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ORIGINAL ARTICLE

Induction of endothelial nitric oxide synthase in perivascular mast cells in rat neurohypophysis after ischemia

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Abstract An electron microscopic immunocytochemical study was performed in order to determine the expression pattern of endothelial nitric oxide synthase (eNOS) in rat neurohypophysis after ischemia. In basal conditions eNOS was found to be weakly expressed on the endothelial cells and on single activated neurohypophyseal mastocytes. After cerebral ischemia, the number of mast cells increased in the neurohypophysis. The eNOS immunolabelling of mast cells was strongly enhanced between 10 min and 3 h after ischemia and declined at 24 h after ischemia. eNOS labelling was also enhanced in endotethelial cells between 30 min and 3 h after ischemia. Ultrastructurally, eNOS labelling was restricted to the granules of activated mast cells and the cytoplasm of endothelial cells. This study suggests that cerebral mastocytes are an important source of eNOS in neurohypophysis during ischemia and contribute to nitric oxide production in the perivascular space.

Abbreviations

CNS central nervous system
NO nitric oxide
NOS nitric oxide synthase
PBS phosphate-buffered saline

Introduction

Mast cells are present in the central nervous system (CNS) of many species, including rodents [1] and humans [2]. These cells are found within the leptomeninges, the cerebral cortex, dorsal thalamic nuclei, the subfornical organ, the olfactory peduncles, the infundibulum, and the area postrema [3, 4]. Mast cells respond to a wide range of stimuli including behavioral factors or hormones [1], but the significance of those responses remains virtually unknown. It has been speculated that mastocytes play a role in the neuroinflammatory conditions such as encephalomyelitis by facilitating the entry of lymphocytes to the CNS and releasing the proinflammatory cytokines [5].

In this study we investigated expression of nitric oxide synthase (NOS) in the neurohypophysis of the rat subjected to total cerebral ischemia. Ischemia is a factor stimulating nitric oxide (NO) production by activating nitric oxygen synthase (NOS) in the brain. NO causes neuronal death by activating glutamate receptors but may also be beneficial by causing vasodilatation and improving cerebral blood supply. There is thus a delicate balance between the deleterious and beneficial effects of NO in brain ischemia, and inappropriate NO production may result in excessive neuronal death following cerebrovascular stroke [6].

The isoforms of NOS (neuronal-nNOS, endothelial-eNOS, inducible-iNOS) seem to play different roles during brain ischemia [7]. Studies employing transgenic knock-out mice have conclusively shown that nNOS and iNOS are deleterious, whereas eNOS appears to protect against brain ischemia [8]. This unique role of eNOS seems to be due to an early activation and NO production near capillaries causes vasodilatation and improved blood perfusion [9].

In the earlier studies, including those originating from this laboratory, it has been shown that total brain ischemia evokes characteristic ultrastructural changes in rat neurohypophysis and hypothalamic nuclei [10, 11]. Neurohypophysis provides an interesting model for investigating the effects of ischemia because this region is devoid of functional blood-brain barrier. Moreover, the model is clinically relevant, since ischemic stroke of the pituitary is known to be a cause of diabetes insipitus. We present evidence that ischemia causes a rapid influx of mast cells into neurohypophysis and that those cells are involved in NO production in this endocrine organ.

Materials and Methods

Sixteen adult male Wistar rats (250–300 g) were subjected to experimental cerebral ischemia following the cardiocirculatory arrest, as described previously [12]. After 10 minutes the animals were resuscitated by means of heart massage and artificial ventilation. For ultrastructural and immunocytochemical studies the rats were anesthetized with ether and perfused intracranially with 0.9% NaCl (1 min) followed by 0.5% glutaraldehyde and 2.5% paraformaldehyde in PBS, pH 7.4, 10 min, 30 min, 3 h, and 24 h after ischemia. The animals were handled according to the guidelines of the local ethics committee for experimentation on animals.

Blocks of tissue were taken from neurohypophysis, rinsed for 2 h in PBS, treated with 1% OsO4 for 1 h, dehydrated in ethanol gradient and finally embedded in Epon. Semi-thin sections were stained with azure II or acidic toluidine blue to visualize mast cell granules in light microscope. For electron microscopy, ultrathin sections were processed according to the post-embedding immunogold procedure. Briefly, the sections were mounted on the formvar-coated nickel grids, incubated 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. The anti-eNOS antibody (Transduction Laboratories, cat. no. N.30020) was diluted to 1:20 in PBS and applied on tissue slices for 2 h at 37°C. After washing, the grids were exposed to goat anti-mouse IgG conjugated with 10 nm colloidal gold particles (Janssen Pharmaceutica, Beerse, Belgium) diluted to 1:15 in PBS, for 30 min in darkness. The material was 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 the anti-eNOS antibody. The sections were examined and photographed in the JEOL 1200EX electron microscope.

To determine eNOS labelling, the number of gold particles was counted over dark and clear granules in mast cells in 5 different tissue sections (total 15 granules of each kind per section) of each experimental group. Then, the area of the granules was determined and the results were expressed as number of gold particles/μm2 with standard deviations (SD). For quantification of endothelial cell labelling, the number of gold particles was counted over the whole cell (in total 5 different sections, 10 cells in each section) and also expressed as number of particles/μm2. Mast cells were quantified in the light microscope in toluidine blue-stained sections. The sections were inspected at magnification ×600, photographed, and

the mean number of mastocytes determined in 5 different regions, each encompassing 100 μm2. Student's t-test was used for statistical analysis.

Results

Normal rat neurohypophysis contains single (2 cells per 200/μm2) mast cells localized in the perivascular space, having distinctive, metachromatically-labelled granules. Ultrastructurally, mast cells were characterized by extensive rough endoplasmic reticulum, well-developed Golgi complex, and numerous secretory granules. Both light and dark granules were labelled with eNOS antibody (Fig. 1). In control preparations where anti-eNOS antibody was replaced with murine serum, the gold labelling was negligible. Labelled mastocytes showed broadened processes protruding towards the neurosecretory processes which could indicate activation. eNOS labelling of endothelial cells was much weaker than that of mast cell granules (Table 1). Few gold particles were diffusely distributed in the cytoplasm and occasionally in the plasmalemma, cytoplasmic vesicles, or basement membrane of endothelial cells of fenestrated capillaries.

In experimental animals an increase in the number of perivascular mast cells took place already 10 min after ischemia (Fig. 2). eNOS labelling of mast cells was strong and the density of gold particles over the secretory granules was higher than in the control animals (Fig. 3a, Table 1). eNOS labelling was restricted to the granules and other organelles

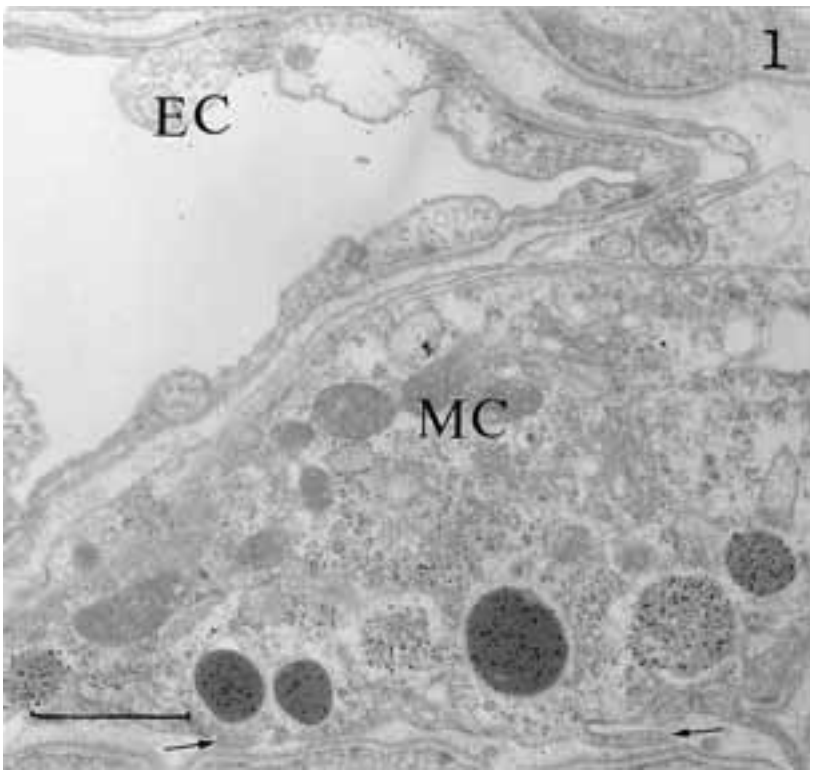


Fig. 1. eNOS immunolabelling of mast cells in rat neurohypophysis (control animal). Note mast cells (MC) with characteristic processes (arrows) and distinctly labelled clear and dark secretory granules. Bar, 1 µm.

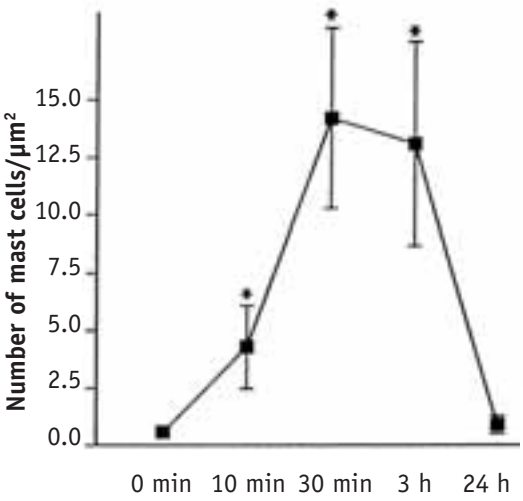


Fig. 2. Increase in mast cell number in neurohypophysis after global cerebral ischemia. Rats (n=2 for the control group 0 min, n=4 for experimental groups) were subjected to total brain ischemia and their neurohypophyses were analysed at different times after resuscitation. The number of mast cells was determined from the semi-thin sections stained with toluidine blue or azure A. Bars show standard deviations, asterisks show statistically significant differences from the control "0 min" group.

Table 1.

	Number of gold particles / µm2				
	0 min	10 min	30 min	3 h	24 h
Endothelial cells	3±2	3±3	24±8*	29±7*	4±3
Mast cells					
dark granules	395±39	1451±119*†	3980±254*	4270±319*	473±100
clear granules	413±48	839±74*	4155±333*	4007±536*	429±68

*statistically different from control (o min), p<0.001, Student's t-test
†statistical difference in labelling between dark and clear granules, p<0.001, Student's t-test

remained virtually unlabelled. Dark granules appeared to be more heavily labelled than the clear ones, in contrast to the situation in the control animals where the degree of labelling was similar. In some cells exocytosis of individual granules containing immunolabelled material was present. Labelling of endothelial cells was not elevated comparing to the control material (Table 1).

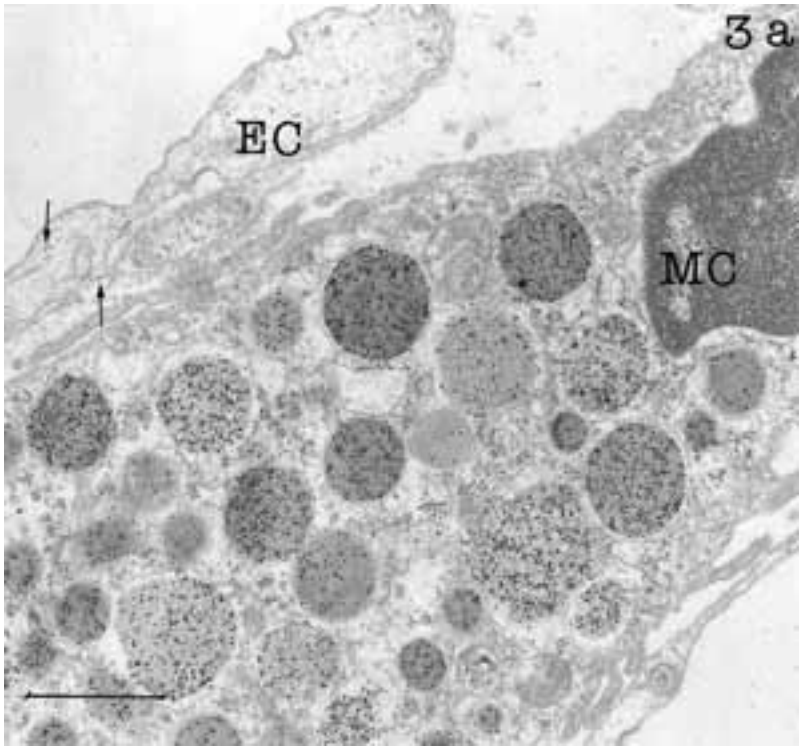
There was a further increase in the number of mastocytes in the pituitary 30 min after ischemia (Fig. 2). A strong labelling for eNOS was present in the mast cell secretory granules. Labelling of dark and clear granules was approximately equal (Table 1). Large numbers of giant, heavily labelled granules were exocytosed into the perivascular space (Fig. 3b). Endothelial cells which demonstrated typical ischemic alterations (edema, denudation, microvilli, and sometimes apoptosis) were also strongly labelled for eNOS.

Labelling intensity and the number of mastocytes 3 h after ischemia were comparable to the situation seen after 30 min (Fig. 2). Exocytosis of the granules was still prominent in some cells. High eNOS labelling was also present in the endothelium (Table 1).

The number of mast cells decreased to the pre-ischemic levels 24 h after ischemia (Fig. 1). Mastocytes showed a baseline level of eNOS labelling. The labelling of endothelial cells returned also to the levels seen in control animals (Table 1).

Discussion

Using immunocytochemical methods for eNOS detection in electron microscopy, we found that this isosyme is constitutively and specifically expressed in the secretory granules of pituitary mastocytes and in lower concentration in the endothelium. Furthermore, global brain ischemia induces a rapid migration of mast cells to neurohypophysis and these cells are heavily labelled with the anti-eNOS antibody over the granules. In the pre-ischemic conditions the labelling of dark and clear granules was equal and was shifted towards higher labelling of dark granules 10 min after ischemia. The



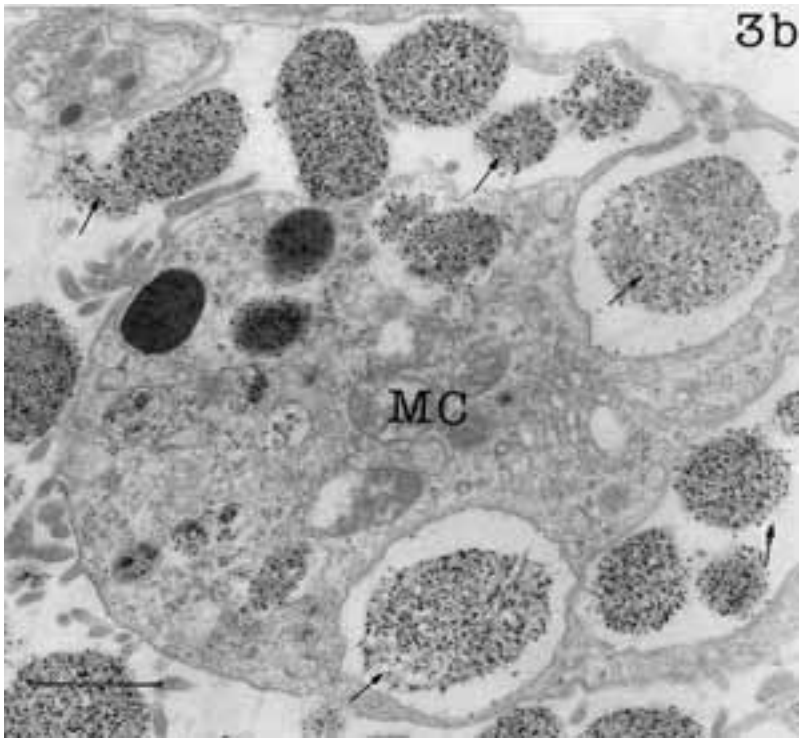


Fig. 3. eNOS immunolabelling of mast cells in rat neurohypophysis after ischemia. (**a**) 10 min after ischemia: note a large number of strongly labelled clear and dark secretory granules. Other cellular elements of mast cell (MC) are unlabelled. A scantily labelled endothelial cell (EC) is indicated by an arrow. (**b**) 30 min after ischemia: fragment showing prominent exocytosis of granules containing immunolabelled material (arrows). Bars, 1 μm.

degree of granule labelling was again equal at 30 min after ischemia. An ischemia-induced increase in eNOS labelling was also seen in endothelial cells, albeit not so vigorous as in the case of mast cell granules.

These results strongly suggest that mast cells may be an important source of NO during pituitary ischemia. However the mechanisms leading to the increase in eNOS labelling are not clear. De novo synthesis of eNOS protein seems not to be a plausible explanation taking into account the very short times (10 min) between the stimulus (ischemia) and the appearance of heavily labelled mastocytes in the pituitary. It is more likely that ischemia causes a very rapid migration of mastocytes with high baseline expression of eNOS from the periphery to the neurohypophysis. This hypothesis implies that there is a subpopulation of NO-producing mast cells which can rapidly migrate to the sites of ischemic stress in the neurohypophysis. Our data showing an increase in the absolute numbers of mast cells parallel to the increase in eNOS labelling supports this hypothesis.

Taking into account that early NO synthesis by eNOS diminishes post-ischemic cerebral damage [8, 9], eNOS-expressing mast cells provide a first-line of defence against the deleterious effects of brain ischemia. This hypothesis is reinforced by current data showing strategic positioning of mast cells in the perivascular space. Such a localization would facilitate the diffusion of NO to the vessel wall causing vasodilation and improvement in blood flow. There is also a possibility that NO is produced extracellularly by mastocyte granules exocytosed to the perivascular space. Thus, the influx of mast cells into the pituitary may provide an important regulatory mechanism in cerebral ischemia.

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