Cell Tissue Res (1996) 285:157–163

Cell & Tissue Research

Localization of constitutive isoforms of nitric oxide synthase in the gastric glandular mucosa of the rat

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Received: 31 July 1995 / Accepted: 5 March 1996

Abstract. Nitric oxide has been implicated in the regulation of blood flow, mucosal integrity and mucus secretion in the gastric mucosa. An antiserum directed against the C-terminal hexadecapeptide of rat brain nitric oxide synthase (NOS) and monoclonal antibodies to the neuronal and endothelial forms of NOS were used to establish the location of isoforms of NOS in rat gastric glandular mucosa. Antibodies to the neuronal form of NOS reacted with a band of 160 kDa on immunoblots of brain and gastric mucosa, and the addition of the hexadecapeptide inhibited recognition by the antipeptide antiserum. The antibody to endothelial NOS detected a band of 140 kDa on protein blots of samples of intestinal mesentery and gastric mucosa. Immunohistochemistry using these antibodies demonstrated that material related to neuronal NOS was present in surface cells of the gastric mucosa, and showed a similar localization to intense NADPH diaphorase activity. The antibody to endothelial NOS did not stain the surface of the gastric mucosa but recognized blood vessels in the lower region of the gastric glands and in the sub-mucosa. This study suggests that nitric oxide might act both as an intra- and inter-cellular messenger to regulate mucus release, and that the NOS present in surface cells is related more closely to the neuronal than to the endothelial isoform.

Key words: Nitric oxide – Nitric oxide synthase – Gastric mucosa – Stomach – Immunohistochemistry – Rat (Wistar)

Introduction

Nitric oxide (NO) is implicated in mechanisms maintaining the integrity of the gastric epithelium (Whittle et al. 1990) and regulates gastric mucosal blood flow (Whittle 1994). NO may directly affect gastric mucus secretion by activating soluble guanylate cyclase and raising intracellular cyclic GMP. Thus, NO donors, and analogues of cyclic GMP, stimulated mucus secretion by both the intact rat stomach and isolated mucosal cells (Brown et al. 1992a, 1993), and stimulation of mucus secretion in vivo by carbachol was prevented by inhibitors of NO synthase (NOS; Price et al. 1994). The gastric mucosal surface is covered by a continuous layer of mucus, into which bicarbonate is secreted from the epithelial cells. This mucus/bicarbonate barrier makes an important contribution to the protection of the mucosa from the damaging effects of acid and pepsin (Allen et al. 1993).

When gastric mucosal cells were separated by centrifugal elutriation, Ca^{2+}-dependent NOS activity was enriched in a fraction containing cells of medium size in which periodic acid-Schiff-positive mucous cells were predominant (Brown et al. 1992b). The elutriation data do not allow discrimination between mucous epithelial cells and mucous neck cells as sites of NOS, nor do they exclude the possibility that NOS might not be in mucous cells but in a minor cell population of similar size. Since the NO involved in mucus secretion could potentially be generated from within the mucous epithelial cells or from another cell-type, the precise localization of NOS within the gastric mucosa is required to define the role played by NO in gastric mucus secretion. Localization of NOS activity may also be important for understanding the participation of NO in the gastroprotective actions of agents such as sucralfate and aluminium hydroxide (Konturek at al. 1993), and in the response to damage by intragastric 1 M sodium chloride (Takeuchi et al. 1994).

Two forms of Ca^{2+}-dependent NOS have been identified. Neuronal NOS (nNOS; also known as type I) is a 161-kDa protein and endothelial NOS (eNOS; type III) has a molecular mass of 133 kDa. Neither nNOS nor eNOS are restricted to neuronal or endothelial cells, respectively. For example, bronchial epithelial cells contain material recognized by antibodies to nNOS (Schmidt et al. 1992) while renal tubules stained with antibody to

This work was supported by a Project Grant from MRC, UK.

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eNOS (McKee et al. 1994). A third form, inducible NOS (iNOS), is present in the gastric mucosa of rats pre-treated with lipolysaccharide, but is absent from control animals (Brown et al. 1994). In the present work antibodies to the forms of NOS were used to investigate the location of NOS within the rat corpus mucosa and thereby to elucidate further the involvement of NO in the regulation of gastric mucosal integrity and secretory activity.

Materials and methods

Animals

Male Wistar rats (200 g) were obtained from Bantin & Kingman, Hull, UK. Rats were fed with a rodent breeder economy diet (Special Diet Services, Witham, UK) and were kept at 20–21°C on a 0800–2000 hours light and 2000–0800 h dark schedule. The Principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985) were followed as well as those pertaining to the use of animals in the UK. All experiments, involving 45 animals, were performed under terminal anaesthesia with sodium pentobarbitone (60 mg/kg i.p.).

Materials

The C-terminal hexadecapeptide (Affiniti, Exeter, UK) of rat brain NOS (residues 1414–1429), was synthesized with a cysteine residue added to the amino-terminus of the sequence. The peptide was coupled to keyhole-limpet haemocyanin via reaction with the cysteine sulphydryl, and antibodies were raised in rabbits. Rat pituitary cell tumour extract, mouse monoclonal antibodies to a 22.3-kDa fragment corresponding to amino acids 1095–1289 of human brain NOS, to a 20.4-kDa fragment corresponding to amino acids 1030–1209 of human endothelial NOS and to a fragment corresponding to residues 961–1144 of mouse macrophage NOS were obtained from Transduction Laboratories (Lexington, Ky., USA). Purified, recombinant rat brain nNOS was obtained from Affiniti, Exeter, UK.

Immunoblotting

Tissues were boiled with electrophoresis sample buffer (0.0625 M TRIS pH 6.8, 10% v/v glycerol, 2% w/v sodium dodecylsulphate), the protein concentration was determined by the bicinchoninic acid procedure (kit from Pierce, Rockford, Ill., USA), and the sample made 0.1 M with dithiothreitol and reboiled. Samples were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (8% acrylamide) and were transferred to nitrocellulose. Blots were blocked for 1 h at room temperature with non-fat dried milk (5% w/v), dissolved in 10 mM TRIS (pH 7.5) 100 mM NaCl and 0.1% (v/v) Tween 20 (TBS-Tween), and were then exposed to nNOS polyclonal antiserum at a dilution of 1/1250 or to nNOS or eNOS monoclonal antibodies at concentrations of 1 μg/ml. All antibodies were diluted into blocking medium. After washing in TBS-Tween, binding of primary antibodies was detected with an appropriate anti-species IgG antibody conjugated with horseradish peroxidase followed by enhanced chemiluminescence detection (kit from Amersham, Little Chalfont, Bucks, UK).

Preparation of tissue sections

Tissue was fixed by perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.2 and room temperature, followed by immersion in 4% paraformaldehyde in PBS for 4 h at 4°C, and transfer to PBS containing first 10% then 20% and finally 30% (w/v) sucrose, or by immersion in 4% paraformaldehyde in PBS at 4°C followed by cryoprotection with sucrose. Tissue was frozen in isopentane and cooled in liquid nitrogen; 10 μm sections were cut with a cryostat and were dried at room temperature onto slides coated with Vectabond (Vector Laboratories, Pe-

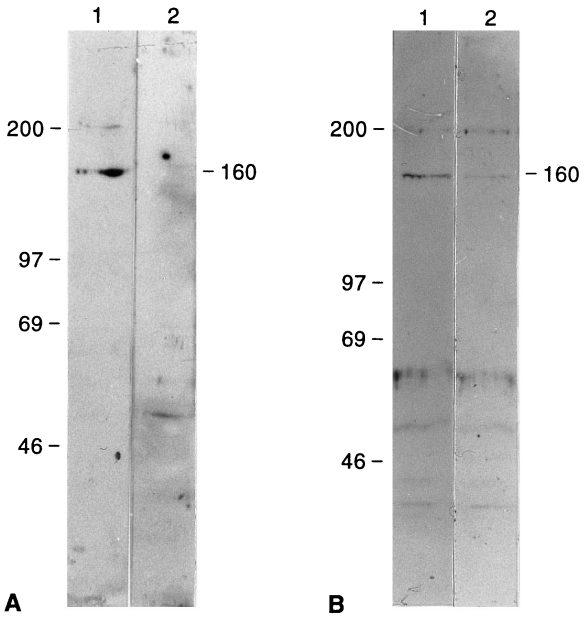

Fig. 1A, B. Immunoblotting using antiserum to the C-terminal hexadecapeptide of rat nNOS. *Lane 1*, blot exposed to antiserum alone; *lane 2*, to antiserum + 100 μg/ml peptide. **A** 50 μg protein from rat brain in both lanes. **B** 100 μg of protein from gastric glandular mucosa in both lanes

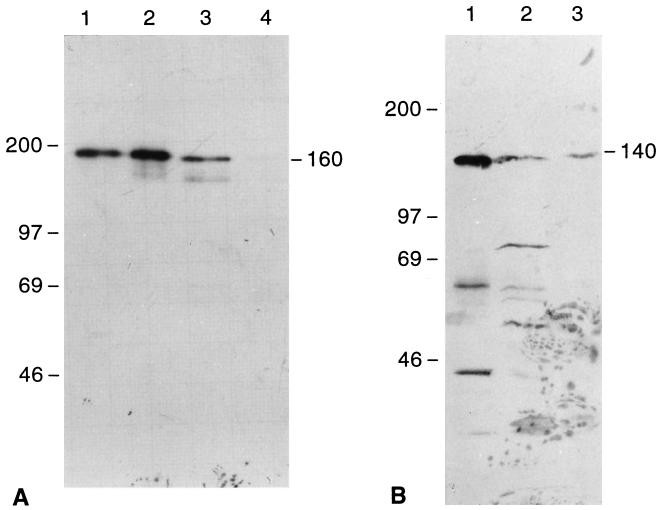

Fig. 2. Immunoblotting with monoclonal antibodies to nNOS (**A**) and to eNOS (**B**). **A** *Lane 1*, 50 μg protein from rat brain; *lane 2*, 5 μg of protein from rat pituitary cells; *lane 3*, 100 μg protein from rat gastric glandular mucosa; *lane 4*, 100 μg protein from rat mesentery. **B** *Lane 1*, 100 μg protein from rat mesentery, *lane 2*, 50 μg protein from rat brain; *lane 3*, 100 μg protein from rat gastric glandular mucosa

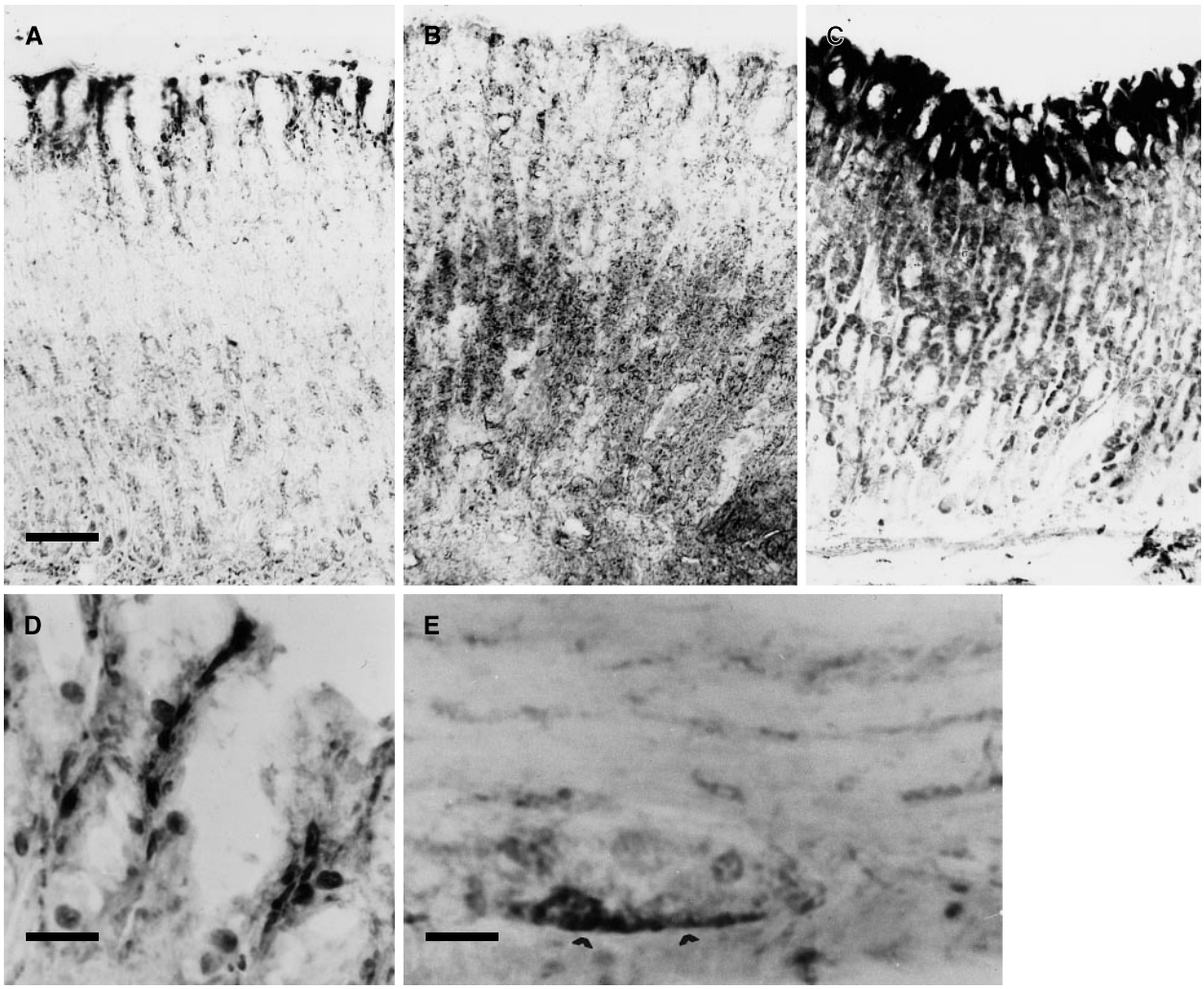

Fig. 3. Immunocytochemistry of rat corpus glandular mucosa (**A, B, D**), duodenal muscle layers (**E**), and NADPH diaphorase staining of the mucosa (**C**). All tissues were preserved by fixation with 4% paraformaldehyde. **A, D, E** Monoclonal antibody to nNOS. **B** Monoclonal antibody to nNOS plus purified nNOS (10 μg/ml). *Bar* in **A**=100 μm with the same magnification for **B** and **C**. **D** Enlarged surface of the epithelium with the *bar*=25 μm. *Bar* in **E**=25 μm, with *arrows* indicating myenteric plexus

terborough, UK). Alternatively unfixed tissue was frozen in liquid nitrogen and 10 μm sections were cut at –20°C using a cryostat and dried onto slides. These sections were fixed by immersion in acetone at 4°C for 5 min.

Immunolocalization

Sections were treated with 0.75% (v/v) hydrogen peroxide for 30 min to inhibit endogenous peroxidase, washed in PBS, and non-specific binding blocked by exposure for 20 min to 5% goat serum in PBS. The section was then exposed to antibodies to NOS, for 30–120 min at room temperature. Rabbit antiserum to nNOS was used at a dilution of 1/320 in blocking medium, with similarly diluted pre-immune serum, or antiserum plus 100 μg/ml of peptide, as controls. Monoclonal antibodies to nNOS and eNOS and iNOS were diluted in blocking medium to give concentrations of 5 , 10 and 10 μg/ml, respectively. Appropriately diluted mouse monoclonal antibody of irrelevant specificity was used as a control. Also purified recombinant rat brain NOS, at a concentration of 10 μg/ml, was added to the nNOS monoclonal antibody as a further control.

After washing with PBS, sections were incubated with the appropriate biotinylated anti-species IgG and binding of the secondary antibody was detected by using avidin-biotinylated-peroxidase reagent (Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK) with diaminobenzidine as the peroxidase substrate.

NADPH diaphorase

Sections were exposed to 50 mM TRIS buffer (pH 8.0) containing 1 mM NADPH, 0.2% (v/v) Triton X-100 and 0.5 mM nitroblue tetrazolium for 30 min at 37°C. NADPH was omitted from the controls.

Results

Characterization of antibodies

The rabbit antiserum raised against the C-terminal hexadecapeptide of rat brain NOS reacted with a 160-kDa protein on nitrocellulose blots of an extract of rat brain. This recognition was prevented if the peptide was added with the antiserum (Fig. 1A). Pre-immune serum did not react with any material on blots of brain extracts. Immunoblot-

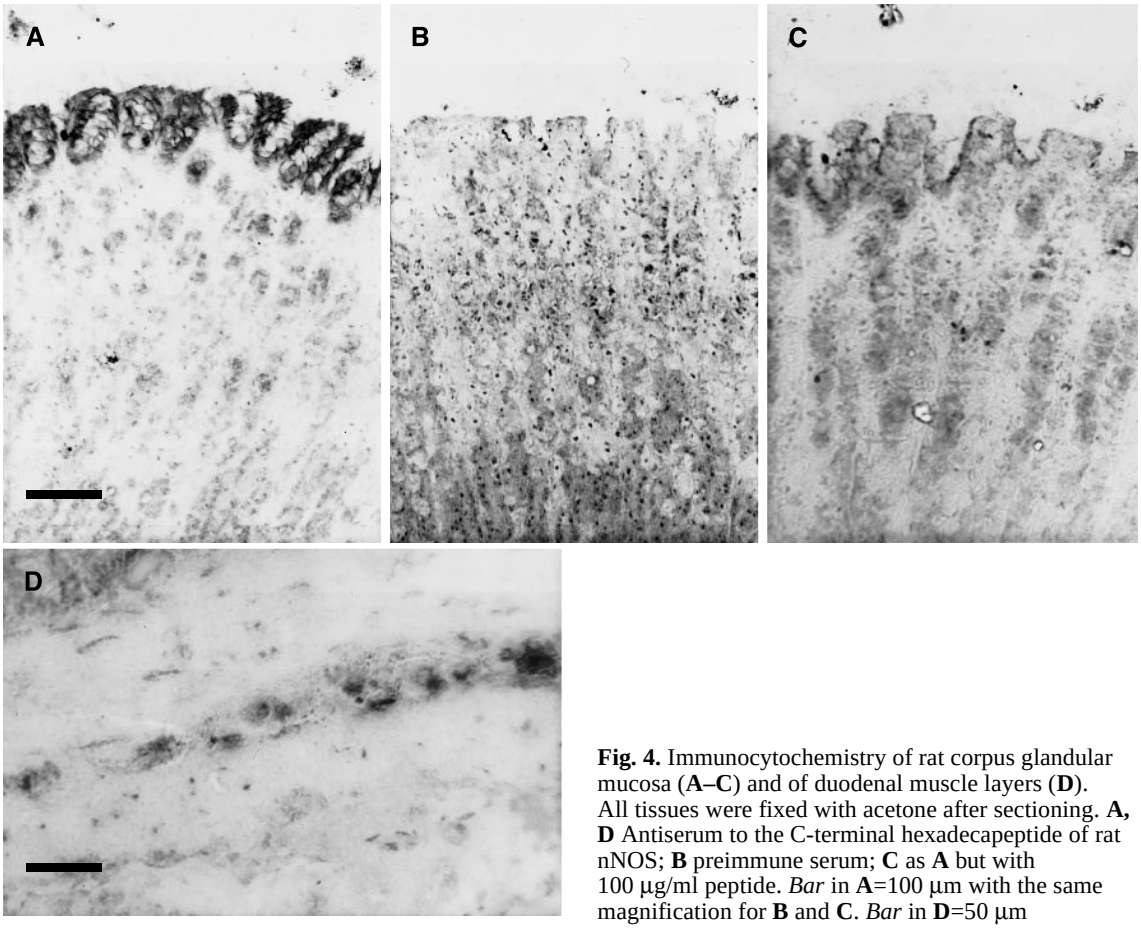

Fig. 4. Immunocytochemistry of rat corpus glandular mucosa (**A–C**) and of duodenal muscle layers (**D**). All tissues were fixed with acetone after sectioning. **A, D** Antiserum to the C-terminal hexadecapeptide of rat nNOS; **B** preimmune serum; **C** as **A** but with 100 μg/ml peptide. *Bar* in **A**=100 μm with the same magnification for **B** and **C**. *Bar* in **D**=50 μm

ting of extracts of gastric mucosa with the antipeptide antiserum also gave a band at 160 kDa, which was substantially reduced by the addition of the peptide to the antiserum (Fig. 1B). The monoclonal antibody directed against nNOS recognized a protein of molecular mass 160 kDa in extracts of rat brain, rat pituitary tumour cells and gastric mucosa but no such band was obtained with an extract of rat intestinal mesentery (Fig. 2A). The monoclonal antibody directed against eNOS gave an intense band at 140 kDa in extracts of rat small intestinal mesentery with fainter bands for extracts of rat brain and gastric mucosa (Fig. 2B). The monoclonal antibody to eNOS also gave a dense band at 140 kDa with immunoblots of extracts of human aortic endothelial cells which was absent if the antipeptide antiserum against nNOS was used.

Immunolocalization with antibodies directed against nNOS

The monoclonal antibody to nNOS stained surface epithelial cells in corpus mucosa which had been fixed by exposure to 4% paraformaldehyde (Fig. 3). Staining of this region did not occur if mouse IgG of irrelevant specificity replaced the antibody, if the secondary antibody was removed or if the monoclonal antibody was applied in the presence of purified recombinant rat brain nNOS (Fig. 3). Mucous-neck, parietal or chief cells did not exhibit specific staining by the nNOS monoclonal antibody. The antibody also stained neuronal elements within duodenal muscle layers (Fig. 3).

The polyclonal antiserum raised against the carboxyl-terminal hexadecapeptide of rat brain nNOS reacted very poorly with corpus mucosa fixed with 4% paraformaldehyde, but staining of surface cells was observed in tissue sections pre-treated with acetone (Fig. 4). Staining was absent when pre-immune serum was used, when the secondary antibody was omitted or when the peptide was added with the antiserum (Fig. 4). The antipeptide antiserum recognized myenteric plexus in the duodenum (Fig. 4), while controls using pre-immune serum were negative.

NADPH diaphorase

The surface of the corpus mucosa fixed in 4% paraformaldehyde exhibited intense diaphorase staining with the remainder of the mucosa staining lightly (Fig. 3). Sections treated with acetone exhibited a similarly intense staining of the surface but the zone of lighter staining went only halfway down the gastric glands.

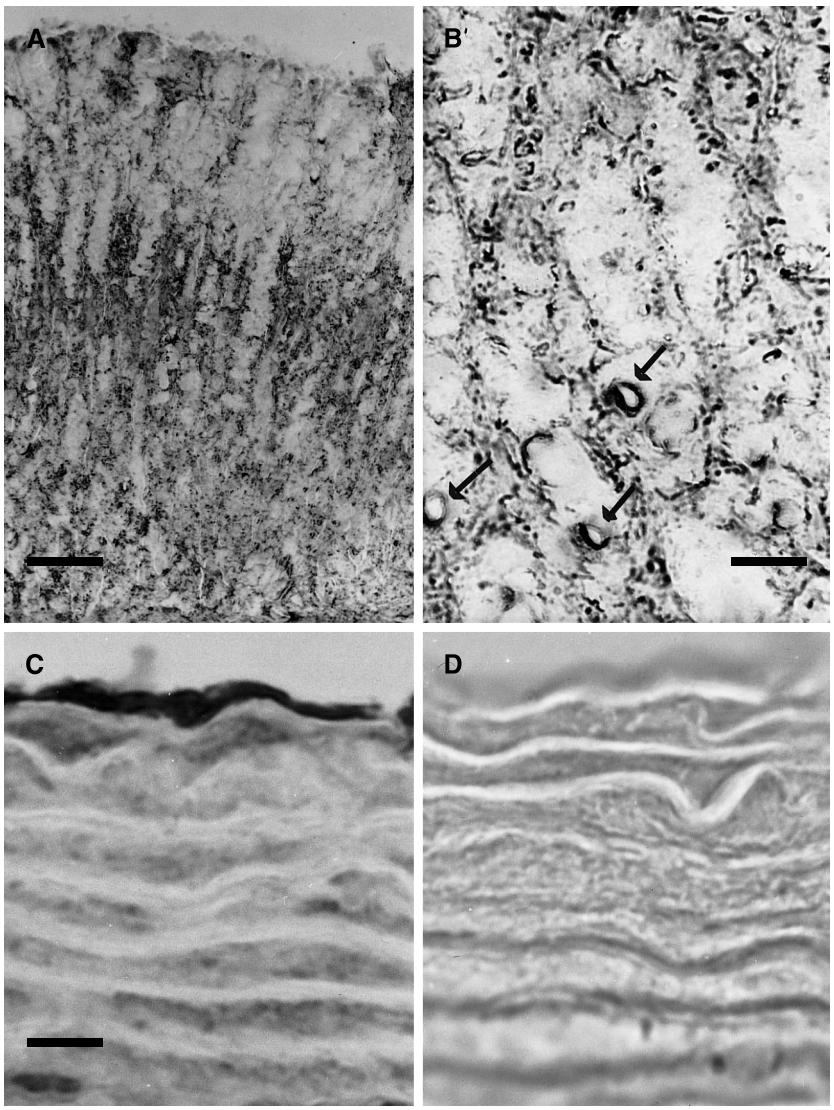

Fig. 5A–D. Immunocytochemistry with eNOS antibody. **A** Rat corpus glandular mucosa (*bar*=100 μm); **B** vessels (*arrows*) in the lower part of the glandular mucosa (*bar*=50 μm) and **C** rat aorta (*bar*=10 μm). **D** Rat aorta incubated with mouse IgG (same magnification as **C**)

Antibody to eNOS

In tissues fixed with paraformaldehyde the monoclonal antibody to eNOS stained the endothelium of rat aorta but did not stain the surface of the rat corpus mucosa (Fig. 5). Blood vessels in the lower half of the gastric glands (Fig. 5) and in the sub-mucosa was stained by this antibody. No such staining occurred if the secondary antibody was omitted or if the primary antibody was replaced by mouse IgG. In mucosal sections treated with acetone the antibody directed against eNOS stained fewer blood vessels and again did not stain the mucosal surface.

Antibody to iNOS

No staining of the mucosa was observed after exposure of sections treated with acetone to a mouse monoclonal antibody directed against iNOS.

Discussion

Specificity of the antibodies

The antiserum raised against the C-terminal hexadecapeptide of rat brain NOS and the monoclonal antibody to human nNOS appeared to recognize rat nNOS. Thus in immunoblots a band of the appropriate molecular mass was detected in extracts of rat brain, gastric mucosa and pituitary cells. Furthermore, the myenteric plexus and neuronal structures in the duodenum, an established site of nNOS (Schmidt et al. 1992; Alm et al. 1993; Nichols et al. 1993), were stained by the polyclonal antiserum and by the monoclonal antibody. The polyclonal antiserum showed very poor activity in tissue which has been treated with 4% paraformaldehyde. A reduction in immunoreactivity of NOS with a polyclonal antibody raised against rat brain NOS following treatment with paraformaldehyde has been reported previously (Schmidt et al. 1992). In the present case reaction of ly-

sine side-chains in the carboxyl-terminal region with formaldehyde may have been responsible for loss of antigenicity of NOS in the tissue. Although the monoclonal antibody directed against nNOS was raised against a peptide derived from the human nNOS sequence, the region used showed 97% homology with the corresponding region from rat nNOS; so it is not surprising that this antibody recognized rat nNOS. The antibody to eNOS recognized a band of the appropriate molecular mass on immunoblots of extracts of rat intestinal mesentery which will contain endothelial material. The presence of a band at the same position on immunoblots of brain extracts has been found by others (Pollock et al. 1993) and can be explained by the presence of eNOS in brain capillaries and in hippocampal pyramidal cells (Dinerman et al. 1994; Tomimoto et al. 1994).

The monoclonal nNOS antibody did not cross-react with eNOS, because no band was produced with the extract of rat mesentery. The faint bands below the main ones for extracts of pituitary cells and gastric mucosa may therefore derive from partial proteolysis during preparation of the extracts. The antiserum directed against the C-terminal sequence of rat brain NOS is unlikely to cross-react with eNOS because this sequence is absent from eNOS, and indeed immunoblots of the extract of rat brain did not exhibit any band in the 140-kDa region. The abolition, or the substantial reduction, in the 160-kDa band on immunoblots of brain and gastric mucosa obtained by addition of the peptide to the rabbit polyclonal antiserum is further evidence of the specificity of this antiserum.

The eNOS antibody did not cross-react with nNOS because blots of brain extracts exposed to the eNOS antibody did not show immunoreactive material in the 160-kDa region. This eNOS antibody has been used by others to successfully stain LLC-PK_1 cells (McKee et al. 1994), an established source of eNOS (Tracey et al. 1994).

Localization of NO synthase

The zone of staining of the surface cells of the rat corpus mucosa with the nNOS antiserum coincided with a region of high NADPH diaphorase reactivity. NADPH diaphorase activity is characterized by the NADPH-dependent reduction of a soluble nitroblue tetrazolium salt to an insoluble formazan dye. NOS is one of the enzymes which exhibits NADPH diaphorase activity, and thus all regions positive for NOS should potentially be NADPH-diaphorase-positive although fixation may affect the intensity of NADPH diaphorase staining associated with NOS activity (Matsumoto et al. 1993; Spessert and Layes 1994). However, NADPH diaphorase staining does not always indicate the presence of NOS (Matsumoto et al. 1993; Tracey et al. 1993). Thus the weak NADPH diaphorase staining present throughout the gastric glands of tissue fixed with paraformaldehyde did not correspond with staining for NOS.

Taken together, these results indicate that surface cells of the corpus mucosa exhibit NOS activity, and are compatible with previous results that centrifugal elutriator fractions containing mucous cells are enriched in NOS (Brown et al. 1992b). The form of NOS present in the surface cells resembles nNOS in its immunoreactivity rather than eNOS. eNOS was, however, detected in blood vessels in the lower region of the gastric glands and in the sub-mucosa, which is compatible with an involvement of NO in regulation of gastric mucosal blood flow (Whittle 1994).

The staining seen with antibodies directed against eNOS and nNOS is unlikely to have occurred due to cross-reaction with the Ca^{2+}-independent, inducible form of iNOS (Cho et al. 1992). Firstly, no Ca^{2+}-independent NOS enzyme activity was detectable in gastric mucosa from normal rats (Brown et al. 1994). Secondly, an antibody directed against iNOS did not stain gastric mucosa. Thirdly, if iNOS had been present in gastric mucosa and had cross-reacted with antibodies to nNOS or to eNOS then a band of 130 kDa (Cho et al. 1992) should have been visible on immunoblots of extracts of gastric mucosa. No such band was evident.

Localization of NOS to surface cells indicates that NO is probably involved in the regulation of mucus secretion by the gastric epithelial cell in which it is generated. However, unlike other intracellular messengers, NO generated within one cell also has the potential to modify activity in adjacent cells because of the ease with which it can pass through cell membranes. Thus activation of NOS in one mucous cell could conceivably trigger secretory activity in adjacent cells. Secretion of bicarbonate by surface cells is stimulated by cyclic GMP (Flemstrom 1994), and consequently NO, via stimulation of guanylate cyclase, may also be involved with the regulation of gastric bicarbonate secretion. NO could therefore have effects on gastric mucosal integrity via actions on the microcirculation (Whittle 1994) and on the surface mucus-bicarbonate barrier. Furthermore, calculations suggest that NO generated from a point source over a few seconds may be active over distances up to and beyond 200 μm (Wood and Garthwaite 1994). NO release from surface cells could therefore potentially influence the functioning of other gastric cells deeper in the mucosa.

The forestomach of the rat exhibits a stratified squamous epithelium which is morphologically distinct from the glandular stomach. NOS has been detected in this region either throughout the epithelium (Schmidt et al. 1992) or in specialized brush or caveolated cells in the cardiac fold (Kugler et al. 1994). Together with the present data, these results suggest the presence of NOS over a large part of the gastric luminal surface.

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