Ultrastructural localization of NADPH-diaphorase and colocalization of nitric oxide synthase in endothelial cells of the rabbit aorta

Andrzej Loesch, Abebech Belai, Geoffrey Burnstock

Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, Gower Street, London WC1E 6BT, UK

Received: 1 April 1993 / Accepted: 30 April 1993

Abstract. This is the first report on the ultrastructural pattern of distribution of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) in endothelial cells, using the rabbit aorta, and its colocalization with the neuronal isoform (type I) of nitric oxide synthase. About 30% of the endothelial cells showed a positive reaction for NADPH-d compared to about 6% for nitric oxide synthase immunoreactivity. Simultaneous double histochemical-immunoerytochemical labelling procedures indicate that all of the cells displaying nitric oxide synthase-positive reactivity also contained NADPH-d; the remainder of NADPH-d-positive endothelial cells were negative for this isoform of nitric oxide synthase. Nitric oxide synthase-immunogold labelling was mostly associated with free ribosomes, while NADPH-d activity was distributed largely in patches in the cytoplasm and in association with the cell membrane.

Key words: NADPH-diaphorase – Nitric oxide synthase – Colocalization – Aortic endothelium – Rabbit

Introduction

Nitric oxide synthase (NOS) is a highly active enzyme involved in the synthesis of nitric oxide (NO from L-arginine) which has been suggested to be the endothelium-derived relaxing factor (EDRF) (Palmer et al. 1987, 1988; Moncada et al. 1991). There is now evidence that NOS may exist as several forms (isoforms) that have been found in neurons of the brain, the endothelium and also in other cell types (see: Forstermann et al. 1991). All forms of NOS require nicotinamide adenine dinucleotide phosphate (NADPH) as an essential cofactor (Bredt et al. 1991; Dawson et al. 1991; Moncada et al. 1991). Furthermore, evidence suggests that NADPH-diaphorase (NADPH-d) and NOS activities are different properties of the same molecule and that NADPH-d activity may thus be used as a marker for NOS, as both these compounds were colocalized at the light-microscopic level in the brain and peripheral nervous system (Bredt et al. 1991; Dawson et al. 1991; Hope et al. 1991; Afework et al. 1992; Belai et al. 1992; Hassall et al. 1992; Saffrey et al. 1992; Young et al. 1992).

In the present study, we have examined intact endothelial cells of the rabbit aorta at the light- and electron-microscopic levels. Histochemical method of detection of NADPH-d activity, and immunocytochemical methods of detection of immunoreactivity to NOS (neuronal isoform, type I) were applied to detect the pattern of NADPH-d- and NOS-labelling, respectively. Combined histochemical-immunocytochemical labelling (double labelling utilizing colloidal gold method) was also applied to detect whether NADPH-d and NOS are colocalized in endothelial cells.

Materials and methods

Adult (6-month-old) male New Zealand White rabbits were killed by an overdose of sodium pentobarbitone (Sagatal, RMB Animal Health Ltd, Dagenham, U.K.) followed by exanguination. Thoracic aortas were dissected, immersion-fixed for 3 h at 4°C with fixative containing 4% paraformaldehyde and 0.25% glutaraldehyde (in 0.1 M phosphate buffer at pH 7.4), then transferred to phosphate buffer and stored overnight at 4°C. Aortas were next cut longitudinally to produce 5 to 7 mm-long strips, which were then processed for the pre-embedding histochemistry of NADPH-d, pre-embedding immunocytochemistry of NOS, and post-embedding immunocytochemistry of NOS.

Histochemistry of NADPH-d

NADPH-d activity in aortic endothelial cells was examined histochemically by a modified method of Vincent et al. (1983) (see: Belai et al. 1992; Hassall et al. 1992; Saffrey et al. 1992). Briefly, strips of fixed aorta were rinsed in phosphate-buffered saline (pH 7.3) and then incubated in the dark for 2 h at 37°C in 0.1 M TRIS-HCl buffer (pH 7.4) containing 1.2 mM β-NADPH, 0.24 mM nitroblue

Correspondence to: G. Burnstock
tetrozolium, 15.2 mM L-malic acid (all Sigma, Poole, Dorset, U.K.) and 0.1% Triton X-100. The specimens were postfixed in 1% osmium tetroxide, dehydrated in ethanol, embedded in Lowicryl K4M (Chemische Werke Lowi GmbH, Waldkraiburg, Germany) at −35°C, and photopolymerized overnight at −35°C by long-wavelength ultraviolet diffuse light. Semithin circumferential sections were examined with a Zeiss RS light microscope. Ultrathin circumferential sections were stained with uranyl acetate and lead citrate and subsequently examined using Philips-300 and JEM-1010 electron microscopes.

Pre-embedding immunocytochemistry

For localization of NOS in endothelial cells, strips of aorta were processed for the peroxidase-antiperoxidase (PAP) immunocytochemistry using the same steps of the procedure as previously reported (Loesch and Burnstock 1993). The rabbit NOS antiserum was used in this study at a 1:1000 dilution. The specimens were osmicated, dehydrated in ethanol and embedded in Araldite. Semithin and ultrathin circumferential sections were examined with a Zeiss 111 RS light microscope and a JEM-1010 electron microscope, respectively.

Post-embedding immunocytochemistry

For localization of NOS in endothelial cells, strips of aortas were osmicated, dehydrated and embedded in Lowicryl K4M as described above. Localization of NOS was examined in ultrathin circumferential sections of the specimens (on uncoated nickel grids) utilizing the immunogold labelling technique (adapted from De Mey 1983). The steps of the procedure were the same as previously reported (Loesch and Burnstock 1993). Briefly, the sections were incubated for 24 h at 4°C with a rabbit antibody to NOS at a dilution of 1:75–100, labelled for 2.5 h at room temperature with a goat antirabbit immunoglobulin G serum-coated colloidal gold probe, coupled to 15-nm gold particles (AuroProbe EM, Amersham International, Amersham, U.K.) at a dilution of 1:25–40. Specimens were stained with uranyl acetate and lead citrate and examined using Philips-300 and JEM-1010 electron microscopes.

NADPH-d/NOS double-labelling

For combined localization of NADPH-d activity and NOS immunoreactivity, the post-embedding immunogold labelling procedure for NOS (procedure as described above) was performed on ultrathin sections of the specimens previously stained for NADPH-d activity by pre-embedding histochemical procedure.

Controls

For control of NADPH-d activity in endothelial cells, β-NADPH was omitted from the incubation medium. This resulted in elimination of NADPH-d-reaction product in endothelial cells. For the immunocytochemical procedures, the rabbit polyclonal NOS antiserum 6761–8 (rabbit 6761, 8th post-immune bleed), prepared by the Abbott Laboratories in Chicago, Ill., USA, was kindly provided by Dr. U. Forstermann (Abbott Laboratories, Department 47 S, Abbott Park, Ill. 60064, USA). It was raised against soluble NOS type I purified from rat cerebellum and tested for specificity as described previously (Forstermann et al. 1991; Schmidt et al. 1991). In the present study, the specificity of the immunolabelling was tested by omission of the NOS antiserum and anti-rabbit immunoglobulin G serum independently, as well as by using non-immune normal goat serum or non-immune normal rabbit serum (Nordic Immunology, Tilberg, The Netherlands) at a dilution of 1:75–1000 instead of the NOS antiserum. No labelling was observed in these control experiments. Preabsorption (22 h at 4°C) of the NOS antiserum (diluted 1:1000) with 20 μg ml⁻¹ soluble NOS type I purified from rat cerebellum also resulted in elimination of positive labelling in the pre-embedding PAP immunoprocessure.

Results

Light microscopy

NADPH-d. Endothelial cells displaying NADPH-d activity by the histochemical procedure were observed in semithin sections of the aortas examined (Fig. 1a). These cells were characterized by the presence of patchily distributed NADPH-d-reaction product (bluish colored in original preparations). The NADPH-d activity was present both in proximal and distal regions of endothelial cell profiles. About 30% of all the observed cells displayed NADPH-d activity.

NOS. Endothelial cells displaying NOS immunoreactivity by the pre-embedding PAP method were also present in semithin sections of the aortas examined (Fig. 1b). About 6% of the cells examined were positive, in keeping with our earlier report (Loesch and Burnstock 1993). They were characterized by the presence of a dense immunodeposit (a rust/brownish precipitate in original preparations). NOS-positive cells were distributed in the manner similar to that concerning NADPH-d-positive cells. In contrast, however, the intensive NOS-labelling was seen throughout the whole cell cytoplasm, including perinuclear and distal regions of the cells.

Controls. Examples of the specimens processed for control of NADPH-d histochemistry and NOS immunocytochemistry, showing lack of activity to NADPH-d and lack of immunoreactivity to NOS, are illustrated in Fig. 1c, d and e.

Electron microscopy

NADPH-d. Endothelial cells displaying NADPH-d activity by histochemical procedure were observed in ultrathin sections of the aortas examined (Fig. 2a, b). These cells displayed moderate-density amorphous deposits which were patchily distributed in the cytoplasm and in association with the cell membrane. The richest accumulation of NADPH-d-reaction product was found in the perinuclear cytoplasm of the cells (Fig. 2a). The NADPH-d activity was also present in the cell processes (Fig. 2b). With this histochemical method a relation of the labelling to intercellular junctions was also observed (Fig. 2b).

NOS PAP-labelling. NOS-positive endothelial cells by pre-embedding PAP method displayed black immunoprecipitate throughout the whole cytoplasm and in association with the membranes of intracellular organelles.