Biomedical Research 2003; 14 (1): 1-5

Special issue: Autonomic Nervous System
Dedicated to Professor Geoffrey Burnstock

Nitric oxide synthase and endothelin in cerebrovascular nerves of the basilar artery in pregnant rat

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Key words: Pregnancy, cerebrovascular nerves, nitric oxide, endothelin, electron-immunocytochemistry

Abstract

Nitric oxide synthase- and endothelin-containing cerebrovascular nerves/axons were investigated by electron-immunocytochemistry in the basilar artery of the rat in late pregnancy. In both types of nerves, similar changes were observed involving the accumulation of synaptic vesicles and the presence of dense bodies in the axon varicosities of the adventitial-medial border. Results suggest either an excessive formation of nitric oxide and endothelin with possible damage to the varicosities, or the neural inhibition of the release of these agents.

Introduction

This contribution is dedicated to Professor Geoffrey Burnstock, who for many decades has been a dynamic force in autonomic neuroscience (see Burnstock 1976, [1]), using a variety of approaches to investigate phenomena associated with the autonomic nervous system. Among the subjects of interest to him are the cerebral vessels, in particular the plasticity of their autonomic innervation in various circumstances e.g. development, ageing, hypertension or denervation.

In this study, performed in Prof. Burnstock's laboratory some time ago, an ultrastructural-immunocytochemical approach was employed to investigate the pregnancy-related changes in cerebrovascular nerves coded by nitric oxide (NO) and endothelin-1 (ET-1). The involvement of these nerves in cerebral vessels in pregnancy is poorly understood. NO is mostly known for its role as a premier endogenous vasodilator, whilst ET-1 is a major endogenous vasoconstrictor [2,3]. The actions of these agents are interrelated; NO exerts an inhibitory influence on the production of ET-1, whilst ET-1 activates NO synthesis and hence vasorelaxation [4].

Cerebral vessels are innervated by parasympathetic, sympathetic and sensorimotor autonomic nerves coded by a variety of agents involved in neurotransmission, co-transmission and neuromodulation [1,5-8]. Among the agents involved are monoamines, amino acids, neuropeptides, adenosine 5'-triphosphate and NO [9-14]. The recent localisation of endothelin-1 (ET-1) in cerebrovascular nerves opens up the possibility that ET-1 of neural origin plays a role in cerebral vessels, together with the ET-1 derived from cerebrovascular endothelium [15-18].

In pregnancy, various changes have been reported in the autonomic nervous system, some of which may affect vascular physiology [19]. The possibility cannot be ruled out that NO and ET-1 from cerebrovascular nerves contribute to such changes. It is well known that increased NO derived from the vascular endothelium in pregnant humans and rats contributes to generalised vasodilation, where a fall in peripheral vascular resistance and systemic blood pressure occurs despite a big rise in cardiac output [20-22]. The role of endothelial ET-1 in this process is less clear. The aim of this study was to examine ultrastructural changes in NO- and ET-1-producing cerebrovascular nerves of the basilar artery of the rat in late stage pregnancy. In order to identify NO-producing nerves, immunocytochemistry of neuronal NO synthase (nNOS) - an enzyme synthesising NO in neurones [23] was performed.

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Materials and Methods

Animal treatment was in compliance with the UK Animals Scientific Procedures Act (1986). Four virgins and four first time pregnant (19-days gestation) Sprague Dawley rats were used in this study. Rats were anaesthetised with sodium pentobarbitone (Sagatal, 60-mg kg^{-1} i.p.) and perfused through the heart with 4% paraformaldehyde and 0.2% glutaraldehyde (in 0.1M phosphate buffer, pH 7.4). The brains were removed and the basilar arteries dissected out. These were then processed for the pre-embedding peroxidase-antiperoxidase (PAP) immunocytochemistry of nNOS and ET-1 using a standard protocol previously reported [24]. In brief, immunoreagents were used as follows: 10% blocking normal goat serum, diluted 1:10 (Nordic Immunology, Tilber, the Netherlands); rabbit polyclonal antibodies to nNOS (a gift from Dr. U. Förstermann; the antigen was purified and the antibody generated and characterised by Schmidt and colleagues, 1992 [25]) and ET-1 (Cambridge Research Biochemicals, Cambridge, UK), both diluted 1:1000; goat-antirabbit immunoglobulin G serum, diluted 1:50 (Biogenesis, Bournemouth, UK); PAP diluted 1:100 (DAKO, Glostrup, Denmark); 3,3'-diaminobenzidine (Sigma, Dorset, UK). Specimens were postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in Araldite. The ultrathin sections were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1010 transmission electron microscope. Controls were performed with omission of the primary antibodies and IgG steps. No labelling was observed in these preparations. For more information about the specificity of nNOS and ET-1 antibodies used, see [24-26].

Results

Virgin rats

At the electron microscope level, both nNOS- and ET-1-positive axon varicosities were observed amongst immuno-negative axon profiles at the adventitial-medial border of the basilar artery of virgin rats (Fig. 1a, b). Both types of immunolabelled axons contained agranular and granular vesicles; agranular vesicles dominated in nNOS-positive varicosities.

Pregnant rats

In the last stage of pregnancy (19^{th} day of gestation), there were changes in both nNOS- and ET-1-positive varicosities (Figs 1c-f). In some immunopositive varicosities, there were notably more agranular vesicles which were clustered or densely packed (Fig. 1c, d). Other labelled varicosities were in striking contrast to those of the virgin rats, and contained dense bodies/material of about 250-300 μm diameter (Fig. 1d-f); in some cases dense bodies occupied a great deal of varicosity (Fig. 1f). Also, present were nNOS-positive varicosities containing vesicular structures of various sizes (Fig. 1d). ET-1-positive varicosities were at times very close (60-80 nm) to vascular smooth muscle.

Discussion & Conclusion

The present study demonstrates striking changes in both nNOS- and ET-1-positive cerebrovascular nerves of the basilar artery of rats at late pregnancy compared with virgin rats. This suggests that parasympathetic nerves (nNOS-containing) as well as sensory and sympathetic nerves (ET-1-containing) that supply the basilar artery were involved. Some of the changes observed in the present study were notably common for both nNOS- and ET-1-positive nerves. These include the 'accumulation' of synaptic vesicles in varicosities and the appearance of dense bodies/material. It may be speculated that altered varicosities represented only one type of nerve; co-localisation studies would help to resolve this.

According to Milner and colleagues [16], changes such as the presence of dense bodies or clustering of synaptic vesicles in varicosities may be indicative of degenerative or neuroprotective processes. Such changes have been observed in ET-1-positive varicosities of the basilar artery of spontaneously hypertensive rats, where increased immunoreactivity for ET-1 was revealed in the neuronal cell bodies of the trigeminal, superior cervical and sphenopalatine ganglia (the ganglia that send projections to cerebral vessels) [16,17].

Immunocytochemistry and/or in situ hybridisation and denervation studies revealed that NO/nNOS-containing cerebrovascular nerves of the main cerebral vessels (rat) originate from cranial parasympathetic ganglia; in particular, the sphenopalatine ganglion [12], whilst those containing ET-1 may originate from sensory trigeminal ganglia and the sympathetic superior cervical ganglia [17]. NO from cerebrovascular nerves has been shown to mediate cerebrovascular vasodilatation [10], which can be rapidly evoked [8]. The role of neural ET-1 in cerebral vessels is currently unknown, however a role of ET-1 in neurogenic vasoconstriction seems likely. Noticeably, cerebral arteries are more sensitive to ET-1 than systemic arteries, suggesting the importance of this peptide in the control of cerebral blood flow [27]. An impairment of the balanced action of endogenous ET-1 (via relevant ET receptors) and NO may contribute to abnormal changes in the cerebral vascular tone and blood flow [28]. The present study shows that cerebrovascular nerves may play a role in such changes, since these nerves might be the sources of endogenous ET-1 and NO.

NO/nNOS is often colocalised with vasoactive intestinal polypeptide (VIP) in parasympathetic nerves [8], in cluding those projecting into cerebral arteries [12].

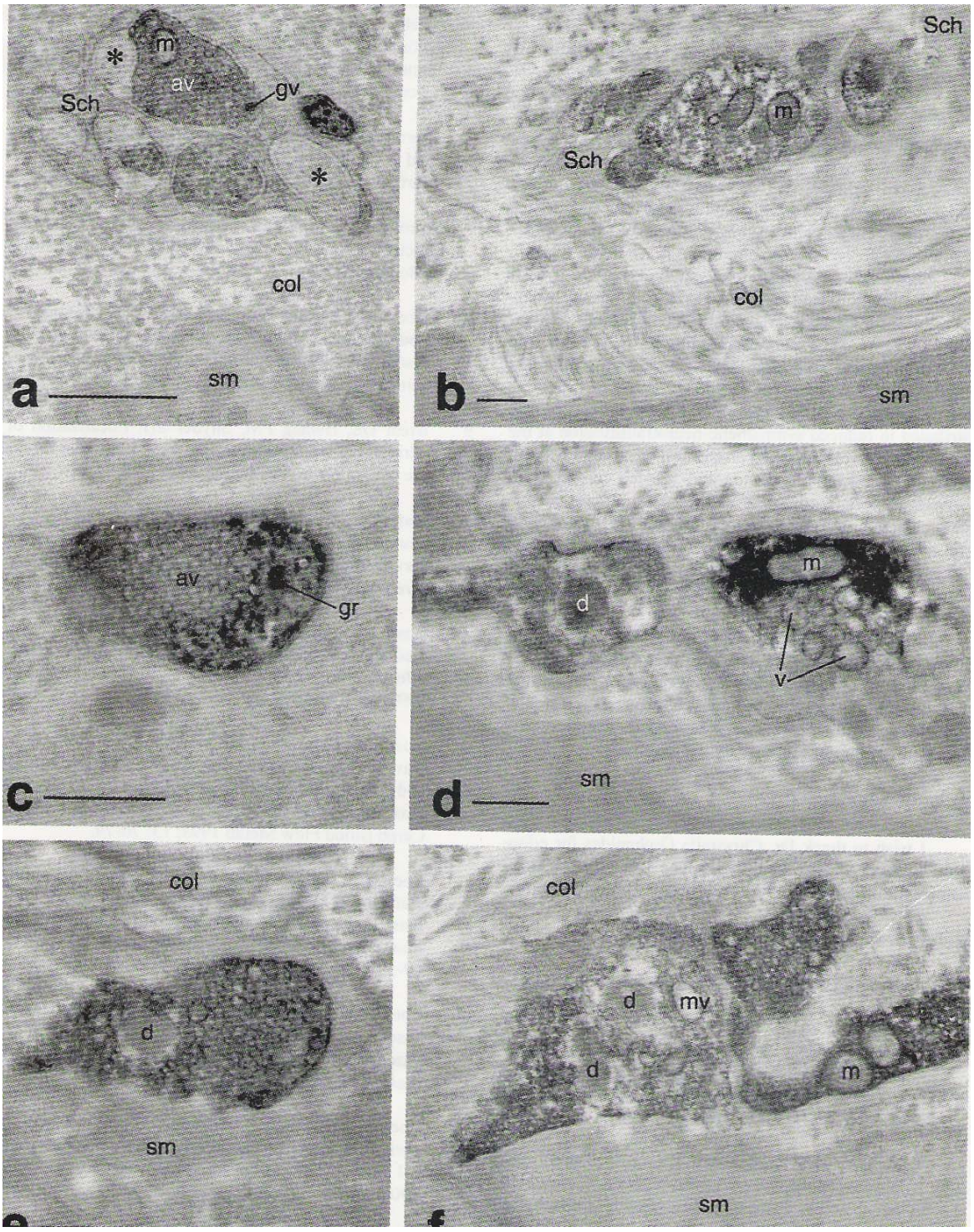

Fig. 1a-b. *Cerebrovascular nerve fibres of the adventitial-medial border of the basilar artery of virgin rats* ***(a, b)*** *and 19 day pregnant* ***(c-f)*** *labelled (black 'stain') for nNOS* ***(a, c, d)*** *and ET-1* ***(b, e, f).*** ***a*** *A nerve bundle showing four nNOS-positive axons, one of which is a varicosity containing numerous agranular vesicles (av) and one granular vesicle (gv); unlabelled axons are also seen (asterisks). Sch-Schwann cell, sm-smooth muscle, col-collagen.* ***b*** *A group of ET-1-positive axons; mitochondria are seen in varicosity.* ***c*** *Clustered agranular vesicles are seen at the centre of nNOS-positive varicosity while the immunolabelling is mostly located in the surrounding axoplasm that also contains a granular vesicle/material (gr).* ***d*** *Two nNOS-positive varicosities. One contains a dense body (d); the other shows vesicular structures of various sizes (v) and unevenly distributed immunoprecipitate.* ***e*** *An ET-1-positive varicosity abutting smooth muscle is filled mostly with agranular vesicles; it also contains a dense body/material.* ***f*** *ET-1-positive varicosities close to smooth muscle; one varicosity contains dense bodies/material, a multivesicular body (mv). Bars: 0.5 µm.*

This suggests the importance of parasympathetic co-transmission of NO and VIP in cerebral vessels [29]. Studies of sphenopalatine ganglia innervating cerebral arteries via parasympathetic projections showed that some of its VIP- and nNOS-positive neurones also co-store ET [16]. The t
trigeminal ganglia responsible for the sensory projections into cerebral arteries contain a subpopulation of calcitonin gene-related peptide- and substance P-positive neurones that also co-store NADPH-diaphorase and may account for nNOS [30]. These point to the complexity of parasympathetic and sensorimotor innervation in cerebral arteries.

Plasticity of innervation of cerebral vessels is well recognised. One example is the increase in density of innervation of the basilar artery by sympathetic nerves containing noradrenaline and neuropeptide Y that precedes the onset of hypertension and associated medial hypertrophy in spontaneously hypertensive rats [31]. It should be noted, however, that decreased systemic resistance may also be attributed to reduced sympathetic neurotransmission at the pre-synaptic level, enhanced production of an endothelium derived hyperpolarising factor and blunted responses to vasoconstrictors, including ET-1 [32-34]. Feline and canine studies showed that ET-1 that affects the tone of cerebral arteries derives from the adventitial but not from the intimal side of the vascular wall and hence it may be involved in the pathogenesis of vasospasm of cerebral vessels [35, 36].

In conclusion, both nNOS- and ET-1-positive cerebrovascular nerves of the basilar artery of the rat showed ultrastructural changes in late stage pregnancy. These changes may influence the mechanisms controlling cerebral circulation. Further studies are needed to examine why both types of nerve react in a similar fashion.

Acknowledgements

I wish to thank Dr. Chrystalla Orphanides for editorial assistance. I also thank Miss Jane Pendjiky for assistance with images.

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