Journal of Neurocytology **25**, 525–534 (1996)

Ultrastructural study of perivascular nerve fibres and endothelial cells of the rat basilar artery immunolabelled with monoclonal antibodies to neuronal and endothelial nitric oxide synthase

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Received 31 October 1995; revised 22 February and 7 June 1996; accepted 10 June 1996

Summary

The ultrastructural distribution of neuronal (type I) and endothelial (type III) isoforms of nitric oxide synthase in perivascular axons and endothelial cells was examined in the Wistar rat cerebral basilar artery, employing monoclonal antibodies specific either to type I or type III of nitric oxide synthase in pre-embedding peroxidase-antiperoxidase immunocytochemistry. Both neuronal and endothelial nitric oxide synthase were localized in similar proportions of perivascular axons (25.2% ± 2.8 and 28.7% ± 6.5, respectively) but in different proportions in vascular endothelial cells (6.2% ± 0.9 and 27.1% ± 2.4, respectively). The intracellular distribution of immunoreactivity to neuronal and endothelial nitric oxide synthase was similar both in axons and endothelial cells; e.g. the labelling of the membranes of mitochondria and synaptic/cytoplasmic vesicles. However, the intensity of immunoreactivity was most prominent in profiles positive for endothelial nitric oxide synthase. The neuronal and endothelial nitric oxide synthase-positive axon varicosities were characterized by the presence of small spherical agranular vesicles; agranular vesicles in nerve varicosities positive for neuronal nitric oxide synthase were significantly larger than those in nerve varicosities positive for endothelial nitric oxide synthase (43.2 nm ± 0.5 versus 37.7 nm ± 0.8; $p < 0.001$). Varicosities positive for endothelial nitric oxide synthase also contained large granular vesicles (116.6 nm ± 5.9) with labelled cores. In conclusion, the present data demonstrate that monoclonal antibodies to neuronal and endothelial nitric oxide synthase immunoreact with subpopulations of both perivascular axons and endothelial cells of rat basilar artery. The significance of neuronal and endothelial isoforms of nitric oxide synthase for the basilar artery is discussed.

Introduction

Nitric oxide (NO) has been shown to have a wide variety of biological effects, including its ability to act as a potent vasodilator (see Moncada *et al.*, 1991; Bredt & Snyder, 1994; Knowles & Mocada, 1994). Nitric oxide is synthesized from L-arginine by nitric oxide synthase (NOS), a highly active enzyme in neurons (nNOS/brain NOS, isoform type I) and endothelial cells (eNOS, isoform type III) (see: Knowles & Moncada, 1994). Both nNOS and eNOS are constitutive and dependent on Ca^{2+}/calmodulin (Bredt & Snyder, 1990; Moncada *et al.*, 1991). NO acts as a non-adrenergic, non-cholinergic neurotransmitter in several parts of the CNS and PNS and as an endothelium-derived relaxing factor (EDRF) in cerebral and peripheral blood vessels (Palmer *et al.*, 1987, 1988; Bredt *et al.*, 1990; Toda & Okamura, 1990; Garthwaite, 1991; Rand, 1992; Toda *et al.*, 1993). Nitric oxide from cerebral perivascular vasodilator nerves (axons) and cerebrovascular endothelium may therefore be involved in the neural and endothelial regulation of cerebral blood flow (see Faraci & Brian, 1994).

Light and/or electron microscopic studies employing polyclonal antibodies to nNOS enzyme from rat brain have revealed that nNOS-immunoreactivity is localized in perivascular axons and endothelial cells of cerebral blood vessels of man and rat (Nozaki *et al.*, 1993; Loesch *et al.*, 1994; Gorelova *et al.*, 1996). It has also been shown that the NOS-containing perivascular axons of rat main cerebral vessels arise from

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the sphenopalatine ganglion and/or the ophthalmic division of the trigeminal ganglion (Nozaki *et al.*, 1993).

nNOS-immunoreactivity in endothelial cells using polyclonal nNOS antibody has also been observed in ultrastructural preparations of non-cerebral blood vessels, including rabbit aorta (Loesch & Burnstock, 1993; Loesch *et al.*, 1993), rat coronary and pulmonary arteries and pulmonary vein (Loesche & Burnstock, 1995, 1996) as well as human umbilical vessels (Sexton *et al.*, 1995). The above studies suggest the presence of nNOS enzyme in a subpopulation of vascular endothelial cells. However, it is not known whether the polyclonal anti-nNOS antibody used in these studies also detected the eNOS enzyme in endothelial cells or whether the labelling was associated with the nNOS-containing cells exclusively. In the present study, therefore, we employed monoclonal antibodies specific to rat nNOS and eNOS to examine the ulstrastructural localization of nNOS- and eNOS-immunoreactivity in perivascular axons and endothelial cells of the Wistar rat basilar artery.

Materials and methods

Four adult (6-month-old) male Wistar rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.; Sagatal, RMB Animal Health Ltd, Dagenham, UK) and perfused through the heart (left ventricle) with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, the basilar arteries dissected and immersion-fixed for 3 h at 4° C with the same fixative. Arteries were then transferred to phosphate buffer and stored overnight at 4° C. The following day arteries were cut longitudinally to produce strips. For localization of n/eNOS in perivascular axons and endothelial cells, strips of arteries were processed for the peroxidase-antiperoxidase (PAP) immunocytochemistry of monoclonal nNOS and eNOS antibodies adapting the steps of the procedure previously reported. Initially, however, the specimens were exposed for 30 min to 0.03% H_2O_2 and sodium azide for blocking of endogenous peroxidases (peroxidase inhibitor, DAKO, High Wycombe, UK), washed in 0.05 M Tris-buffered saline (TBS, DAKO) at pH 7.6 and then exposed to normal goat serum (Nordic Immunology, Tilberg, The Netherlands) diluted 1:30 in TBS containing 0.1% sodium azide (this buffer was also used for the dilution of primary and secondary antibodies) for 1.5 h. After rinsing in TBS the specimens were incubated for 48 h at 4° C either with a mouse monoclonal antibody to nNOS or eNOS at a dilution of 1:250 (1 µg of antibody per ml of incubation buffer). After washing in TBS, the specimens were then exposed for 1.5 h to goat-antimouse immunoglobulin G serum (reacting with mouse IgC subclasses G1, G2a, G2b and G3) (Sigma, Poole, UK) diluted 1:40, washed in TBS and incubated for 3 h with a mouse PAP soluble complex (Sigma) diluted 1:200, washed in TBS, and next treated with 3, 3′-diaminobenzidine (Sigma) and 0.01% H_2O_2. After washing in TBS and phosphate buffer, the specimens were postfixed in 1% OsO_4 for 1 h at 4° C, washed in phosphate buffer, dehydrated in a graded series of ethanol and embedded in Araldite. The ultrathin circumferential sections were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1010 electron microscope.

Controls

Monoclonal mouse anti-nNOS (N31020) and anti-eNOS (N30020) antibodies used were manufactured and characterized by Transduction Laboratories, Lexington, USA (distributed by Affiniti, Exeter, UK). The anti-nNOS antibody (IgG2a isotype) reacts with human and rat nNOS. The anti-eNOS antibody (IgG1 isotype) reacts with human and rat eNOS. The nNOS and eNOS isoenzymes have been shown to be distinct gene products of about 155 kDa and 140 kDa, respectively; enzymes from the human and rat show 93% identity of the sequences of amino acids (Bredt *et al.*, 1991; Janssens *et al.*, 1992; Marsden *et al.*, 1992; Nakane *et al.*, 1993; see also: Förstermann *et al.*, 1994; Knowles & Moncada, 1994). To avoid the possibility of cross-reactivity, the anti-nNOS and anti-eNOS antibodies were used at an optimal dilution of (1 µg ml^{-1}) according to the manufacturer's directions. Immunoblotting studies have also shown that monoclonal anti-nNOS antibody (N31020) reacts with expressed inducible NOS enzyme (iNOS, macNOS) in addition to nNOS when substantially higher concentrations of the antibody are used than those recommended by the manufacturer and distributor (Affinity, direct communication); it is well known that the iNOS enzyme is neither expressed in normal perivascular nerves nor vascular endothelial cells – those which have not been induced by some means (see Förstermann *et al.*, 1991b). Using monoclonal anti-iNOS antibody (N32020, Transduction Laboratories), neither the endothelial cells nor the perivascular nerves of the rat basilar artery examined showed signs of immunolabelling. Preabsorption of the anti-eNOS antibody with the human endothelial cell lysate (recommended as a positive control in Western blotting experiments) prevented positive immunolabelling in endothelial cells (Golgi complex) as shown in submucosal blood vessels from the guinea pig ileum (O'Brien *et al.*, 1995). Our own unpublished preabsorption experiments with human endothelial cell lysate indicate a substantial reduction in eNOS immunoreactivity in endothelial cells of rat carotids. Also control preabsorption of the anti-eNOS antibody with the human endothelial cell lysate and anti-nNOS antibody with the cell lysate from rat pituitary tumour cell line (recommended as positive control in western blots (Transduction Laboratories) indicates in hamster blood vessels elimination of eNOS and nNOS immunoreactivity, respectively. In the present PAP study, no immunolabelling was observed, when the monoclonal nNOS and eNOS antibodies were omitted from the incubation medium and/or replaced with non-immune normal goat serum (Nordic) and non-immune normal mouse serum (BioCell, Cardiff, UK) at a dilution of 1:250, as well as when goat-antimouse immunoglobulin G serum (Sigma) was omitted.

Measurements

Limited quantitation has been made on axon varicosities and endothelial cells. In order to calculate the diameter of

synaptic vesicles that appeared within the nNOS- and eNOS-positive axon varicosities, two measurements, at right angles to each other, were taken for each vesicle from electron micrographs; differences in vesicle size between the nNOS- and eNOS-positive varicosities were analysed statistically using Student's *t*-test. A p value of < 0.05 was taken as being significant. (There were some difficulties with the estimation of the 'real' size of vesicles due to the obscuring effect of immunoprecipitate. This estimation, however, concerned all labelled vesicles examined regardless of the type of varicosity labelled and the type of antibody used). In order to establish the percentage of perivascular axon and endothelial cell profiles positive and negative for nNOS and eNOS, these profiles were counted in ultrathin sections taken from different levels of specimens separated from each other by the distance of at least 20 μm (average size of endothelial cells) to avoid double counting of the same cells. Axons were counted by examination with the electron microscope and examination of the electron micrographs, while endothelial cells were counted with the electron microscope exclusively. Results are expressed as mean ± standard error of the mean (SEM) of four basilar artery preparations from four rats.

Results

Perivascular nerve fibres (axons) and vascular endothelial cells displaying nNOS and eNOS immunoreactivity as a 'black' immunoprecipitate, were observed in ultrathin sections of the Wistar rat basilar artery (Figs 1–4).

Perivascular axons

Perivascular axons (axon varicosities and intervaricosities) displaying either nNOS or eNOS immunoreactivity were present at the adventitial-medial border. (Figs 1 and 2). Immuno-negative axon profiles were also seen. The nNOS- and eNOS-positive axons were seen either in association with, or free from, Schwann cell processes. The labelled axons displayed particles of immunoprecipitate in association with cytosolic sites of the membranes of synaptic vesicles and mitochondria in varicosities (Figs 1c and 2b,c) and microtubules in intervaricosities (Fig. 1b). However, the labelling of membranous structures was more

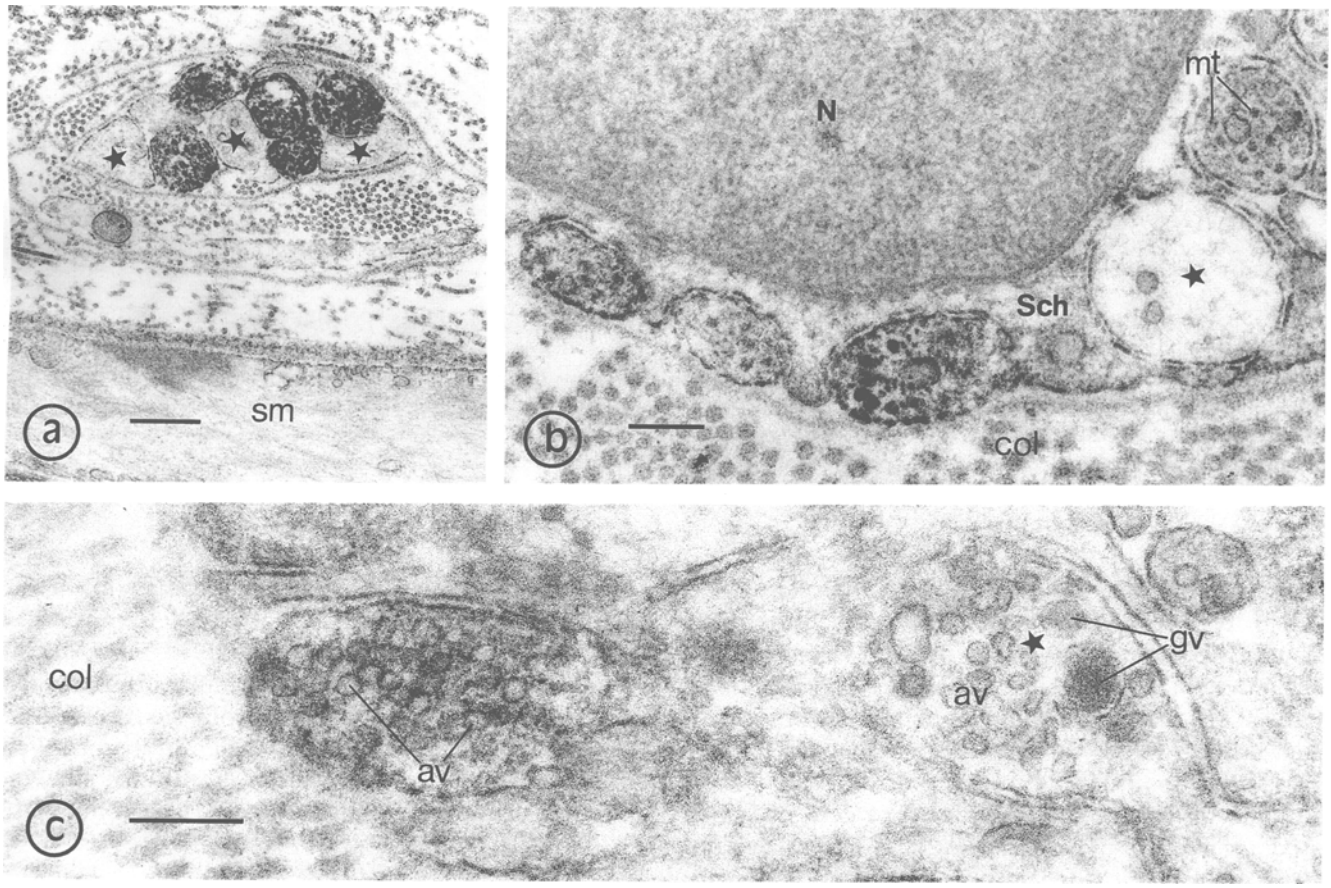

Fig. 1. Perivascular nerve fibres of the basilar artery labelled for nNOS. (a) Five axon profiles in a nerve bundle are nNOS-positive. Unlabelled axons (black stars) can also be seen. sm, smooth muscle. Scale bar = 0.5 μm. A nerve bundle showing nNOS-positive and nNOS-negative axon profiles and an unlabelled Schwann cell (Sch). N, nucleus; mt, microtubules; col, collagen fibres. Scale bar = 0.2 μm. (c) A higher magnification example of an nNOS-positive varicosity containing small agranular vesicles (av); the particles of immunoprecipitate are attached to the membrane of the vesicles. In a neighbouring unlabelled varicosity, note both small agranular vesicles and large granular vesicles (gv). Scale bar = 0.2 μm. (a) ×20 000; (b) ×50 000; (c) ×75 100.

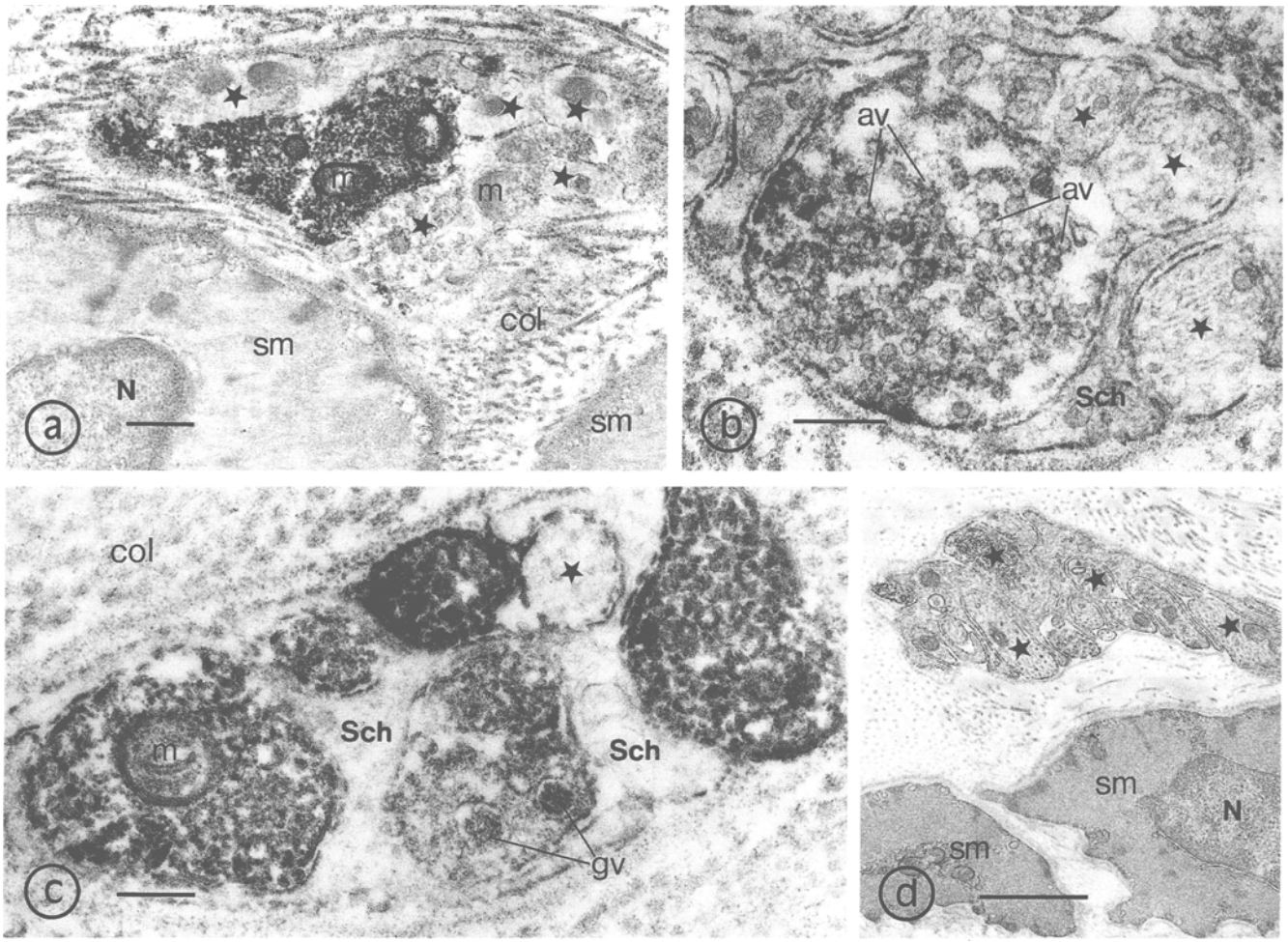

Fig. 2. Perivascular nerve fibres of the basilar artery labelled for eNOS (a–c) and a control specimen (d). (a) One eNOS-positive and several eNOS-negative (black stars) axon profiles are seen in a nerve bundle close to smooth muscle cells (sm). The separation between the eNOS-positive profile and the smooth muscle is about 0.5–0.6 μm. N, nucleus; m, mitochondria; col, collagen fibres. Scale bar = 0.5 μm. (b) A higher magnification example of an eNOS-positive varicosity showing immunolabel attached to the membrane of small agranular vesicles (av). Sch, Schwann cell. Scale bar = 0.2 μm. (c) At least five axon profiles in a nerve bundle are heavily labelled for eNOS. Note large granular vesicles (gv) displaying labelled cores. Scale bar = 0.2 μm. (d) Perivascular nerve fibres in control specimen (omission of NOS antibody) showing no labelling. Scale bar = 1 μm. (a) ×19 000; (b) ×62 100; (c) ×56 700; (d) ×14 700.

prominent in eNOS-positive axons (Fig. 2c). The nerve varicosity types associated with nNOS-immunoreactivity contained small spherical agranular vesicles (Fig. 1c). The diameter of these vesicles was 43.2 nm ± 0.5 ($n = 50$; number of varicosities examined in four rates = 7). In eNOS-positive varicosities, the small agranular vesicles (37.7 nm ± 0.8; $n = 54$; number of varicosities examined in four rats = 5) predominated, but a few large granular vesicles (116.6 nm ± 5.9; $n = 16$; number of varicosities examined in three rats = 4) were also observed showing immunoprecipitate associated with the granular cores (Fig. 2c). In nNOS-positive varicosities, the diameter of agranular vesicles was significantly larger ($p < 0.001$) than the diameter of agranular vesicles observed in eNOS-positive varicosities. Not all perivascular axon profiles were immunoreactive. Less than 30% of the perivascular axon profiles were positive for nNOS (25.2% ± 2.8; 107 out of a total of 430 axon profiles examined in four rats) and eNOS (28.7% ± 6.5; 132 out of a total of 520 axon profiles examined in four rats). An example of control axon profiles processed for immunocytochemistry, where NOS antibodies were omitted from the incubation medium, showing absence of immunoprecipitate, is illustrated in Fig. 1c.

Endothelial cells

Immunoreactivity for nNOS and eNOS was localized in endothelial cells of the basilar artery including its branches (Figs 3 and 4). Not all endothelial cells examined were immunoreactive for nNOS and eNOS. the nNOS-positive endothelial cells were less frequently represented (6.2% ± 0.9; 31 out of a total of 506 cells examined in four rats) than those positive for

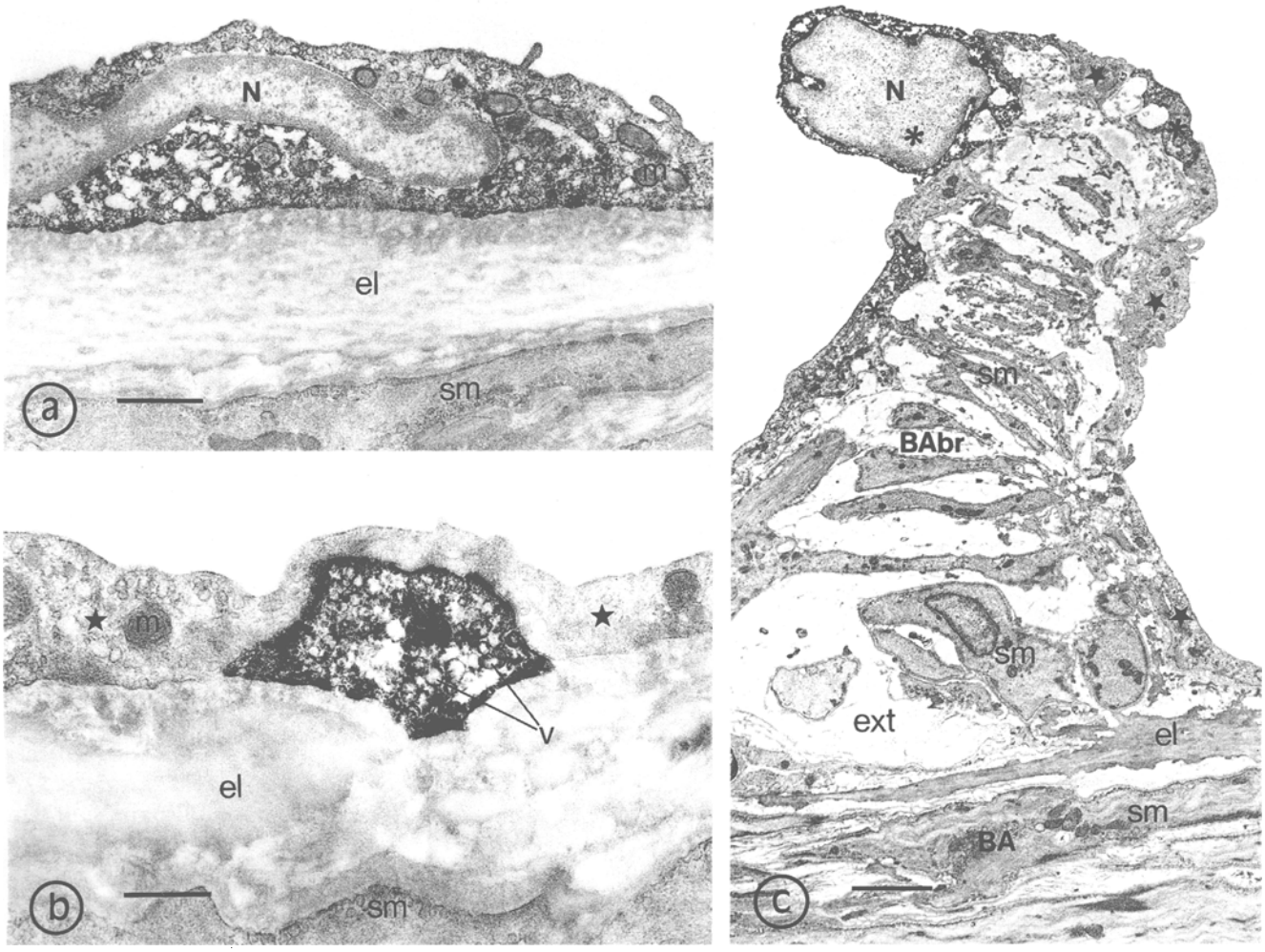

Fig. 3. Endothelial cells of the basilar artery labelled for nNOS. (a) The cytoplasm of a cell body of nNOS-positive endothelial cell displays 'black' particles of immunoprecipitate. N, nucleus; m, mitochondria; el, elastic lamina; sm, smooth muscle. Scale bar = 1 μm. (b) A process of an nNOS-labelled endothelial cell covered by nNOS-negative endothelium (black stars) at the luminal side; the process shows cytoplasmic vesicles. Scale bar = 0.5 μm. (c) A fragment of a branch (BAbr) of basilar artery (BA) contains nNOS-positive endothelial cells (black asterisks). ext, extracellular matrix. Scale bar = 2 μm. (a) ×12 900; (b) ×23 900; (c) 5500.

eNOS (27.1% ± 2.4; 105 out of a total of 382 cells examined in four rats). The nNOS- and eNOS-positive endothelial cells often appeared singly, but eNOS-positive cells were also seen in groups. Immunoreactivity to nNOS and eNOS was similar. It was distributed in the cytoplasm and in association with the membranes of intracellular organelles and structures, such as mitochondria, endoplasmic reticulum and cytoplasmic vesicles. However, the eNOS-positive axons (Fig. 2c) and eNOS-positive endothelial cells (Fig. 4a,b) were frequently more intensely labelled than those nNOS-positive. Endothelial cells of control specimens showed lack of labelling to nNOS and eNOS (Fig. 2d).

Discussion

The present ultrastructural study employing monoclonal antibodies to nNOS and eNOS demonstrates that in the Wistar rat basilar artery, both perivascular axons and endothelial cells exhibit immunoreactivity to nNOS and eNOS. These results, therefore, extend our previous ultrastructural observations of Sprague-Dawley rat basilar artery using polyclonal antibody to nNOS (Loesch *et al.*, 1994) and support the considerable evidence for neuronal and endothelial involvement in the NO-associated mechanisms regulating cerebrovascular tone (Morin & Stanboli, 1993; Toda, 1993; Toda *et al.*, 1993; Tomimoto *et al.*, 1994; Faraci & Brian, 1994).

An important question raised by the present results is whether specific functional implication may be associated with the presence of both isoforms of NOS (type I and type III), in subpopulations of perivascular axons and endothelial cells of cerebral blood vessels.

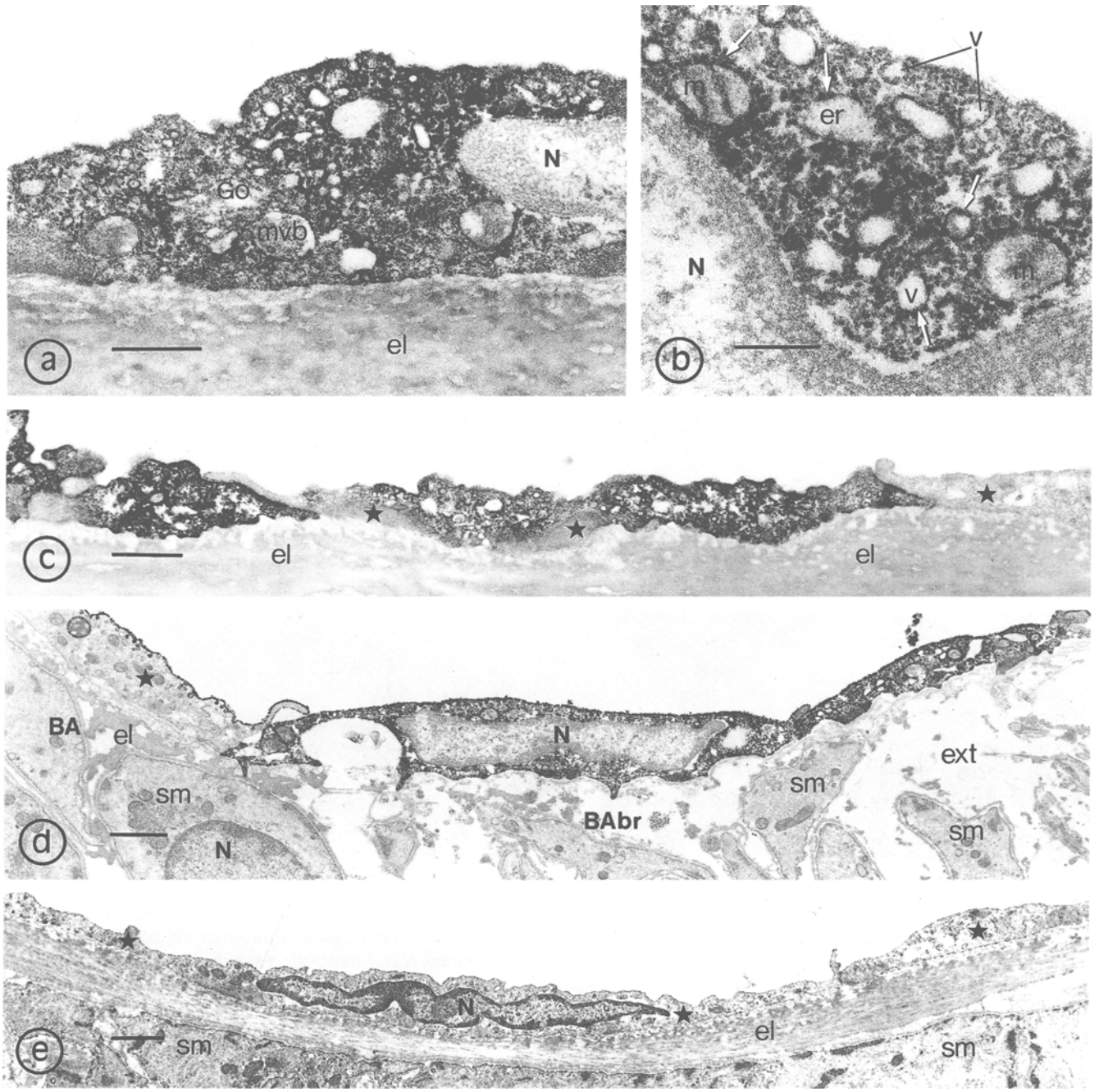

Fig 4. Endothelial cells of the basilar artery labelled for eNOS (a–d) and a control specimen (e). A cell body of an endothelial cell showing intense immunoreactivity to eNOS. N, nucleus; Go, Golgi complex; mvb, multivesicular body; el, elastic lamina. Scale bar = 0.5 μm. (b) Fragment of an eNOS-labelled endothelial cell showing immunoprecipitate throughout the cytoplasm and in association (white arrows) with the membranes of endoplasmic reticulum (er), cytoplasmic vesicles (v) and mitochondria (m). Scale bar = 0.2 μm. (c) A group of eNOS-positive endothelial cells; eNOS-negative cells are also seen (black stars). Scale bar = 1 μm. (d) A branch (BAbr) of basilar artery (BA display eNOS-positive endothelial cells. sm, smooth muscle; ext, extracellular matrix. Scale bar = 1 μm. (e) Endothelial cells in control specimen (omission of NOS antibody) showing no labelling. Scale bar = 1 μm. (a) ×30 000; (b) ×7600; (c) ×12 100; (d) ×10 100; (e) ×8300.

Although being different gene products, nNOS and eNOS are substantially similar (both in human and rat) sharing 57% amino acid identity and containing consensus NADPH-, FMN-, FAD, calmodulin-binding sites and a consensus PKA phosphorylation site (see Förstermann *et al.*, 1994; Knowles & Moncada, 1994). Both isoforms produce NO which may be implicated in a similar physiologic process, e.g. relaxation of vascular smooth muscle (see Förstermann *et al.*, 1991a,b; Moncada *et al.*, 1991). The NO derived from perivascular axons and vascular endothelial cells would therefore be involved in the mechanisms

influencing basal cerebral vascular tone by acting on sites of neurotransmission and endothelial cells (Palmer *et al.*, 1987; Bult *et al.*, 1990). There are indications that (i) the neuronally derived NO may mediate a local increase in cerebral blood flow such as during increases in cerebral metabolism and (ii) the impairment of endothelium-dependent relaxation may contribute to some pathological conditions such as cerebral ischaemia or stroke (Faraci & Brian, 1994). The increased NO synthesis has been shown to be crucial for the increase in cerebral vasodilatory response to hypercapnia but not to hypoxia (Pellegrino *et al.*, 1993). Therefore, the possibility exists that hypercapnia acts mostly by increasing NO production in the non-adrenergic, non-cholinergic vasodilator nerves (Pellegrino *et al.*, 1993) but not in endothelial cells, since hypercapnia-induced relaxation of cerebral arteries is unaffected by removal of endothelium from the vessel wall (Toda *et al.*, 1989). In some regions of the brain (rat), where both nNOS and eNOS were detected in neurons, the NO generated by eNOS may also act as a retrograde messenger of long term potentiation (Dinerman *et al.*, 1994).

It has been shown that more than 90% of eNOS is related to particulate fraction (membrane-bound), presumably membranes derived from the endoplasmic reticulum and plasma membranes, and that NO formed at these sites is more likely to be released into the extracellular environment than NO formed in cytosol (Förstermann *et al.*, 1991a; Michel *et al.*, 1993; Hecker *et al.*, 1994). Unlike the nNOS (and inducible NOS), the eNOS has a unique N-terminal myristate responsible for myristoylation at the N-terminal glycine and binding mostly eNOS to the cell membranes (Förstermann *et al.*, 1991a; Busconi & Michel, 1993). In contrast, the nNOS is predominantly a cytosolic enzyme (Bredt & Snyder, 1990; Schmidt *et al.*, 1992) although the nNOS immunoreactivity may appear in both supernatant and particulate fractions (Dinerman *et al.*, 1994). In endothelial cells, the eNOS may undergo substantial translocation from particulate to cytosol fractions, following the bradykinin stimulation of the enzyme phosphorylation resulting in enzyme reduced catalytic activity (Busconi & Michel, 1993; Michel *et al.*, 1993; Fukuda *et al.*, 1995). In contrast, the phospholipid components of the plasma membrane enhance the activity of eNOS (Ohashi *et al.*, 1993). It seems, therefore, that at least in endothelial cells these changes in eNOS activity (via translocation) might have a functional significance such as a decrease in NO production capacity, hence representing a feedback mechanism for the agonist-induced NO release (Hecker *et al.*, 1994). The present findings indicate that particles of both the nNOS- and eNOS-immunoprecipitate are related to the cytoplasm and to the membrane of intracellular organelles and structures rather than the content (lumina) of the synaptic vesicles in axons and cytoplasmic vesicles in endothelial cells. However, in some eNOS-positive varicosities the content (core) of large granular vesicles was also immunolabelled. In fact, the eNOS-labelling of the axonal/endothelial membranous systems as well as mitochondria and vesicles was frequently more intense than those labelled for nNOS, which might reflect the presence of myristoylated N-terminus of eNOS. The membranous localization of eNOS-immunoreaction product in the perinuclear region and diffuse cytoplasmic immunoreactivity in the periphery of the perikarya of endothelial cells was previously observed in some large cerebral arteries of rat using monoclonal antibody to eNOS in avidin-biotin complex (ABC) method for electron microscopy (Tomimoto *et al.*, 1994). Using monoclonal anti-eNOS antibody (N30020) to post-embedding colloidal-gold electron immunocytochemistry of the human cerebral and superficial temporal arteries, Fukuda and colleagues (1995) demonstrated the possibility of translocation of eNOS from the plasma membranes of cytoplasmic organelles to the cytosol in the endothelium in response to bradykinin stimulation of the enzyme phosphorylation. In their light microscopic study of NADPH-diaphorase (staining for cNOS) in cultured endothelial cells from bovine cerebromicrovasculature, Morin and Stanboli (1993) demonstrated a striking change in the subcellular localization of the labelling (from the juxtanuclear position to the cytoplasm) and its intensity after treatment with colchicine. In non-cerebrovascular endothelium, differences and/or similarities in the distribution of eNOS-labelling was also observed. For example, O'Brien and colleagues (1995) demonstrated eNOS-labelling associated with the membrane of Golgi complex and cytoplasmic vesicles in endothelial cells of submucosal arterioles from the guinea-pig ileum. Using monoclonal anti-eNOS antibody (H32) to pre-embedding ultrastructural study of human umbilical vein, Pollock and colleagues (1993) demonstrated immunoreactivity in the cytoplasm of endothelial cells which was also associated with the plasma membrane and cytoplasmic vesicles; the authors also demonstrate the variety of the pattern of intracellular labelling. It seems that the existing differences and/or similarities of PAP/ABC immunolabelling of eNOS reported by various authors (above) can also be related to the thickness of the sections, the type of blood vessels as well as the age and type of the species examined.

It is premature to speculate whether nNOS and eNOS examined in the present study were colocalized in axons and/or endothelial cells. However, both nNOS and eNOS were localized in similar proportions in perivascular axons (25.2% and 28.7%, respectively). The labelled axons also displayed similar ultrastructural features; the varicosities containing a predominance of small spherical agranular synaptic vesicles.

However, the small agranular vesicles in nNOS-positive varicosities were significantly larger than those in eNOS-positive ones (43.2 nm versus 37.7 nm) suggesting the presence of two NOS-positive populations (nNOS and eNOS) of perivascular nerve fibres. In fact, due to the obscuring effect by immunoprecipitate the measurement of 'real' size of synaptic vesicles was restricted; this concerned all the vesicles examined. Nevertheless, the data presented provides a guide to the size of synaptic vesicles as well as to the ultrastructural differences between the varicosities labelled either for nNOS or eNOS. The size of small agranular vesicles displayed by nNOS-positive varicosities was in concert with ultrastructural observations of NOS-positive varicosities in Sprague-Dawley rat basilar artery and human middle cerebral artery using polyclonal nNOS antibody (Loesch *et al.*, 1994; Gorelova *et al.*, 1996). In contrast, the presence of labelled large granular vesicles (116.6 nm) in eNOS-positive varicosities is a new observation. The possibility of coexistence of nNOS and eNOS in neurons has recently been presented based on the immunohistochemical study of the rat cerebellum and olfactory bulb (Dinerman *et al.*, 1994).

Our study suggests that there are more endothelial cells positive for eNOS than are positive for nNOS. It has previously been shown that there were more endothelial cells and perivascular axons positive for the nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) – the co-factor of the NOS enzyme (Dawson *et al.*, 1991) than were positive for nNOS in rat basilar artery when immunoprocedure involved polyclonal antibody to nNOS (Loesch *et al.*, 1994). Therefore, some of the endothelial cells and perivascular axons labelled for NADPH-d seemed not to be related to the nNOS-labelled cells, exclusively. In fact, this discrepancy in the proportions of NADPH-d- and NOS-labelled perivascular axons and endothelial cells (Loesch *et al.*, 1994) may be explained by the possibility that the polyclonal anti-nNOS antibody used did not label the eNOS-containing cells. In the present study, the overall proportion of eNOS-labelled perivascular axons and endothelial cells examined was higher than that previously observed in Sprague-Dawley rat basilar artery using polyclonal antibody to nNOS (Loesch *et al.*, 1994). Using the same polyclonal nNOS antibody, a relatively small proportion of immunoreactive endothelial cells were also demonstrated in rabbit aorta and human umbilical blood vessels (Loesch & Burnstock, 1993; Sexton *et al.*, 1995). However, in the coronary and pulmonary arteries of the newborn rats, particularly rich immunoreactivity to nNOS has been displayed in the endothelium and vascular smooth muscle (Loesch & Burnstock, 1995). The present finding of nNOS- and eNOS-labelling of endothelial cells is in concert with a study of human middle cerebral artery showing that the immunoreactivity to nNOS and eNOS has been displayed in subpopulations of endothelial cells (Gorelova *et al.*, 1996). However, from the limited quantitation of endothelial cells reported in the present study, only about 6.2% were immunopositive to nNOS and 27.1% to eNOS. This is in contrast to the almost total immunostaining of the cerebrovascular endothelium seen by light microscopy using polyclonal antibodies to the nNOS (Bredt *et al.*, 1990; Springall *et al.*, 1992; Nozaki *et al.*, 1993). This apparent discrepancy may be explained by the more precise immunolocalization afforded by electron microscopical techniques. However, a number of factors including technical inconsistency and/or the different immunocytochemical procedures used which may produce different results may also be responsible for the variability in the proportions of NOS-immunolabelled cells. In the present study, the possibility cannot be completely excluded that some labelling was a result of cross-reactivity of the antibodies used. In any case, in view of the results reported in this paper, the nomenclature of nNOS and eNOS does not appear to be appropriate, and the nomenclature suggested by Knowles (1994) would appear to be preferable. A study employing antisense oligodeoxynucleotides complementary to mRNA sequences specifically encoding either nNOS or eNOS strongly indicates the presence of both nNOS and eNOS in endothelial cells of mouse cerebral microvasculature (Rosenblum, 1996).

In summary, the present study indicates the presence of nNOS- and eNOS-immunoreactivity in perivascular nerve fibres and endothelial cells in rat basilar artery, providing further immuno-ultrastructural evidence for sources of NO which may participate in the control of the cerebral circulation.

Acknowledgements

The authors thank Dr D. Christie for editorial assistance and Mr S. Miah for skilful technical assistance. Financial support of the British Heart Foundation is gratefully acknowledged.

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