenterological Association meeting in San Diego, California that also showed NADPH diaphorase activity in colonic and other gastrointestinal mucosal cells (see Liu et al. 1995).

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