FOLIA NEUROPATHOL. 1997, 35, 3
PL ISSN 0028-3894

BARBARA GAJKOWSKA, MIROSŁAW J. MOSSAKOWSKI

ENDOTHELIAL NITRIC OXIDE SYNTHASE IN VASCULAR ENDOTHELIUM OF RAT HIPPOCAMPUS AFTER ISCHEMIA: EVIDENCE AND SIGNIFICANCE

Laboratory of the Ultrastructure of the Nervous System, Medical Research Centre, Polish Academy of Sciences, Warszawa

Electron microscopy immunocytochemical study was performed to clarify ultrastructural localization and role of endothelial nitric oxide synthase (EC-NOS) in the endothelial cells (EC) of rat hippocampal vessels after transient cerebral ischemia. EC-NOS immunoreactivity was found in the endothelial cells in association with plasma membrane, sub-plasmalemmal vesicles, basal membrane and in cytosol (cytoplasm free of subcellular organelles). A sharp transient increase in immunoreactivity of NOS was observed at 10 min up to 1 hour after ischemia. The results of the present study indicate that NO, as a potent vasodilator, may play a protective role in ischemic brain damage.

Key words: *endothelial nitric oxide synthase, immunoelectron microscopy, endothelial cell, hippocampus, ischemia*

Nitric oxide (NO) is an important vascular and neuronal messenger molecule, first identified as having endothelium-derived relaxing factor (EDRF) activity (Moncada, Higgs 1993; Dawson, Synder 1994). NO is also formed in macrophages and other peripheral blood cells and modulates immune responses (Nathan 1992; Marletta 1993). NO and L-citrulline are produced in the two-step oxidation of L-arginine by the three isozymes of nitric oxide synthase: inducible NOS (i-NOS), endothelial NOS (e-NOS), and neuronal NOS (n-NOS) (Fostermann et al. 1991; Stuehr, Griffith 1992). Endothelial NOS has been localized in the endothelium of the blood vessels in the nervous system as well as other body organs and systems. Endothelial NOS was found to be concentrated in the hippocampus (Dinerman et al, 1990). The best characterized forms of biological reactions controlled by NO include vasodilation and regulation of normal vascular tone, inhibition of platelet aggregation, neuronal transmission, and cytostasis. Recent data suggest that NO is involved in the regulation of cerebral circulation and may play an important role in selected vasodilator responses of cerebral circulation. There is increasing evidence that NO is involved in the mechanisms of cerebral ischemic damage (Iadecola et al. 1994). Although the role that NO plays in the mechanisms of ischemic damage is not entirely clear, the experimental up to date studies suggest that NO can play both beneficial and deleterious roles. In this paper the effect of cerebral ischemia on NO synthesis and on the expression of EC-NOS is examined with the use of immunoelectron microscopy. Several electron microscopic studies on ultrastructural localization of EC-NOS in the blood vessels in the rat brain revealed the membranous localization of EC-NOS expressed by perinuclear granular structures observed in immunocytochemistry for EC-NOS as well as NADPH diaphorase histochemistry (Pollock et al 1993; Loesch et al. 1994; Tomimoto et al. 1994). In order to clarify the ultrastructural EC-NOS localization in endothelium , particularly in blood vessels of rat hippocampus we undertook electron immunocytochemical studies using EC-NOS antibodies. Effects of ischemia on NOS localization in rat hippocampus were investigated.

Material and methods

Twenty adult male Wistar rats (250-300 g) were used. Sixteen animals were subjected to experimental 10 min global cerebral ischemia. The experiment procedure was described in the previous papers (Gajkowska et al. 1989; Gajkowska, Mossakowski 1995). The experiment was performed according to the guidelines of the local ethic committee for experimentation on animals that approved the applied experimental protocol. For ultrastructural and immunocytochemical studies the animals were decapitated 10, 30 min, and 1, 3, 24 hours after ischemia. Then rats were anesthetized with ether and perfused intracardially with 0.9% NaCl (1 min) followed by 0.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M of sodium phosphate buffer

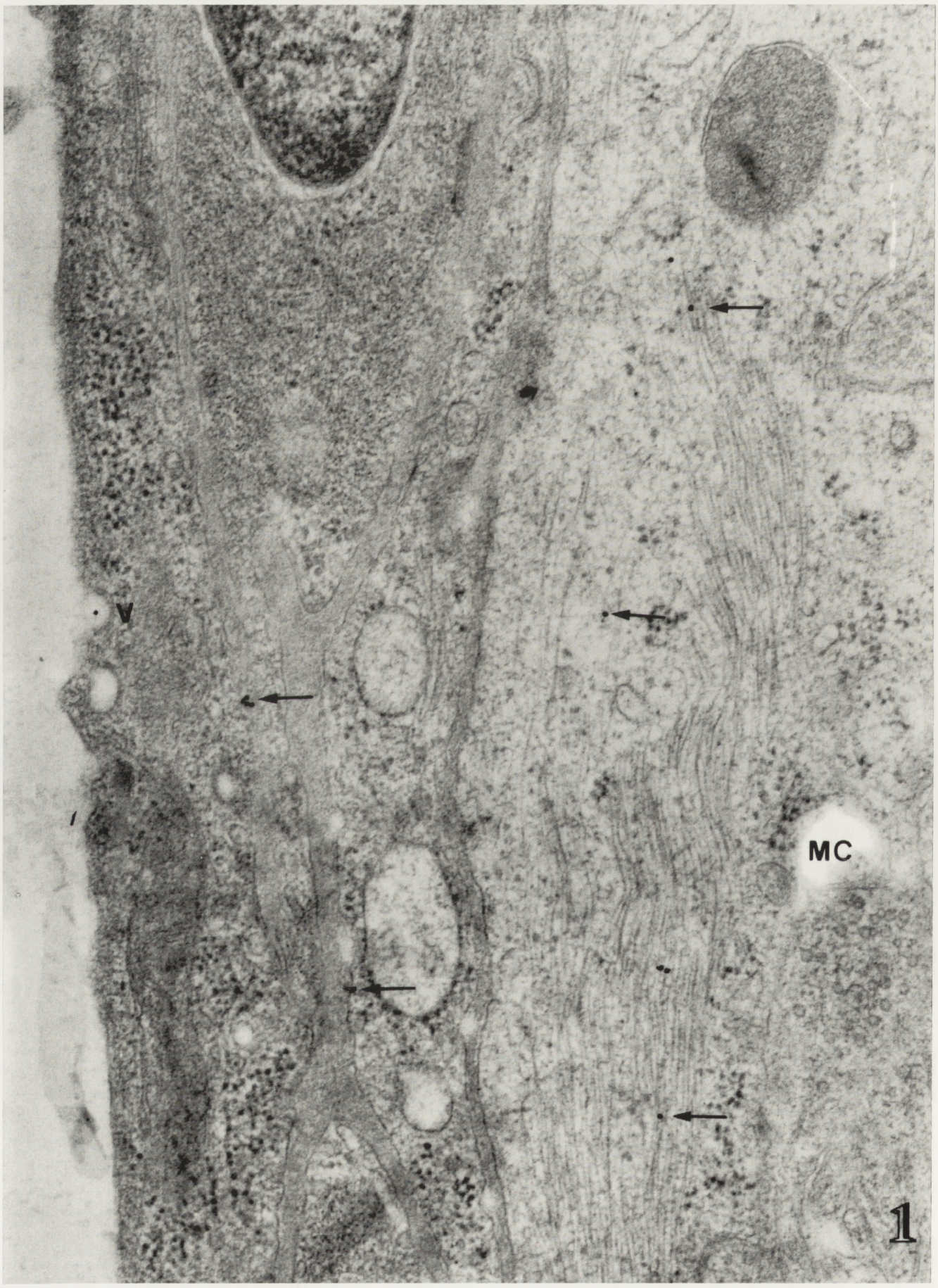

Fig. 1. Endothelial cells of rat hippocampal vessel in control rat labeled with EC-NOS antibody. Gold particles are found in association with a subplasmalemmal vesicle (v) in the cytoplasm near the basement membrane (arrows) and in the smooth muscle cell (Mc). × 58,000

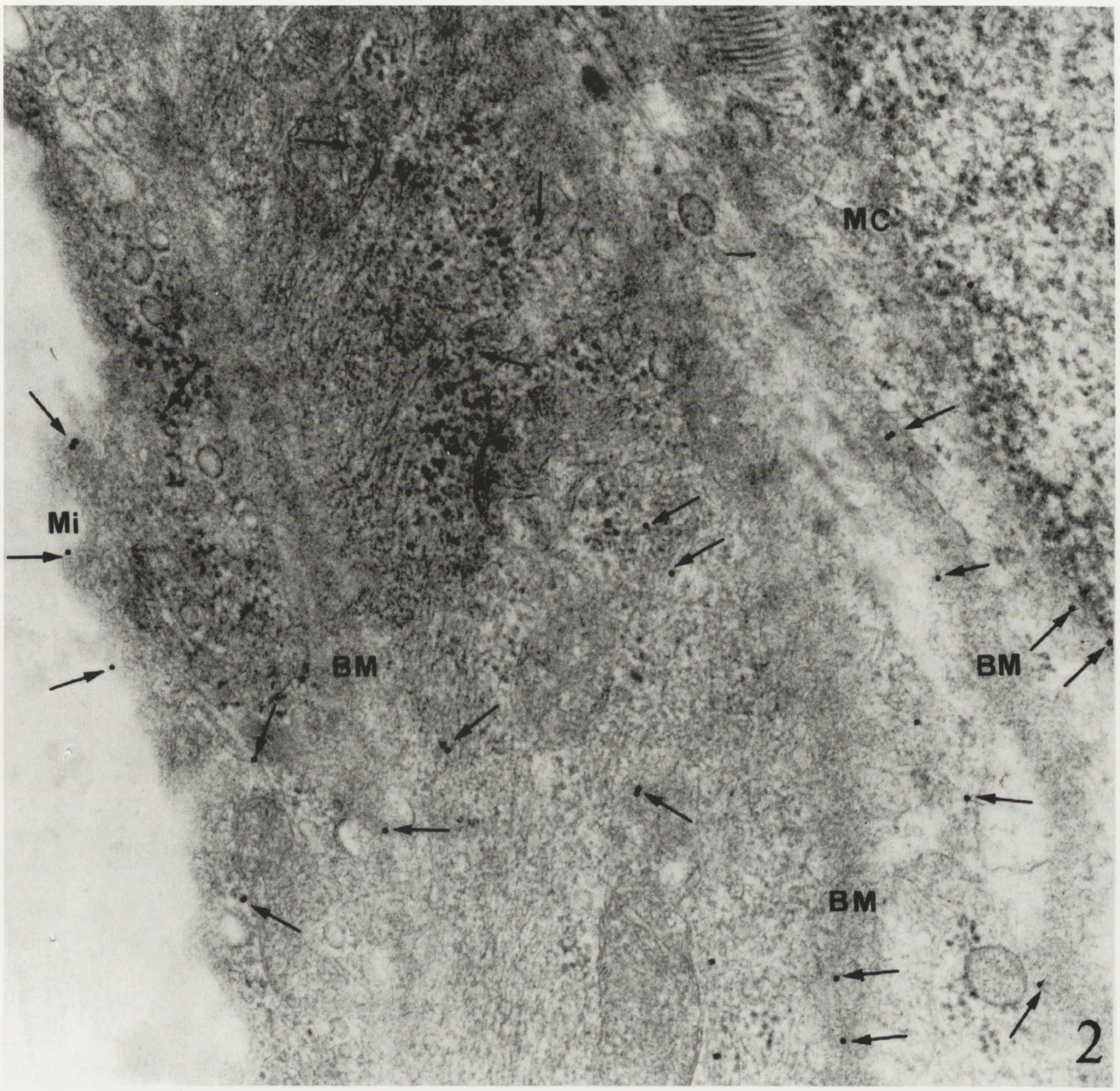

Fig. 2. Endothelial cells of rat hippocampus vessel 10 min after ischemia labeled with EC-NOS antibody. Arrow indicate particles in microvilli (Mi), in the cytosol and in extracellular regions, such as the basement membrane (BM) as well as in the smooth muscle cell (Mc). ×72,000

(PBS) of pH 7.4 (30 min). Blocks of the tissue were taken from hippocampal CA1 area, rinsed for 2 hours in PBS, treated with 1% sodium tetroxide for 1 hour, dehydrated in ethanols of increasing gradients and finally embedded in Epon. Ultrathin sections were processed according to the post-embedding immunogold procedure. Briefly, the sections were mounted on the formwar-coated nickel grids, placed in 10% hydrogen peroxide for 10 min, rinsed in PBS for 15 min and exposed for 15 min to 5% bovine serum albumin in PBS. Anti-EC-NOS antibody (Transduction Laboratories, Catalog no. N30020) used in the present study was an IgG1 monoclonal antibody raised against rabbit vascular endothelial – NOS and purified from mouse ascites with chromatographic techniques. This antibody was diluted 1:20 in PBS and applied on the slices for 2 hours in 37°C. The grids were washed in PBS for 30 min and exposed to goat anti-rabbit IgG conjugated with colloidal gold particles of 10 nm diameter (Janssen Pharmaceutica, Beerse, Belgium) diluted 1:15 in PBS. After incubation for 30 min in darkness, the grids were washed with PBS for 15 min and then with distilled water for 15 min. Tissue slices were air-dried, stained for 10 min with 4.7% uranyl acetate and for 2 min with lead citrate. Control sections were prepared using normal murine serum instead of anti-EC-NOS antibody; no NOS

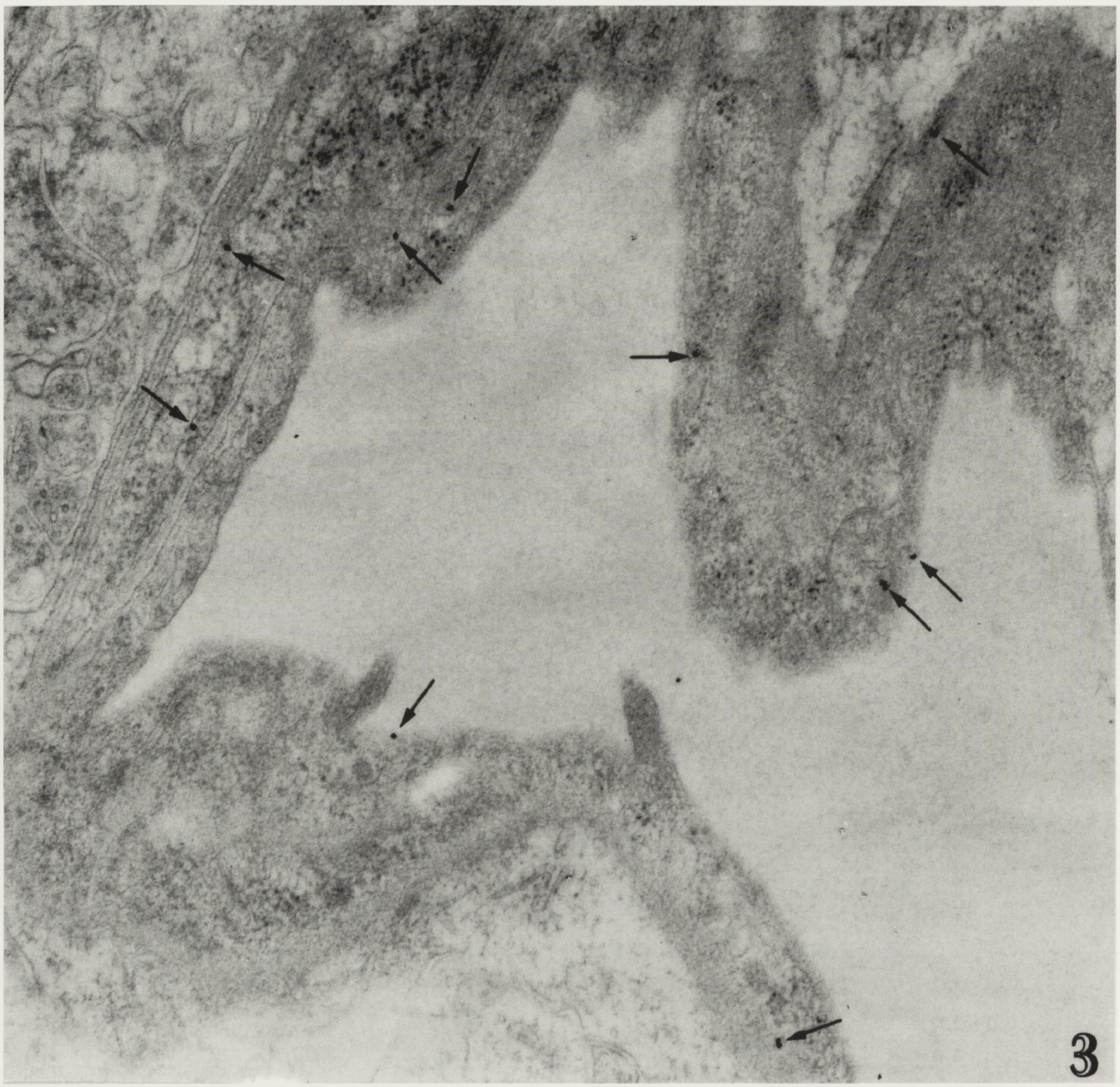

Fig. 3. Endothelial cell of rat hippocampal vessel – 30 min after ischemia labeled with EC-NOS antibody. The gold particles are located in cytosol and near the basement membrane. ×49,950

immunoreactivity was detected in them. The sections were examined and photographed in JEOL 1200 EX electron microscope. In each section, gold particles were counted in 50 endothelial cells.

Results

I. EC-NOS immunoreactivity in control animals

Immunoreactivity for EC-NOS was localized in almost all endothelial cells of blood vessels in the CA1 region of hippocampus. There were no reaction products in neuronal and glial cells. In most endothelial cells immunoreactivity for EC-NOS was weak. The endothelial plasma membranes and the cytoplasm were weakly labeled. One or several clusters of the gold particles were distributed in the cytoplasm, with an occasional association with membrane, or with cytoplasmic organelles such as endoplasmic reticulum and sub-plasmalemmal vesicles more at the luminal cell surface than at the side of basal membrane. Weak immunoreactivity was also visible in the cytoplasm with intermediate filament-rich areas of smooth muscle cell (Fig. 1).

II. EC-NOS immunoreactivity in experimental animals

The intensity of immunoreactivity for EC-NOS in endothelial cells was in experimental animals much more pronounced than in control animals. Gold par-

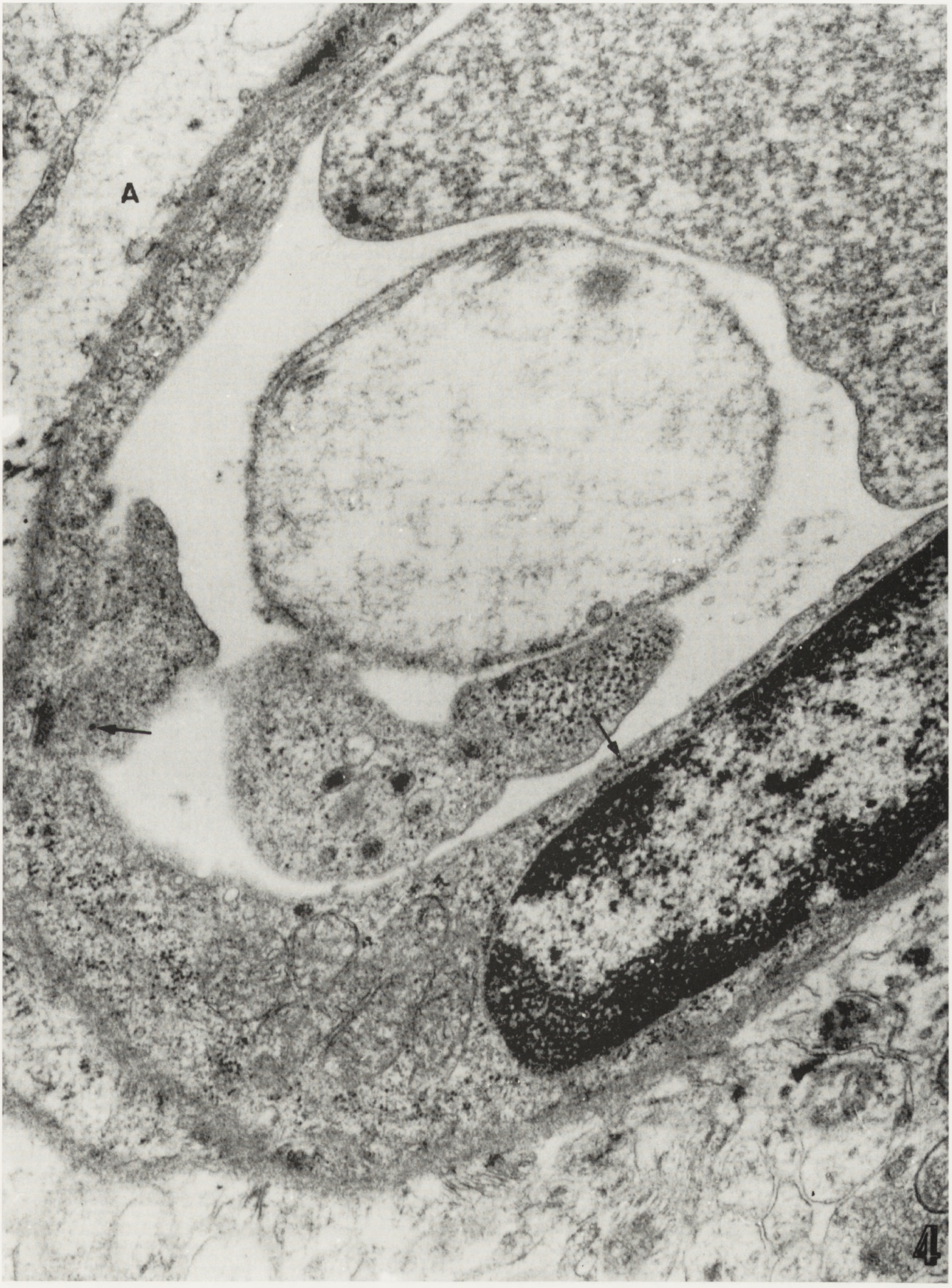

Fig. 4. Endothelial cell of rat hippocampal vessel – 30 min after ischemia weakly labeled with EC-NOS antibody. Note swollen perivascular astrocytic processes (A) and aggregation of morphological elements of the blood. ×43,500

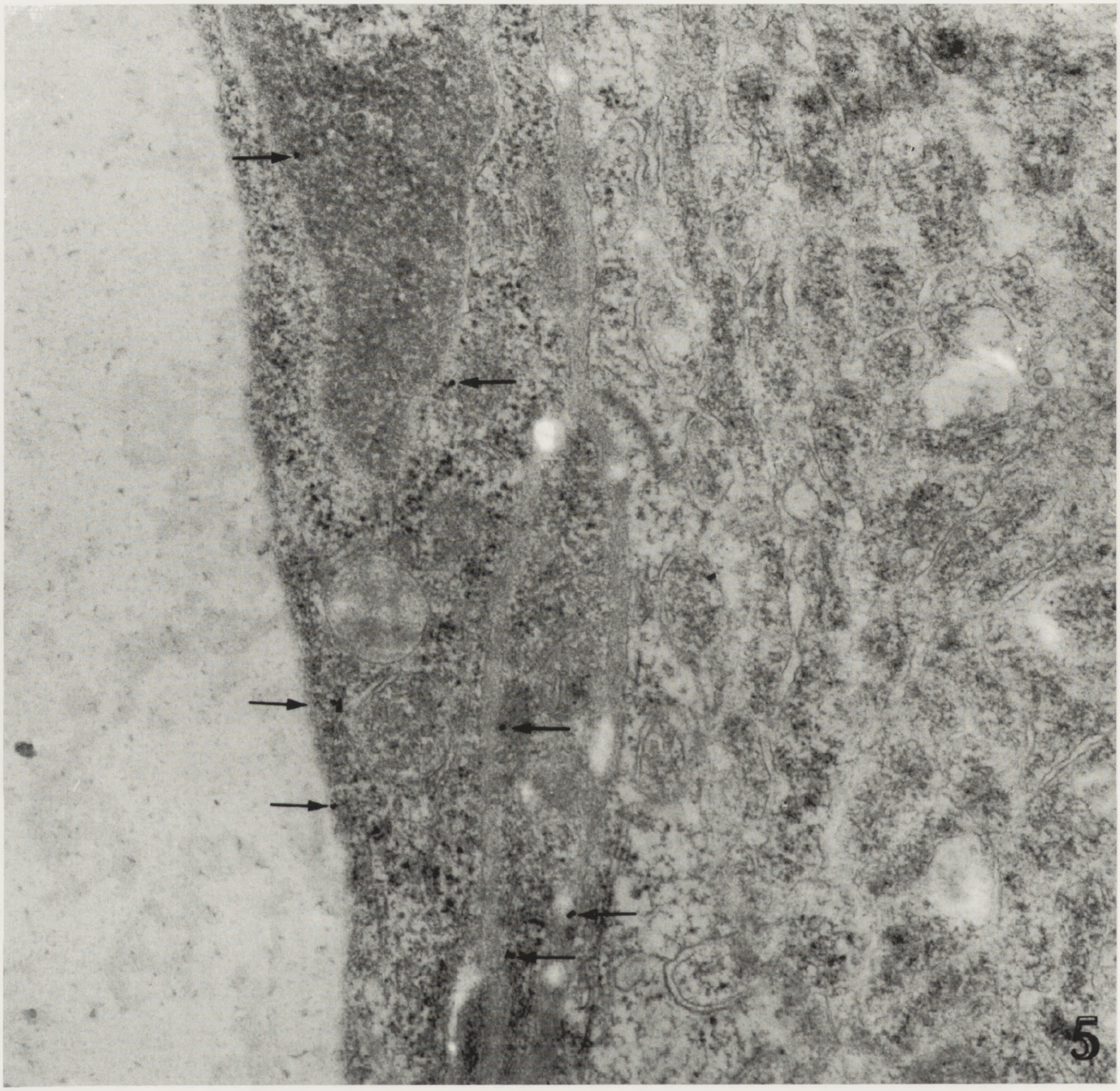

Fig. 5. Endothelial cells of rat hippocampal vessel – 1 hour after ischemia labeled with EC-NOS antibody. Note immunogold particles or their clusters in cytosol, in basement membrane, and in luminal cell membrane. Gold particles are present in nucleus and in perinuclear region. ×49,950

ticles showing immunoreaction for EC-NOS in endothelial cells were found in a considerable number.

Ten min after ischemia several immunogold particles or clusters were found in all observed endothelial cells not only on the membrane of cytoplasmic organelles and on the cell membranes but also in cytoplasm rich in the intermediate filaments containing no cytoplasmic organelles. EC-NOS immunoreactivity was also demonstrated in microvilli of endothelial cells. Immunogold particles were additionally found in non-endothelial structures, such as basement membrane and the smooth muscle cells (Fig. 2) (Table 1). In 50 endothelial cells, examined quantitatively, the total of 189 clusters of immunogold particles was found.

Thirty min after ischemia, the localization and intensity of EC-NOS immunoreactivity in a great majority of endothelial cells were comparable to that seen in endothelial cells of the animals, examined 10 min after ischemia (Fig. 3). In the 50 hippocampal endothelial cells the total of 170 clusters of immunogold particles was found (Table 1). On the other hand, in a few endothelial cells EC-NOS immunoreactivity was weak one or two immunogold particles were found in cytoplasm or plasma membrane of endothelial cell profiles. Weak immunoreactivity for EC-NOS was observed in showing structural abnormalities or adhering platelet to vascular endothelium (Fig. 4) (Table 1). In these ultrastructurally changed 50 endothelial cells, the

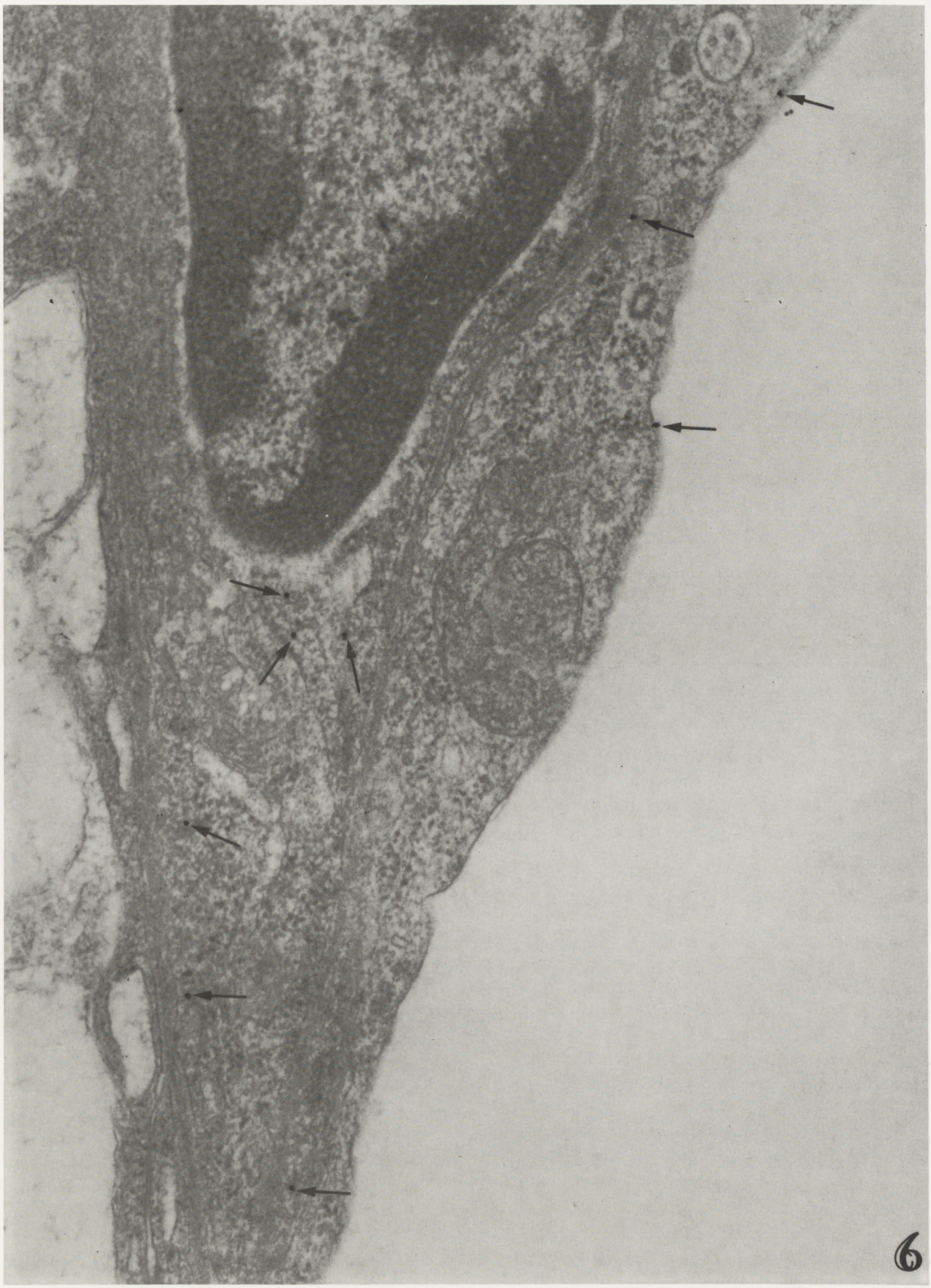

Fig. 6. Endothelial cell of rat hippocampal vessel – 3 hours after ischemia, labeled with EC-NOS antibody. Several gold particles in cytosol not associated with cytoplasm organelles; a weak labeling membrane also present. ×54,450

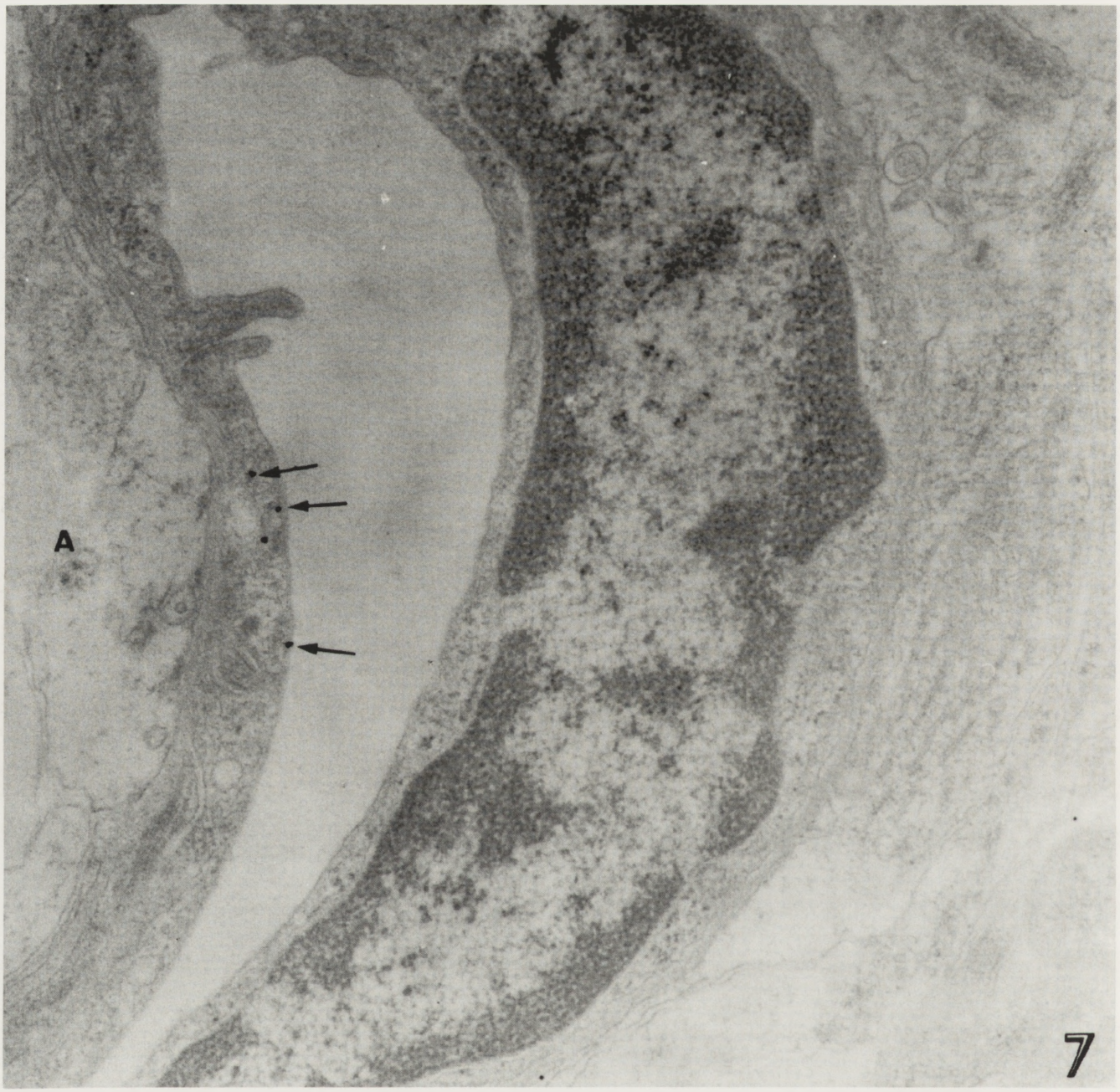

Fig. 7. Endothelial cell of rat hippocampal vessel – 24 hours after ischemia, labeled with EC-NOS antibody. Note weakly labeled cytoplasm and basement membrane of endothelial cell. There is no immunoreactivity in swollen perivascular astrocyte (A). ×43,500

total of 64 clusters of immunogold particles was revealed.

One hour after ischemia, the immunoreactivity for EC-NOS was additionally demonstrated in the periphery of nuclei, in the cytosol (cytoplasm free of organelles), in area associated with cytoplasm organelles and in the luminal membranes of endothelial cells (Fig. 5) (Table 1). In 50 endothelial cells the total of 150 clusters of immunogold particles was observed.

Table 1. Quantification of endothelial-nitric oxide synthase immunolabeling in hippocampus before and after ischemia

Structure	Number of gold partices in 50 endothelial cells*					
	Control n = 4	10′ after ischemia n = 3	30′ after ischemia n = 3	1h after ischemia n = 3	3h after ischemia n =	24h after ischemia n = 4
Blood vessels of hippocampus	68±9	189±43#	170±36#	150±47#	69±33	65±21

* mean ± SD; n shows the number of experimantal animals in a group;
# $p < 0.05$ compared to the control (Student's test)

Three and twenty four hours after ischemia the EC-NOS immunoreactivity was comparable to that seen in the control animals. One or several clusters of gold particles were distributed on plasma membrane or in cytoplasm of endothelial cell profile (Figs. 6, 7) (Table 1). Three hours after ischemia in 50 endothelial cells, the total of 69 clusters of immunogold particles was observed, so was 24 hours after ischemia when in 50 endothelial cells, the total of 65 clusters of immunogold particles was found.

Discussion

Under normal conditions nitric oxide (NO) participates in maintaining the basal tone of cerebral microvessels and the autoregulation of cerebral blood flow (Kobari et al. 1994). However, it was shown that under pathological conditions NO can mediate a breakthrough of cerebral autoregulation mechanisms (Talman, Dragon 1995) leading to protracted cerebral vasodilation. The aim of our studies consisted in establishing possible changes in EC-NOS expression in cerebral microvessels of rat hippocampus after transient global brain ischemia, considering the temporal profile and intensity of cellular localization of EC-NOS as an indicator of NO participation in the development of postischemic microcirculatory disturbances and consecutive tissue abnormalities.

Immunoreactivity for EC-NOS was exclusively in all the endothelial cells of blood vessels of CA1 region of hippocampus. There were no reaction products in the neuron and glial cells both under normal and pathological conditions. Although recent studies of Dawson et al. (1991) showed EC--NOS immunoreactivity in neurons of hippocampus, the absence of neuronal staining in our studies seems result from the differences of antibody tissue processing, and time profile of observation. The specificity of the antibody used by us excluded cross-reactivity with other NOS isoforms.

The most important finding was that EC-NOS was rapidly upregulated at 10 min after ischemia. It progressively increased 1 hour after ischemia. At 3 hours and 24 hours after ischemia the low intensity of EC-NOS staining was a constant feature. The results of the present study may indicate that EC-NOS mediates alteration in the cerebral blood flow in the early postischemic period, as changes coincide well with those described in this experimental model by Kapuściński (1987). The previous investigations performed in other laboratories suggest that the role of NO in the mechanisms of cerebral ischemia is multifarious. It is thought that immediately after cerebral ischemia, NO plays a beneficial role because it promotes vasodilation, inhibits platelet aggregation, inhibits NMDA receptors that may mediate cellular damage, and increases blood flow to penumbral regions at risk for infarction (Izumi et al. 1992; Morikawa et al. 1992; Zhang et al. 1994). After than 2 hours following ischemia, the vascular effects of NO are no longer beneficial (Zhang, Iadecola 1994). At this time, NO owing to its cytotoxic effects, may facilitate the development of ischemic damage (Iadecola et al. 1995a, b). Degenerative change of endothelial cells has been documented in animal models of focal ischemia.

Upregulation of EC-NOS may also be involved in the mechanism of cerebral vascular endothelial damage. Palmer et al. (1992) showed that NO synthesized by inducible NOS in endothelium reduced its viability. Recently, it was hypothesized, that pathological production of NO contributes to blood-brain barrier disruption (Boje 1996; Mayhan et al. 1996). Other investigators have demonstrated that administration of NOS inhibitor can attenuate barrier breakdown (Chi et al. 1994; Janigro et al. 1994). It seems however, that our immunocytochemical observations indicating that NO is induced rapidly after brain ischemia and then returns to normal values, suggest its beneficial early effect by increasing cerebral blood flow.

Wpływ niedokrwienia mózgowego na syntetazę tlenku azotowego w komórkach śródbłonka

Streszczenie

Badania immunocytochemiczne i ultrastrukturalne wykonano w celu ustalenia rozmieszczenia i roli śródbłonkowej syntetazy tlenku azotowego (EC-NOS) w komórkach śródbłonka naczyń hipokampa szczurów po przejściowym niedokrwieniu mózgu. Immunoreaktywność EC-NOS wykazano w błonie plazmatycznej, podbłonowych pęcherzykach, błonie podstawnej i cytosolu komórek śródbłonka. Gwałtowny, przejściowy wzrost immunoreaktywności NOS zaobserwowano w okresie od 10 minut do jednej godziny po niedokrwieniu. Wyniki pracy sugerują, że tlenek azotowy, jako czynnik rozszerzający naczynia może odgrywać rolę ochronną w poniedokrwiennym uszkodzeniu mózgu.

References

1. Boje KMK: Inhibition of nitric oxide synthase attenuates blood-brain barrier disruption during experimental meningitis. Brain Res, 1996, 720, 75-83.
2. Chi OZ, Wei HM, Sinha AK, Weiss HR: Effects of nitric oxide synthase on blood-brain barrier transport in focal ischemia. Pharmacology, 1994, 48, 367-373.
3. Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Synder SH: Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci USA, 1991, 88, 7797-7801.
4. Dawson TM, Synder SH: Gases as biological messengers: nitric oxide and carbon monoxide in brain. J Neurosci, 1994, 14, 5147-5159.

5. Dinerman JL, Dawson TM, Schell MJ, Snowman A, Snyder SH: Endothelial nitric oxide synthase localized to hippocampal pyramid cell: Implications for synaptic plasticity. Proc Natl Acad Sci USA, 1990, 92, 4218.
6. Faraci FM, Breese KR: Nitric oxide mediates vasodilatation in response to activation of N-methyl D-asparte receptors in brain. Circ Res, 1993, 72, 476-480.
7. Fostermann U, Schmidt HHHW, Pollock JS, Sheng H, Mitchell JA, Warner T, Iadecola C, Pelligrino DA, Moskowitz MA, Lassen MA: Nitric oxide synthase inhibition and cerebrovascular regulation. J Cereb Blood Flow Metab, 1994, 14, 175-192.
8. Gajkowska B, Gadamski R, Mossakowski MJ: Influence of short-term ischemia on the ultrastructure of hippocampal gyrus in Mongolian gerbils. Part II. Electron microscope picture of synapses in early postischemic period. Neuropatol Pol, 1989, 27, 359-366.
9. Gajkowska B, Mossakowski MJ: Localization of endothelin in the blood-brain interphase in rat hippocampus after global cerebral ischemia. Folia Neuropathol, 1995, 33, 221-230.
10. Iadecola C, Pelligrino DA, Moskowitz MA, Lassen NA: Nitric oxide synthase inhibition and cerebrovascular regulation. J Cereb Blood Flow Metab, 1994, 14, 175-192.
11. Iadecola C, Zhang F, Xu X: Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. Am J Physiol, 1995, 268, R286-R292.
12. Iadecola C, Zhang F, Xu X, Casey R, Ross ME: Inducible nitric oxide synthase gene expression in brain following focal cerebral ischemia. J Cereb Blood Flow Metab, 1995, 15, 378-384.
13. Izumi Y, Bezn AM, Clifford DB, Zorumski CF: Nitric oxide inhibitors attenuate N-methyl-D-aspartate excitotoxicity in rat hippocampal slices. Neurosci Lett, 1992, 135, 227-230.
14. Janigro D, West GA, Nguyen TS, Winn HR: Regulation of blood-brain barrier in endothelial cells by nitric oxide. Circ Res, 1994, 75, 528-538.
15. Kapuściński A: Cerebral blood flow in the experimental model of clinical death in rats (in Polish). Neuropatol Pol, 1987, 25, 387-398.
16. Kobari Y, Fukuuchi M, Tomita N, Tanahashi , Takeda H: Role of nitric oxide in regulation of cerebral microvascular tone and autoregulation of cerebral blood flow in cats. Brain Res, 1994, 667, 255-262.
17. Loesch A, Belai A, Burnstok G: Ultrastructural localization of NADPH -diaphorase and colocalization of nitric-oxide synthase in endothelial cells of rabbit aorta. Cell Tissue Res, 1994, 274, 539-545.
18. Marletta MA: Nitric oxide synthase structure and mechanism. J Biol Chem, 1993, 268, 12231-12234.
19. Mayhan WG, Scan P, Didion MA: Glutamate – induced disruption of the blood-brain barrier in rats. Role of nitric oxide. Stroke, 1996, 27, 965-970.
20. Moncada S, Higgs: The L-arginine-nitric oxide pathway. N Engl J Med, 1993, 329, 2002-2012.
21. Morikawa E, Rossenblatt S, Moskowitz MA: L-arginine dilates rat pial arterioles by nitric oxide-dependent mechanisms and increased blood flow during focal cerebral ischemia. Brain J Pharmacol, 1992, 107, 905-907.
22. Nathan C: Nitric oxide as a secretory product of mammalian cells. FASEB J, 1992, 6, 3051-3064.
23. Pollock JS, Nakane M, Butterey LK, Martinez A, Springall D, Pollak JM, Forstermann U, Murad F: Characterization and localization of nitric oxide synthase using specific monoclonal antibodies. Am J Physiol, 1993, C1379-C1387.
24. Stuehr DJ, Griffith OW: Mammalian nitric oxide synthases. Adv Enzymol, 1992, 65, 287-346.
25. Talman WT, Dragon DN: Inhibition of nitric oxide synthesis extends cerebrovascular autoregulation during hypertension. Brain Res, 1995, 672, 48-54.
26. Tomimoto H, Akiguchi I, Wakita H, Nakamura S, Kimura J: Histochemical demonstration of membranous localization of endothelial nitric oxide synthase in endothelial cells of the rat brain. Brain Res, 1994, 667, 107-110.
27. Zhang F, White JG, Iadecola C: Nitric oxide donors increase blood flow and reduce brain damage in focal ischemia: evidence that nitric oxide is beneficial in the early stages of cerebral ischemia. J Cereb Blood Flow Metab, 1994, 14, 217-226.
28. Zhang F, Iadecola C: Reduction of focal cerebral ischemic damage by delayed treatment with nitric oxide donors. J Cereb Blood Flow Metab, 1994, 14, 574-580.

Authors address: Laboratory of Ultrastructure of the Nervous System, Medical Research Centre, PASci, 5 Pawińskiego St., 02-106 Warszawa