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Localisation of immunoreactive factor VIII, nitric oxide synthase, substance P, endothelin-1 and 5-hydroxytryptamine in human postmortem middle cerebral artery

E. GORELOVA[1], A. LOESCH[1], P. BODIN[1], L. CHADWICK[2], P. J. HAMLYN[2] AND G. BURNSTOCK[1]

[1] *Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, London, and* [2] *Department of Neurosurgery, Royal Hospitals NHS Trust, London, UK*

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ABSTRACT

This pre-embedding electron-immunocytochemical study investigated the localisation of endothelial (type III) and neuronal (type I) isoforms of nitric oxide synthase, substance P, endothelin-1 and 5-hydroxytryptamine in the human middle cerebral artery taken up to 40 h postmortem. To 'recover' from the anoxic period some of the vessels were incubated in oxygenated Krebs solution prior to the immunoprocedure. At this long postmortem time, immunoreactivity to type III and type I nitric oxide synthase, substance P, endothelin-1 and 5-hydroxytryptamine was found in a subpopulation of intact cells present in the vessel intima; immunoreactivity to type I nitric oxide synthase was also observed in a subpopulation of adventitial perivascular nerve fibres. Cultures of the cells from the intima of the postmortem vessels showed that the cells were proliferating and positive immunoreactivity to factor VIII identified them as endothelial cells. The results therefore indicate that even after up to 40 h postmortem, endothelium of human middle cerebral artery is immunoreactive for a number of vasoactive agents and perivascular nerve fibres show nitric oxide synthase immunoreactivity.

Key words: Vasculature; middle cerebral artery; vasoactive agents.

INTRODUCTION

The vasoactive agents released by perivascular nerve fibres and vascular endothelium are involved in regulation of cerebral blood flow (Burnstock, 1990). The cerebral arteries are richly supplied by nerves containing acetylcholine (ACh), noradrenaline, 5-hydroxytryptamine (5-HT), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), arginine-vasopressin (AVP), neuropeptide Y (NPY) and substance P (SP), as shown using histo/immunohistochemical techniques (Matsuyama et al. 1983; Jojart et al. 1984; Saito et al. 1985; Liu-Chen et al. 1986; Dhital et al. 1988; Mione et al. 1988; Cavanagh et al. 1990; Gaw et al. 1991). Some of the agents are colocalised, for example NPY and VIP, in parasympathetic cerebrovascular nerve fibres (Cavanagh et al. 1990). VIP and CGRP are known to be present separately in nonadrenergic, noncholinergic vasodilator cerebrovascular nerve fibres (Gibbins et al. 1984; Suzuki et al. 1984, 1988; Edvinsson et al. 1987). The cerebral arteries are also supplied by vasodilator nerve fibres originating from the sphenopalatine and trigeminal ganglia containing nitric oxide synthase (type I, nNOS), an enzyme which synthesises nitric oxide (NO), a potent vasodilator agent (Bredt et al. 1990; Nozaki et al. 1993). NO derived from vasodilator nerves and endothelium mediates cerebroarterial relaxation (Toda et al. 1993).

A number of the vasoactive agents cited above were also found within vascular endothelial cells as detected by immunohistochemical techniques (for review, see Ralevic & Burnstock, 1993). Some of the agents, e.g. ACh, AVP and SP act via endothelium-associated receptor(s) to stimulate the production of an endothelium-derived relaxing factor (EDRF/NO) (Vanhoutte et al. 1986; Ignarro et al. 1987; Palmer et al. 1987). Other agents, including endothelin-1 (ET-1),

Correspondence to Professor G. Burnstock, Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, Gower Street, London WC1E 6BT, UK.

are potent vasoconstrictors, released from endothelial cells (Yanagisawa et al. 1988). The immunohistochemical studies on mammalian cerebral vessels localised AVP, SP, ET-1, nNOS and choline acetyltransferase, an enzyme synthesising ACh in the vascular endothelium (Parnavelas et al. 1985; Loesch et al. 1993, 1994).

Only a few articles have reported on the identification of immunoreactive SP in human cerebrovascular endothelium (Linnik & Moskowitz, 1989; Linnik et al. 1989) and SP, VIP, NPY and nNOS in human cerebrovascular nerves (Edvinsson et al. 1985, 1986; Hiroe et al. 1989; Nozaki et al. 1993). In the present study we examined the electron-immunocytochemical localisation of the endothelial isoform of nitric oxide synthase (type III, eNOS), nNOS, SP, ET-1 and 5-HT in the intimal endothelium and adventitial perivascular nerve fibres of the human middle cerebral artery (MCA) taken between 28 and 40 h postmortem. Some of the vessels were also examined after incubation in Krebs solution, which has been shown to (1) 'revitalise' postmortem tissue from the period of anoxia (Ferguson & Richardson, 1978) and (2) sustain both smooth muscle and endothelial-mediated responses of the MCA (L. Chadwick et al., unpublished data). To establish whether the cells present in intima of postmortem MCA were still alive and to confirm that they were endothelial cells, these cells were cultured and labelled for factor VIII.

MATERIALS AND METHODS

Human autopsy specimens of the left M2 segment of the MCA were obtained from subjects (n = 5; age 59–77 y; both sexes) with no apparent cerebrovascular and/or cerebral disease, at postmortem time 28–40 h. The MCA were placed in Hanks solution for about 1 h for transport to the laboratory. The cultured endothelial cells from the MCA were examined by light microscopy and intact endothelial cells by electron microscopy. For light microscopy of cultured endothelial cells, the MCA was cut longitudinally. The luminal side of the open vessel was incubated in a drop of collagenase (1 mg ml^{-1}, CLS type II, Worthington) for 10 min at 37 °C. Endothelial cells were flushed with a stream of M199 medium supplemented with 15% fetal calf serum (FCS). The cells were centrifuged (7 min, 180 *g*) and their viability was assessed with trypan blue. The cells were plated on glass coverslips in M199 medium + 15% FCS. The medium was changed after 2 d and the cells were used after 4 d in culture. Endothelial cells were fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) at 4 °C and then processed for peroxidase–antiperoxidase (PAP) immunocytochemistry according to the protocol previously described (Loesch et al. 1991). The antibody to factor VIII/human von Willebrand factor antigen (Sera Laboratories Ltd, Crawley Down, UK) used in this study was diluted 1:250 in 0.1 M Tris buffer saline (TBS, pH 7.6) containing 0.1% sodium azide. After immunoprocedure, the coverslips were dehydrated in a graded series of ethanol and propylene oxide and mounted culture side down on glass slides with a drop of Araldite. After polymerisation, the specimens were examined with a Zeiss III RS light microscope. In the controls, the antibody to factor VIII and/or immunoglobulin G were omitted from the incubation medium.

For electron microscopy of intact endothelial cells and perivascular nerves, the MCA was cut in two equal parts. One was immersion-fixed immediately after dissection, and the other was fixed after 3 h incubation with oxygenated Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8, gassed with 95% O_2, 5% CO_2 and maintained at 37 °C. This method was used to 'revitalise'/'recover' the postmortem vessels from the period of anoxia (Ferguson & Richardson, 1978). The samples were fixed overnight at 4 °C with fixative containing 4% paraformaldehyde and 0.25% glutaraldehyde (in phosphate buffer). Arteries were then transferred into phosphate buffer, washed in TBS, and stored in TBS overnight at 4 °C. The following day arteries were cut longitudinally to produce 5 mm long strips which were then processed for eNOS, nNOS, SP, ET-1 and 5-HT PAP immunoprocedure adapting the steps of the procedure as previously reported (Loesch & Burnstock, 1988). Specimens were exposed to 0.3% hydrogen peroxide in 50% methanol for 30 min (for blocking of endogenous peroxidase), washed in TBS, and then exposed to normal goat serum (Nordic Immunology, Tilberg, the Netherlands) diluted 1:9 in TBS containing 0.1% sodium azide (this buffer was used for the dilution of antibodies) for 1.5 h. After rinsing in TBS the specimens were incubated for 48 h at 4 °C with a mouse monoclonal antibody to eNOS at a dilution 1:250, and rabbit polyclonal antibodies to nNOS, SP, ET-1 and 5-HT at a dilution of 1:1000. After washing in TBS, the samples labelled for eNOS were then exposed for 1.5 h to goat-antimouse immunoglobulin G serum (Sigma, Dorset, UK), while the samples labelled for nNOS, SP, ET-1 and 5-HT were exposed to goat-antirabbit immunoglobulin G serum (Biogenesis, Bournemouth, UK) diluted 1:40.

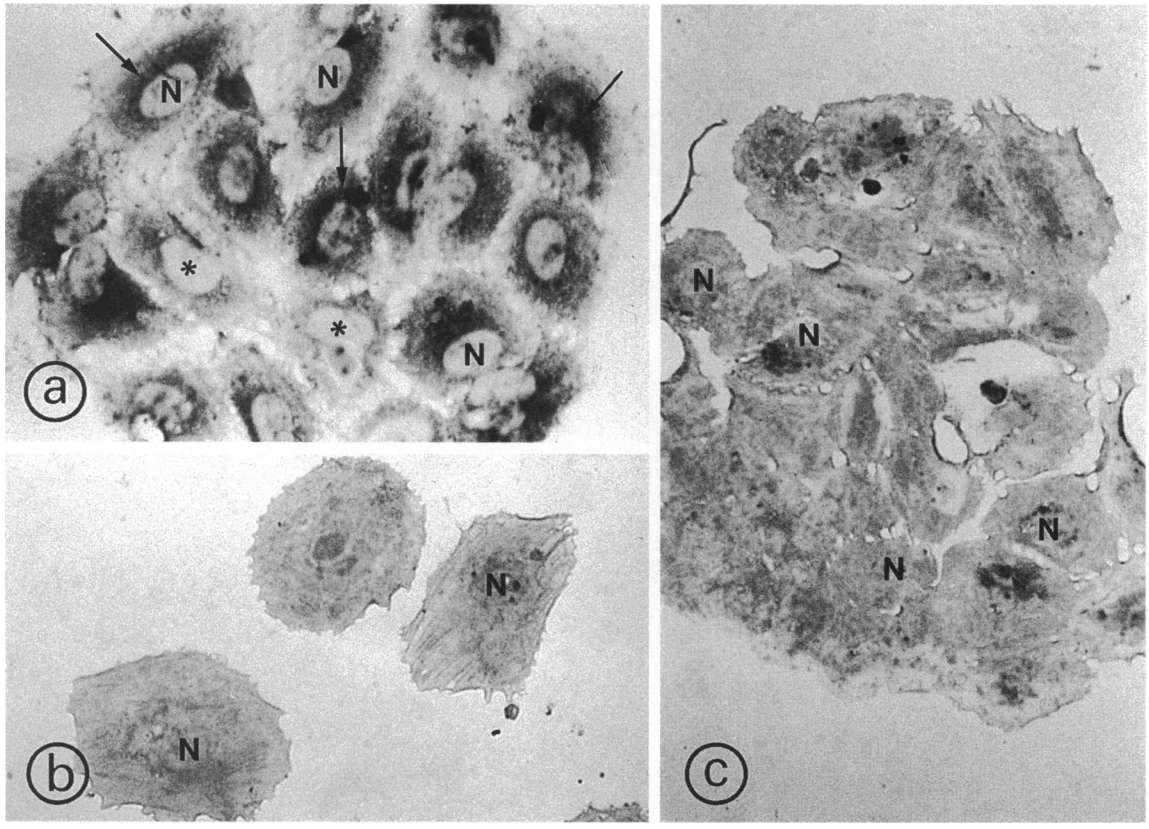

Fig. 1. Light microscopy of cultured endothelial cells from postmortem human MCA labelled for factor VIII (*a*) and control cultures (*b*, *c*). (*a*) The cells display distinctive granular, perinuclear labelling (arrows) for factor VIII. The immunonegative cells (asterisks) can also be seen. Note that endothelial cells grow in groups showing typical cobblestone pattern. N, nucleus (×490). In (*b*) note unlabelled endothelial cells processed for control immunohistochemistry (omission of the IgG) (×490). (*c*) Control immunohistochemistry (omission of the primary antibody) (×490).

After washing in TBS the specimens were incubated for 3 h with a mouse PAP complex (Sigma) diluted 1:150 (for monoclonal antibody) and with a rabbit PAP complex (DAKO, Glostrup, Denmark) diluted 1:75 (for polyclonal antibodies), and next treated with 3,3′-diaminobenzidine (Sigma) and 0.01% hydrogen peroxide. After washing in TBS and phosphate buffer, the specimens were transferred to 1% osmium tetroxide for 1 h, washed in phosphate buffer, dehydrated in a graded series of ethanol and propylene oxide and embedded in Araldite. Ultrathin circumferential sections were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1010 electron microscope.

Controls

The monoclonal eNOS antibody used (Affiniti, Nottingham, UK) was raised in mouse to eNOS from human vascular endothelium as well as from bovine aortic endothelial cells. A 20.4 kDa protein fragment corresponding to amino acids 1030–1209 of human eNOS was used as an immunogen. The antibody was purified from mouse ascities using chromatographic techniques.

The polyclonal nNOS antibody was raised in rabbit against soluble NOS (type I) from rat cerebellum and tested for specificity (Schmidt et al. 1992). Preabsorption of the nNOS antibody (diluted 1:1000) with 20 μg ml^{-1} soluble NOS type I purified from rat cerebellum has been shown to prevent positive labelling in perivascular nerves and also in vascular endothelium in the PAP immunoprocedure (see Loesch & Burnstock, 1993; Loesch et al. 1993, 1994). This antibody also immunolabels human endothelium (Dikranian et al. 1994; Sexton et al. 1995).

The SP antibody to synthetic SP^{1-11} (mammalian sequence) conjugated to bovine serum albumin was raised in rabbit (CRB, Cambridge Research Biochemicals, Cambridge, UK). This antibody cross-reacts with human SP, labelling for example vascular endothelial cells in human ovarian vein (Stones et al. 1995). Preincubation of 1 ml optimally diluted antibody with 5 nmol SP was sufficient to abolish immunostaining (CRB).

The rabbit polyclonal ET-1 antibody to synthetic

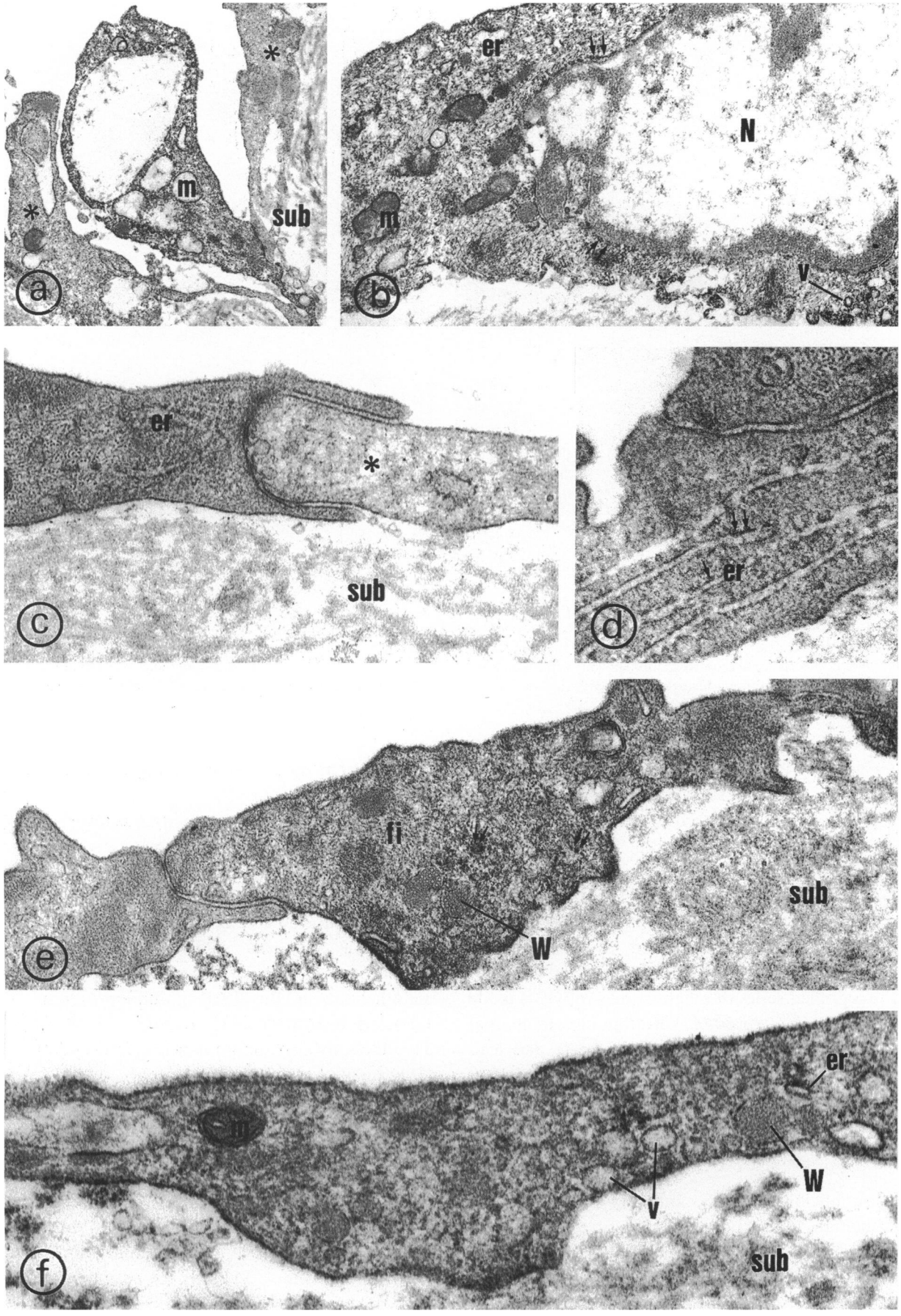

Fig. 2. For legend see opposite.

human ET-1 (CRB) has been used in PAP electron immunocytochemistry of endothelial cells (Loesch et al. 1991) as well as in immunoassay to detect ET levels in the perfusate of freshly harvested endothelial cells (Milner et al. 1990). Preabsorption of ET-1 antibody with its respective antigen (synthetic human ET-1, from CRB) at a concentration of 10^{-4} M eliminated positive labelling in human and animal vascular endothelial cells (Loesch et al. 1991; Loesch & Burnstock, 1995*a*). An inhibition enzyme-linked immunosorbent assay (ELISA) of ET-1 antibody showed that this antibody cross-reacted with proendothelin 39 (7%), ET-2 (15%) and ET-3 (100%) (Bodin et al. 1992). Preincubation of ET-1 antibody with 10 nmol of human ET-1, ET-2 or ET-3 substances per ml of optimally diluted antibody was sufficient to abolish immunostaining (CRB).

The polyclonal 5-HT antibody was generated in rabbit against 5-HT conjugated to bovine serum albumin with paraformaldehyde (Incstar, Minnesota, USA). The immunostaining was completely eliminated by the pretreatment of the antibody (diluted 1:100) with 5-HT/bovine serum albumin (100 μg ml^{-1}) (Incstar).

In this study the specificity of the immunolabelling was investigated routinely by omission of the primary antibody and IgG steps, independently, as well as by replacement of primary antibodies by nonimmune normal mouse serum and normal rabbit serum (both from Nordic). No labelling was observed in these control preparations.

RESULTS

Light microscopy

Cells isolated from the intima of human postmortem MCA were harvested and seeded into dishes. These cells were alive and proliferating in these cultures as demonstrated by incorporation of bromodeoxyuridine (BrdU) and subsequent examination with a fluorescence microscope (data not shown). Using light microscopy these cells appeared flattened, ovoid/polygonal in shape, with a characteristic cobblestone pattern of vascular endothelial cells in culture (Fig. 1a). Immunocytochemical preparations of these cultures examined by light microscopy revealed that approximately 86% of cells (234 out of 273 cells randomly counted) displayed positive immunoreactivity for the factor VIII antigen (Fig. 1*a*). The labelling intensity of the cells positive for factor VIII was considerable. The labelling, a 'brown–black' cytoplasmic immunoprecipitate, was confined mainly to the central regions of the cells. As shown in Figure 1*a*, a granular, perinuclear pattern of the labelling was observed in the factor VIII-positive endothelial cells. No immunoreactivity to factor VIII was found in controls (Fig. 1*b*, *c*).

Electron microscopy

In all specimens examined, both before and after incubation in the Krebs buffer solution, the luminal side of the MCA intima displayed regions containing endothelial cells and regions free of endothelium. After incubation in Krebs solution, however, there were more endothelial cells showing 'improved' ultrastructural preservation. No differences were observed in perivascular nerves. All antigens examined appeared to be present in the MCA both before and after incubation of the arteries in the Krebs buffer. Immunoreactivity for eNOS, nNOS, SP, ET-1 and 5-HT was observed in vascular endothelium (Figs 2–5), whilst in perivascular nerves only immunoreactivity to nNOS was displayed (Fig. 6).

Endothelium

Examination of numerous ultrathin sections taken at different levels of the specimens showed that the endothelial cells immunopositive for any of the substances could be seen distributed between immunonegative cells. The labelled cells appeared singly or in groups. The examples of endothelial cells showing positive labelling for eNOS, nNOS, SP, ET-1 and 5-HT are illustrated in Figures 2, 3, 4 and 5, respectively. The labelled endothelial cells contained 'black' immunoprecipitate throughout the cytoplasm. The im-

Fig. 2. Endothelial cells of MCA labelled for eNOS (*a–d*) and nNOS (*e*, *f*) before (*a*, *b*, *e*) and after (*c*, *d*, *f*) incubation in Krebs buffer. (*a*) Note one endothelial cell with cytoplasmic labelling ('black stain') for eNOS. Two neighbouring endothelial cells (black asterisks) are eNOS-negative. m, swollen mitochondria; sub, subendothelial zone (× 14000). (*b*) The cytoplasm of eNOS-positive endothelial cells contains condensed forms of mitochondria, short strands of endoplasmic reticulum (er) and cytoplasmic vesicles (v). Note particles of immunoprecipitate (arrows) in association with nucleolemma. N, nucleus (× 18000). (*c*) Note a close contact of eNOS-positive and eNOS-negative endothelial cells (× 55000). (*d*) Immunoprecipitate in the cytoplasm and in association with endoplasmic reticulum is shown at higher magnification of an eNOS-positive endothelial cell (× 90000). (*e*) An nNOS-positive endothelial cell shows immunoprecipitate concentrated at the basal portion of the cell. Note abundance of filaments (fi) in the apical portion of the same cell. The cytoplasm also contains Weibel–Palade bodies (W) (× 42000). (*f*) Cytoplasmic vesicles, endoplasmic reticulum and mitochondria are seen at higher magnified portion of an nNOS-positive endothelial cell (× 60000).

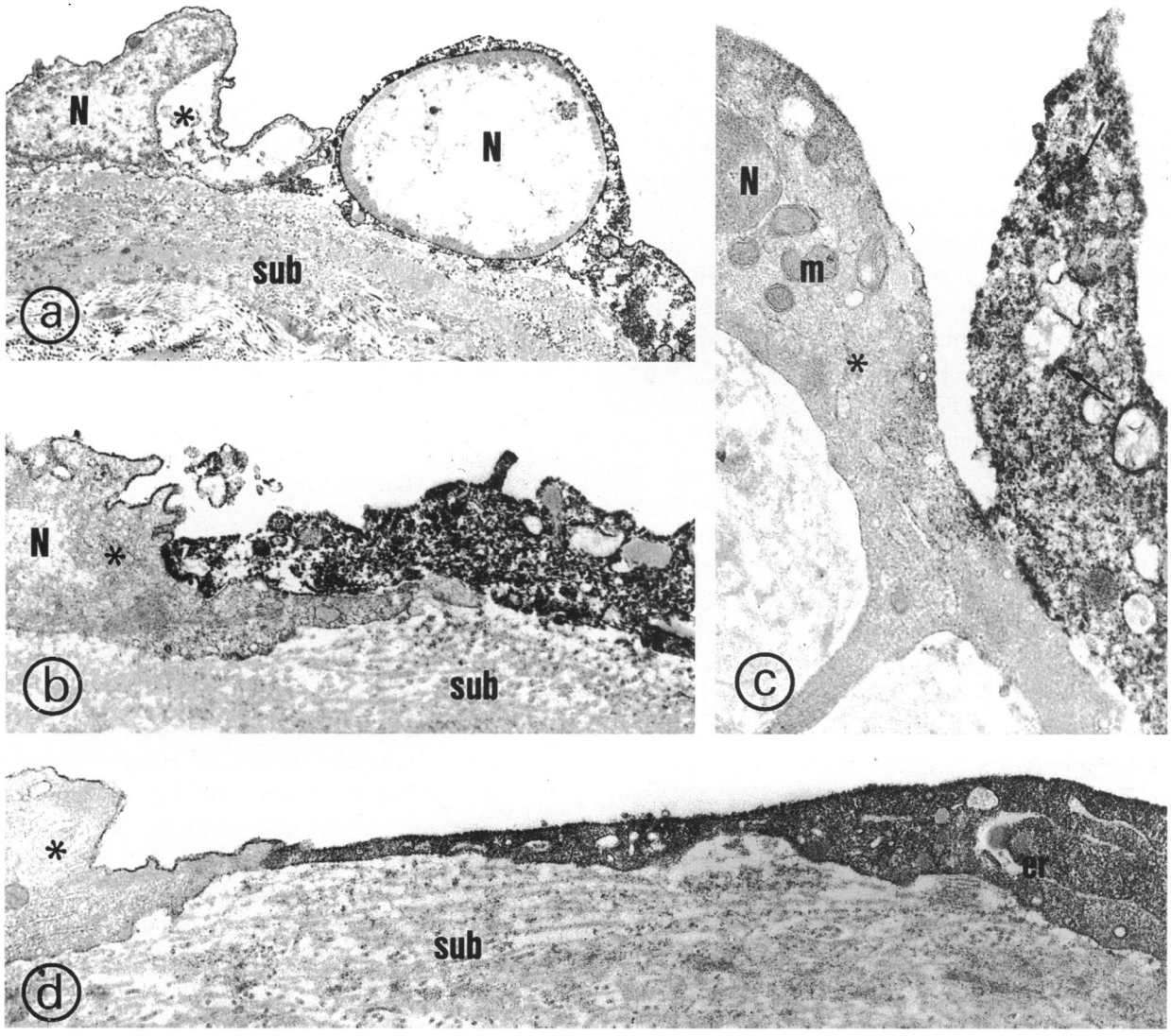

Fig. 3. Endothelial cells of MCA labelled for SP before (*a–c*) and after (*d*) incubation in Krebs buffer. (*a*) Note one SP-positive and one SP-negative (asterisk) damaged endothelial cell. N, nucleus; sub, subendothelial zone (× 8000). (*b*) Note the intense labelling of a SP-positive cell (× 19000). (*c*) Note clusters of immunoprecipitate (arrows) in SP-labelled endothelial cell. m, mitochondria (× 18000). (*d*) Note elongated strands of endoplasmic reticulum (er) in one SP-positive endothelial cell. (× 15000).

munoreactivity was also confined to the outer membranes of mitochondria, nucleolemma, cytoplasmic vesicles and especially endoplasmic reticulum. Along with well preserved endothelial cells, there were also damaged endothelial cells which were positive or negative for a given substance. For example, Figure 3*a* illustrates the SP-positive and SP-negative damaged endothelial cells.

Perivascular nerves

In this study, only immunoreactivity to nNOS was observed in perivascular nerve fibres (Fig. 6*a*, *b*). These were located in the adventitia, sometimes being in close apposition with the media layer. The nNOS-positive axons were seen either in association, or free from, Schwann cell processes. Immunoreactivity to nNOS was observed within the axon varicosities and intervaricosities (Fig. 6*a*, *b*). The nNOS-positive varicosities displayed small agranular vesicles (Fig. 6*b*), while the nNOS-positive intervaricosities displayed microtubules (Fig. 6*a*). The number of nNOS-positive and nNOS-negative axons varied from one nerve bundle to another. 'Black' immunoprecipitate in nerve fibres was associated with the axoplasm and membranes of synaptic vesicles in varicosities (Fig. 6*b*) and with microtubules in intervaricosities (Fig. 6*a*). No immunolabelling to nNOS was observed in the perivascular nerves of the control preparations, as illustrated in Figure 6*c*.

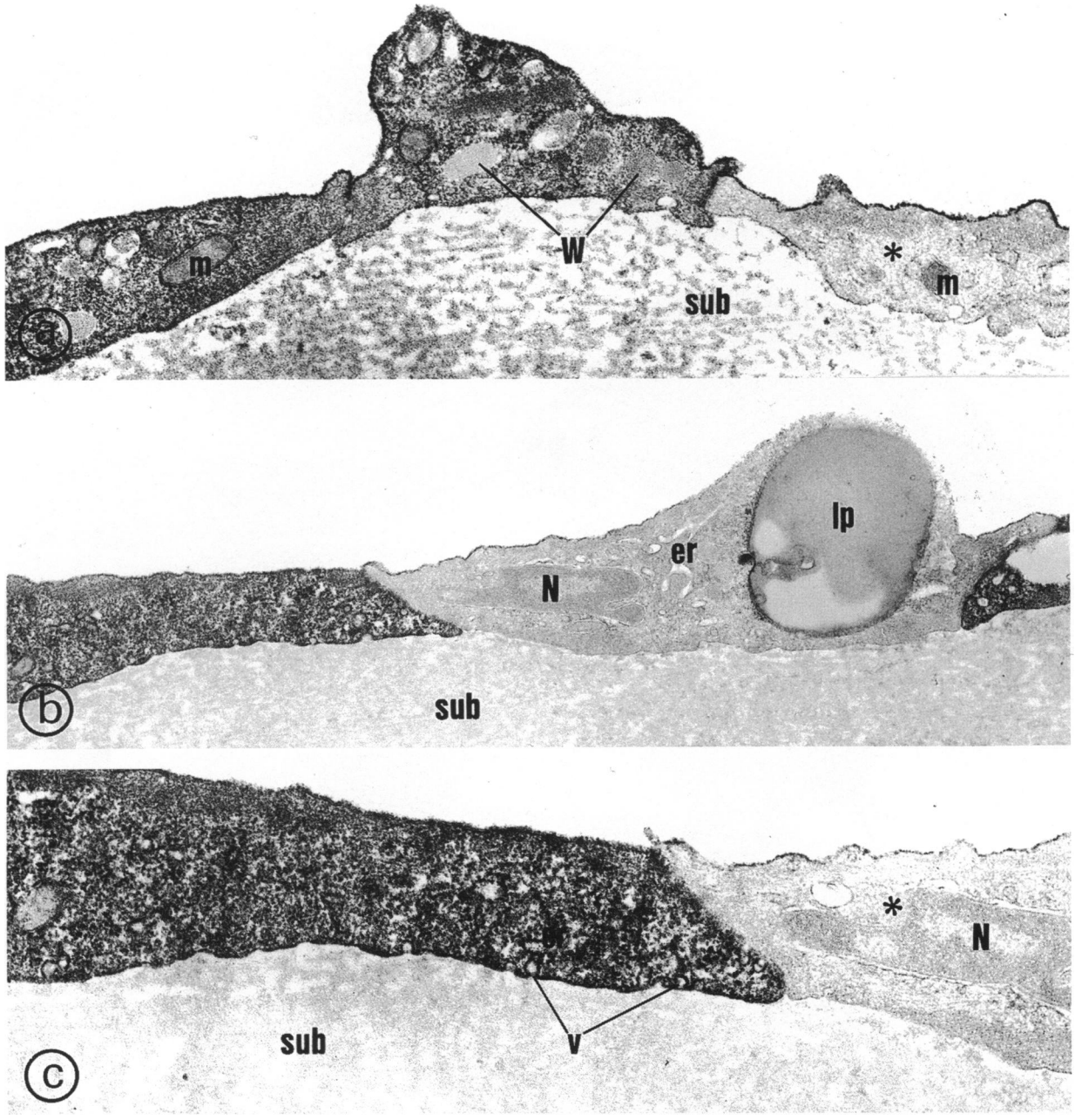

Fig. 4. Endothelial cells of MCA labelled for ET-1 before (*a*) and after (*b*, *c*) incubation in Krebs solution. (*a*) Note the well preserved ET-1-positive endothelial cell containing a number of intracellular organelles including mitochondria (m) and Weibel–Palade bodies (W). One ET-1-negative endothelial cell (asterisk) is also seen (×28000). (*b*) Note 2 ET-1-positive endothelial cell profiles separated by an ET-1-negative endothelial cell containing a large lipid droplet (lp). N, nucleus (×18500). (*c*) A magnified portion of the cells illustrated in (*b*) shows particles of immunoprecipitate densely packed in the cytoplasm. Cytoplasmic vesicles (v) and endoplasmic reticulum (er) are also visible (×35000).

DISCUSSION

The present report presents new data on the human cerebral blood vessels. This is the first time that a pre-embedding immuno-ultrastructural study of a cerebral artery of the MCA from postmortem specimens has been described. We demonstrate that immuno-reactivity to eNOS, nNOS, SP, ET-1 and 5-HT are localised in postmortem human MCA intimal cells. Light microscopy showed that cells from the intima of postmortem MCA can be cultured and display the antigen to factor VIII, the most widely used marker for endothelial cells. This study also showed that this had a pattern of granular perinuclear labelling,

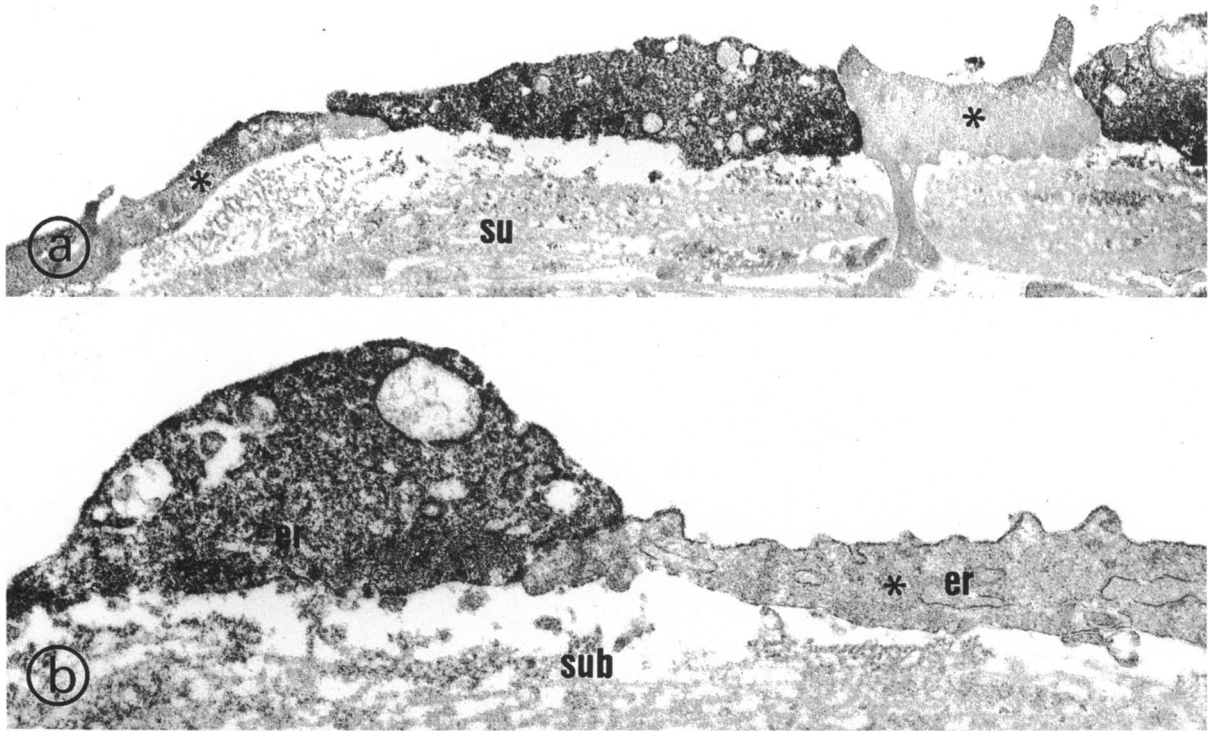

Fig. 5. Endothelial cells of MCA labelled for 5-HT before (*a*) and after (*b*) incubation in Krebs buffer. In (*a*) and (*b*) note the 5-HT-positive and 5-HT-negative (asterisk) endothelial cells. sub, subendothelial zone; er, endoplasmic reticulum (*a*, ×19500; *b*, ×30000).

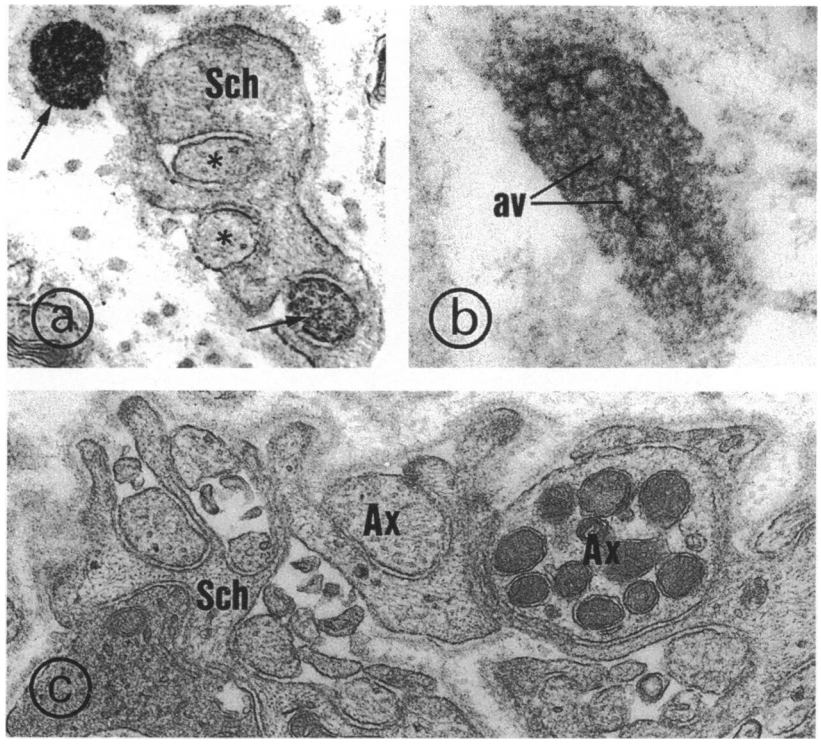

Fig. 6. Perivascular nerve fibres of MCA labelled for nNOS (*a*, *b*) and a control specimen (*c*) after incubation in Krebs buffer. (*a*) Note 2 nNOS-positive (arrows) and 2 nNOS-negative (asterisks) axon intervaricosities. Sch, unlabelled Schwann cell process (×46000). (*b*) A higher magnification example of nNOS-positive varicosity showing small agranular vesicles (av) (×100000). (*c*) Note the ultrastructural preservation of the unlabelled nerve fibre profiles (Ax) in a control specimen (omission of primary antibody) (×32000).

characteristic for factor VIII-containing endothelial cells (Bowman et al. 1984). The nNOS-immunoreactivity was also localised in the nerve fibres of the MCA adventitia. Thus these data are consistent with evidence for both endothelial and neuronal content of vasoactive agents (see Ralevic & Burnstock, 1993).

The immunoreactive endothelial cells were labelled with an immunoprecipitate in association with the cytoplasm and the membranes of intracellular organelles, whilst immunoreactivity in perivascular nerve fibres was associated with the axoplasm, microtubules and membranes of the synaptic vesicles. The nNOS-positive axon varicosities displayed smooth agranular synaptic vesicles. In all specimens examined, both before and after incubation in the Krebs solution, the MCA intima displayed regions covered with endothelial cells and regions free of endothelium. After incubation in Krebs solution, however, there were more endothelial cells showing better ultrastructural preservation, and also more perivascular nerves expressed nNOS-immunoreactivity. Thus the incubation, indeed, 'improved'/'revitalised' the human postmortem cerebral vessels examined, as already described for human tissue after a postmortem delay (Ferguson & Richardson, 1978).

The immunocytochemical finding of this study suggests that subpopulations of endothelial cells contain eNOS, nNOS, SP, ET-1 and 5-HT in human MCA. At this stage, however, it is not possible to state unequivocally whether postmortem conditions within the MCA produced substantial damage to the endothelial cells resulting, for example, in nonspecific immunoreactivity. Electron microscopy of MCA confirmed the relatively good preservation of a number of immunoreactive and immunonegative endothelial cells, in both incubated and nonincubated Krebs solution preparations. Some cells, however, showed ultrastructural changes and displayed either positive or negative immunoreactivity for a given antigen. This simultaneous appearance of immunopositive and immunonegative ultrastructurally changed and/or even damaged (possibly due to postmortem processes) endothelial cells may indicate that the immunolabelling was independent of the damage to the endothelium. In their study on the eNOS in fresh human umbilical cord and postmortem human tissues such as cerebellum, trachea, lung, liver, renal cortex and renal medullae, Pollock et al. (1993) suggested that the differences in the intensity of labelling observed between postmortem and fresh tissues may reflect a time-dependent deterioration of the antigen. The possibility cannot be excluded, however, that some ultrastructural and/or immunocytochemical features observed in the present study were also related to the age of the subject examined.

Evidence for the existence of immunoreactive vasoactive agents in human and other mammalian cerebrovascular endothelial cells comes from elegant studies on tachykinin SP by Linnik & Moskowitz (1989) and Linnik et al. (1989). Using high performance liquid chromatography, SP was detected in measurable amounts within vascular endothelial cells of human large pial cerebral vessels (circle of Willis, basilar artery, and proximal branches of the large projecting arteries), and in cultures of bovine cerebral microvessels. These studies were carried out on human autopsy specimens at a mean postmortem time of 17 h. Our electron-immunocytochemical studies, carried out on specimens at the mean postmortem time of about 30 h (at a nearly twice longer postmortem period than in the studies of SP by Linnik & Moskowitz, 1989), fully support the finding of SP in vascular endothelium and together with findings for ET-1 and 5-HT indicate a possible source of these vasoactive agents in human cerebral vessels. The SP, ET-1 and 5-HT as well as some other vasoactive agents are well recognised as being involved in the mechanisms regulating vascular tone (Ralevic & Burnstock, 1993).

Our laboratory has previously reported on the endothelial content of SP, ET-1 and AVP from normal rabbit cerebral vessels (basilar artery, posterior communicating arteries) and cerebral vessels perfused with the Krebs buffer solution and/or a perfluorocarbon emulsion (Loesch et al. 1993). Employing perfusion with a perfluorocarbon emulsion we have also reported that rabbit cerebral vessels release SP, ET-1 and AVP, as well as adenosine 5′-triphosphate (Domer et al. 1992, 1993). The endothelial content of nNOS was previously reported in cerebral vessels including the middle cerebral artery and basilar artery of the rat (Nozaki et al. 1993; Loesch et al. 1994).

The present eNOS-immunoreactivity in vascular endothelium and nNOS-immunoreactivity in the endothelium and perivascular nerves of the human MCA support the considerable evidence for endothelial and neuronal production of NO and thus involvement in the control mechanisms of vascular tone (Palmer et al. 1987; Moncada & Palmer, 1992; Rand, 1992; Brizzolara et al. 1993; Toda, 1993; Toda et al. 1993). Following incubation in Krebs solution some of the postmortem human MCA showed pharmacological relaxation in response to the endothelium-dependent vasodilators ACh, SP and 2-methylthio ATP (L. Chadwick et al., unpublished data).

Our preliminary studies with monoclonal antibodies also indicate that both eNOS and nNOS are present in a subpopulation of vascular endothelial cells and perivascular nerves of rat cerebral blood vessels (Loesch & Burnstock, 1995*b*). Whether specific

functional implication is associated with the presence of both isoforms of NOS in vascular endothelium and perivascular nerves is unknown at this stage. There is indication, however, that eNOS is rather related to a particulate fraction (presumably plasma membrane), and that NO formed at the plasma membrane is more likely to be released to the extracellular environment than NO formed in cytosol (Michel et al. 1993). Both isoforms constitutively produce NO and therefore may be implicated in the mechanisms influencing basal cerebral vascular tone. It has been suggested that impairment of endothelium-dependent relaxation may contribute to some pathophysiological conditions, e.g. cerebral ischaemia or stroke (Faraci & Brian, 1994). In some regions of the brain (rat), where both eNOS and nNOS were detected in neurons, the eNOS generates NO which may act as a retrograde messenger of long-term potentiation (Dinerman et al. 1994). Neuronally derived NO may mediate a local increase in cerebral blood flow, e.g. during increases in cerebral metabolism (Faraci & Brian, 1994).

In conclusion, the present study demonstrates that eNOS, nNOS, ET-1, SP and 5-HT are localised in intact cells in the intima of postmortem human MCA, either treated or nontreated with Krebs solution. The culturing and immunolabelling of the cells obtained from the MCA intima indicate that these cells were alive and positive for the endothelial marker—factor VIII. The nNOS was also localised in nerve fibres at the adventitia–media border.

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