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The Ultrastructural Localization of NADPH-diaphorase in the Cerebral Arteries of Rats

Masahiro Aoyama

Department of Neurology, School of Medicine, Keio University, Tokyo, Japan

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Abstract. The ultrastructural localization of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), which has been considered to be a neuronal nitric oxide synthase (NOS), was explored in the vascular endothelial cells and perivascular nerves of the cerebral arteries in the rat. In order to detect NADPH-d activity, 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl) tetrazolium chloride was utilized as a substrate for NADPH-d histochemistry at the electron microscopic level. In vascular endothelial cells, NADPH-d positive deposits were observed on the nuclear envelope and the endoplasmic reticulum (smooth or rough surfaced). Positive deposits were seen on distinct membrane portions of the endoplasmic reticulum (ER) in the perivascular nerves (axons), but no positive materials were observed either in the cytoplasm of the endothelial cells or in the axoplasm of the perivascular nerves. It was concluded that NOS is located on the membranes of the ER and the nuclear envelope, and that NOS may play substantial roles in the regulation of the cerebral vessels. (Keio J Med 45 (4): 318–323, December 1996)

Key words: NADPH-diaphorase, nitric oxide synthase, cerebral blood vessels, electron microscope, endoplasmic reticulum

Introduction

Nitric oxide (NO) has been identified not only as an endothelium-derived relaxing factor synthesized by vascular endothelial cells, but also as a neurotransmitter both in the central and peripheral nervous system.^{1–8} NO is synthesized from L-arginine by nitric oxide synthase (NOS).⁵ Recently, neuronal nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) has been proven to be NOS *per se* and NOS-containing fibers around the cerebral blood vessels have been identified by means of NADPH-d histochemistry.^{1,9–13} However, it has not been clarified whether NOS is a cytosolic or “membrane-binding” enzyme. The aim of this study is to elucidate the sites of nitric oxide production at a subcellular level. In this study, by examining the ultrastructural localization of NADPH-d activity in the cerebral arteries of rats using 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl) tetrazolium chloride (BSPT) as a substrate, NADPH-d positive deposits were demonstrated

in the endothelial cells and in the perivascular nerves.

Materials and Methods

Adult male Sprague-Dawley rats, six to ten months-old and weighing 200–300 g, were deeply anesthetized with an intraperitoneal injection of pentobarbital. The perfusion was carried out through the ascending aorta with 250 ml of 0.01M phosphate buffered saline (pH7.4) followed by 300 ml of fixative containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer (pH7.4). After the perfusion, the brain was removed and immersed in a fixative containing 4% paraformaldehyde in 0.1M phosphate buffer (pH7.4) for 2 hours at 4°C. The internal ethmoidal artery (IEA) was removed from the brain parenchyma carefully under a light microscope. All the specimens were processed for NADPH-d histochemistry.^{13–16} Each specimen was briefly incubated at 37°C for 90 minutes in a solution containing 1.2 mM nicotinamide adenine dinucleotide

Fig 1 Electron micrographs of rat cerebral arteries with (a, b, c) and without NADPH-d reaction (d). a; Lower magnification of the rat internal ethmoidal artery ($\times 2000$). The wall of the artery consists of three layers, endothelial cells (EC), smooth muscles (SM), and the tunica adventitia. Some varicosity of perivascular nerve fibers are in the tunica adventitia (arrows). Smooth muscles have large nuclei and some mitochondria (arrow heads). The nucleus of the endothelial cell is shown at the square. Lu; lumen of the artery, Scale bar = $10\ \mu\text{m}$. b; Higher magnification of the area shown by the square in a. In most of the endothelium, the positive linear deposits were observed on the membrane of the nuclear envelope (arrow head) and the smooth- and rough-surfaced endoplasmic reticulum (ER) (arrows). No positive deposits could be detected in the cytoplasm. Nu; nucleus of the endothelial cells, Scale bar = $1\ \mu\text{m}$. c; Higher magnification of the ER in b. Some pairs of linear dense deposits were situated in parallel with each other. The deposits seemed to be located on the membrane of the ER. Scale bar = $200\ \text{nm}$. d; The control study without NADPH-d reaction showed no dense deposits in the rough-surfaced ER (arrow heads) and the nuclear envelope. Scale bar = $1\ \mu\text{m}$.

phosphate (NADPH) (Kojin-Co.), 1.2 mM BSPT (Sigma, dissolved in 3–4 drops of dimethylformamide) and 0.1M phosphate buffer (pH8.0). Several specimens which were incubated without NADPH served as a negative control. After osmification in 2% OsO₄ (diluted in phosphate buffer pH7.4) for 4 hours and dehydration in a graded series of ethanol, the specimens were embedded in resin. After staining with uranyl acetate and lead citrate, ultra thin sections were observed under an electron microscope (JEM 1200EX2, Nihon Denshi), which had an accelerating voltage of 75.0 kv.

Results

The wall of the rat's cerebral artery consists of three layers composed of endothelial cells, smooth muscles, and the tunica adventitia (Fig 1a). In the tunica adventitia occurs some varicosity, which means the bundle of the perivascular nerve fibers (Fig 1a, arrows).

In the major portions of the endothelium, positive linear deposits were observed on the membrane of the nuclear envelope (Fig 1b, arrow head) and the smooth- and rough-surfaced endoplasmic reticulum (ER) (Fig 1b, arrows). As some pairs of linear dense deposits were situated in parallel with each other, the deposits seemed not to be in the ER or the nuclear envelope but on their membranes (Fig 1c). The ER situated closer to the nuclei tended to show an exaggerated staining. No positive deposits could be detected in the cytoplasm. Figure 1d is from the control study (see Materials and Methods) and shows no dense deposits in the rough-surfaced ER and the nuclear envelope.

Some varicosity of the nerve fibers was discovered in the tunica adventitia (Fig 1a). In the axons of the perivascular nerves, the smooth-surfaced ER were presented as NADPH-d positive linear figures (Fig 2, arrow heads). The mitochondria in the axon stained negatively (Fig 2, arrow). Figure 3 shows the nerve fibers in a higher magnification. Neither neurofilaments nor microtubules (Fig 3, arrow) were stained positively. The smooth-surfaced ER in the axon of other representative specimens were positive for NADPH-d as well (Fig 4a, b, c, arrow heads). The ER in the myelinated fiber was also positive (Fig 4d). No dense deposits could be found in the negative control specimen without an NADPH-d reaction (Fig 4e).

Discussion

NOS uses arginine as its substrate, forming citrulline and NO, and it requires NADPH as an electron donor. Thus, NOS can produce NO in an NADPH-dependent fashion in response to changes in intracellular free calcium. NOS initially receives electrons from NADPH in a calcium/calmodulin- and arginine-independent step that presumably involves the reduction of the associated

Fig 2 Perivascular nerves in higher magnification shown by arrows in Fig 1a. In the axons of perivascular nerves, NADPH-d positive deposits were on the smooth-surfaced ER (arrow heads). The mitochondria in the axon (arrow) stained negatively. Scale bar = 1 μ m.

Fig 3 The nerve fibers in the higher magnification shown by the square in Fig 2. NADPH-d positive linear figures were found to be the membrane of ER (arrow heads). Neither neurofilaments nor microtubules (arrow) were stained positively. Scale bar = 500 nm.

flavins. The reduced NOS rapidly reoxidizes in the presence of an appropriate electron acceptor, such as nitro blue tetrazolium (NBT) or BSPT, giving rise to the NADPH-d reaction product. Thus, the reduced type of NOS (a complex of NOS and flavin) is able to change a dye, such as NBT or BSPT, to formazan. Accordingly, NOS activity can be detected as the activity of NADPH-d; *i.e.* NADPH-d histochemistry provides a specific histochemical marker for organs producing NO.¹⁷

Some biochemical or histochemical studies have been carried out in order to investigate the subcellular localization of NOS. Biochemical studies of the endothelial cells of the bovine aorta have shown that some NOS activity could be found in the cytosolic fraction, but most of the activity was associated with the particulate fraction.^{18,19} Some immunohistochemical studies using

Fig 4 Electron micrographs of other representative specimens with (a, b, c, d) and without (e) NADPH-d reaction. The smooth-surfaced ERs in the axon were positive for NADPH-d as well (a, b, c, arrow heads). b shows the higher magnification of the square in a. Mitochondria were also negative for an NADPH-d reaction (a, arrow). The ER in the myelinated fiber was also positive (d, arrow head). No dense deposits could be found in the control study without an NADPH-d reaction (e). Scale bar = 1 μ m (e), 500 nm (a, c, d.), 100 nm (b).

anti-NOS antibodies have proven that NOS is associated with the plasma membrane and the membrane of the cytoplasmic vesicles and is present in the cytoplasm in the endothelial cells of the human umbilical vein.²⁰ In the endothelial cells of rabbit aorta, NOS is reported to be associated with free ribosome.²¹ In the rat basilar artery, NOS activity can be seen throughout the cell cytoplasm and is associated with the membrane of the mitochondria and ER in the endothelium.²² Some studies of NADPH-d histochemistry at the electron microscopic level have also been performed,^{23,24} however the precise subcellular localization of NADPH-d (*i.e.* NOS), has not been fully clarified. This study clearly demonstrated the subcellular localization of NOS with NADPH-d histochemistry using BSPT in rat IEA which had been

demonstrated to be denser with NADPH-d positive nerve fibers than any other main arteries in the circle of Willis at a light microscopic level by the previous study.¹⁷

NBT is a commonly utilized substrate in NADPH-d histochemistry at the light microscopic level. However, NBT is not appropriate for electron microscopic studies because the electron density of the formazan is too faint to be demonstrated.^{14,15} Gabbott *et al*²³ employed a unique technique in the application of NBT in an electron microscopic study but failed to demonstrate NADPH-d activity directly. In this study, BSPT was utilized instead of NBT, in accordance with the method of Wolf *et al*.¹⁶ BSPT itself does not react with osmium tetroxide but, when reduced by NOS activity, it gives off a formazan which produces a dark osmium complex and provides a

high contrast for NOS activity.¹⁴

It is commonly recognized that NOS has several subtypes. The NOS in the brain has been reported to share most of the properties of the isoforms Ia and III.¹⁹ Isoform Ia is a constitutive enzyme purified from the brain and is called "neuronal NOS". Isoform III, endothelial NOS, is a particulate enzyme responsible for most of the NO synthesis in endothelial cells and is also constitutive.¹⁹ In this study, NADPH-d activity shows the localization of constitutive-NOS.

In the endothelial cells the NADPH-d positive deposits as an accumulation of osmium on the nuclear envelope and on the smooth- or rough-surfaced ER were observed. No positive materials were observed in the nucleoplasm and in the cytoplasm. As for the axon of the perivascular nerves, NADPH-d positive linear figures, which were considered to be the smooth-surfaced ER, were observed. The deposit was located not in the ER, but on its membrane. No positive deposits were observed in the axoplasm. It was concluded that NOS is a membrane-binding protein both in the endothelium and perivascular nerve fibers. These results are compatible with the previous study by Tomimoto *et al.*,²⁴ Wolf *et al.*,²⁵ Wurdig and Wolf,²⁸ and Faber and Wolf,²⁷ who reported that NOS was localized on endomembranes, predominantly the ER, in the cerebellar cortex,²⁶ hippocampal cortical synapses²⁷ and cerebral endothelial cells²⁴ of the rat.

The nuclear envelope and the smooth- and rough-surfaced ER are connected with each other. One of the functions of these "membrane-binding" endothelial NOS may be the relaxation of vascular smooth muscle, or participation in the metabolism of the membranous components. The smooth-surfaced ER of the neuron is located in the cytoplasm, axoplasm and the nerve terminal, though its function has not been fully clarified. In general, the smooth-surfaced ER is considered to provide the synaptic terminals with the membrane compound in order to produce the synaptic vesicles.²⁸⁻³⁰ NOS might be transported by the smooth-surfaced ER on its membrane with a relatively slow velocity. Also the membrane of the smooth-surfaced ER changes to the synaptic vesicle at the synaptic terminals.²⁸⁻³⁰ Accordingly, it is suggested that NOS has some relationship to the endomembrane system.

In the present study, it was demonstrated that the endothelial NOS was located on the ER and on the nuclear envelope in the endothelial cells, and that neuronal NOS was located on the smooth-surfaced ER in the axon of the perivascular nerves in the rats' cerebral arteries. In conclusion, it was determined that NOS was predominantly located on the membranous structures as a membrane-binding state rather than as a cytosolic state in the cerebral arteries. The relationship of NOS and the endomembrane system as well as the function of membrane-binding NOS remains to be

elucidated.

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