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Immunolocation of Heme Oxygenases in the Walls of Cerebral Arteries of Various Diameters in Rats

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The distribution of two enzymes involved in the formation of carbon monoxide, heme oxygenases 1 and 2, in the pial branches of orders I-V of the middle cerebral artery basin and in intracerebral vessels was studied in adult Wistar rats. Immunohistochemical studies detected hemeoxygenase-2 in the endothelium of the small pial and intracerebral arterioles and in myocytes of pial branches I-III. Heme oxygenase 1, an inducible form of the enzyme, is normally not expressed in the cerebral vessels, but the enzyme is expressed in response to sodium meta-arsenite. In this case, heme oxygenase markers are detected in myocytes of pial arteries I-II and in the endothelium of small pial and intracerebral vessels. Sodium meta-arsenite is inessential for immunolocation and quantitative distribution of heme oxygenase 2 in the vessels.

Key Words: *pial vessels of various diameters; intracerebral arteries; heme oxygenase 1; heme oxygenase 2; immunolocation*

Carbon monoxide (CO) is involved in the regulation of the tone of the cerebral resistive vessels; its effect is less potent but more lasting than that of another gas transmitter — nitrogen oxide (NO) [9,10,15]. The substrate for endogenous CO generation is heme molecule mainly cleaved by two hemeoxygenase isoforms — 1 and 2 [7,11]. High concentrations of heme oxygenase 1 (HO-1) were found in the CNS of rats (in the hippocampal, hypothalamic, and olfactory bulb neurons) [7,13] and high content of heme oxygenase 2 (HO-2) were found in some nuclei of the hindbrain and spinal cord in humans and rats [1,3]. The data on the distribution of these enzymes in the cerebral vessels are scanty and contradictory. According to some data, HO-1, an inducible form of the enzyme, is normally not expressed in the vessels [4,5,10], while other authors detected it only in smooth myocytes [8] or only in the endothelium [5,12]. The data on immunolocation of the constitutive heme oxygenase form, HO-2, in the vessels are no more definite [2,12,14]. The distribution of these two enzymes, involved in the formation of CO, in the pial arteries of various diameters has never been described.

Here we studied the distribution of HO-1 and HO-2 in the pial arteries of various diameters.

MATERIALS AND METHODS

The study was carried out on adult male Wistar rats (200-240 g) distributed into two groups, control and experimental. Experimental rats (n=6) were subcutaneously injected with HO-1 inductor sodium meta-arsenite in a concentration of 75 mM 1 h before sacrifice. Controls (n=12) were injected with an equivalent volume of saline. The animals were intraperitoneally injected with 3% sodium thiopental solution before decapitation. The study was carried out in accordance with regulations for studies with the use of experimental animals (Order No. 755 of the Ministry of Health of the USSR, August 12, 1977).

Pial branches I-V of the middle cerebral artery basin served the object of the study. For immunohistochemical detection of HO, brain fragments with pial vessels were fixed (1 h) in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. Serial cryostate sections (30 μ) were sliced from brain fragments.

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One of these sections was processed for immunohistochemical detection of HO-1, the other for HO-2 detection. The sections were then incubated in 1% normal equine serum (Abcam) at ambient temperature (1 h), then with murine monoclonal antibodies to HO-1 (1:100) or to HO-2 (1:1000) (Abcam) at 4°C during 18 h, with biotinilated equine antibodies to murine IgG (1:100; VectorLabs) during 2 h, and with avidin-peroxidase complex (Vectastain Elite ABC Kit; VectorLabs) during 1 h at ambient temperature. In order to detect HO-1 reaction products under microscope, the sections were incubated in red-colored substrate for peroxidase detection (VIP Substrate Kit; VectorLabs). For detection of HO-2 the immunoprecipitate was visualized by diaminobenzidene (DAB Substrate Kit for Peroxidase; VectorLabs). The sections were then washed, dehydrated by the standard method, and embedded in polystyrene. The reaction specificity was

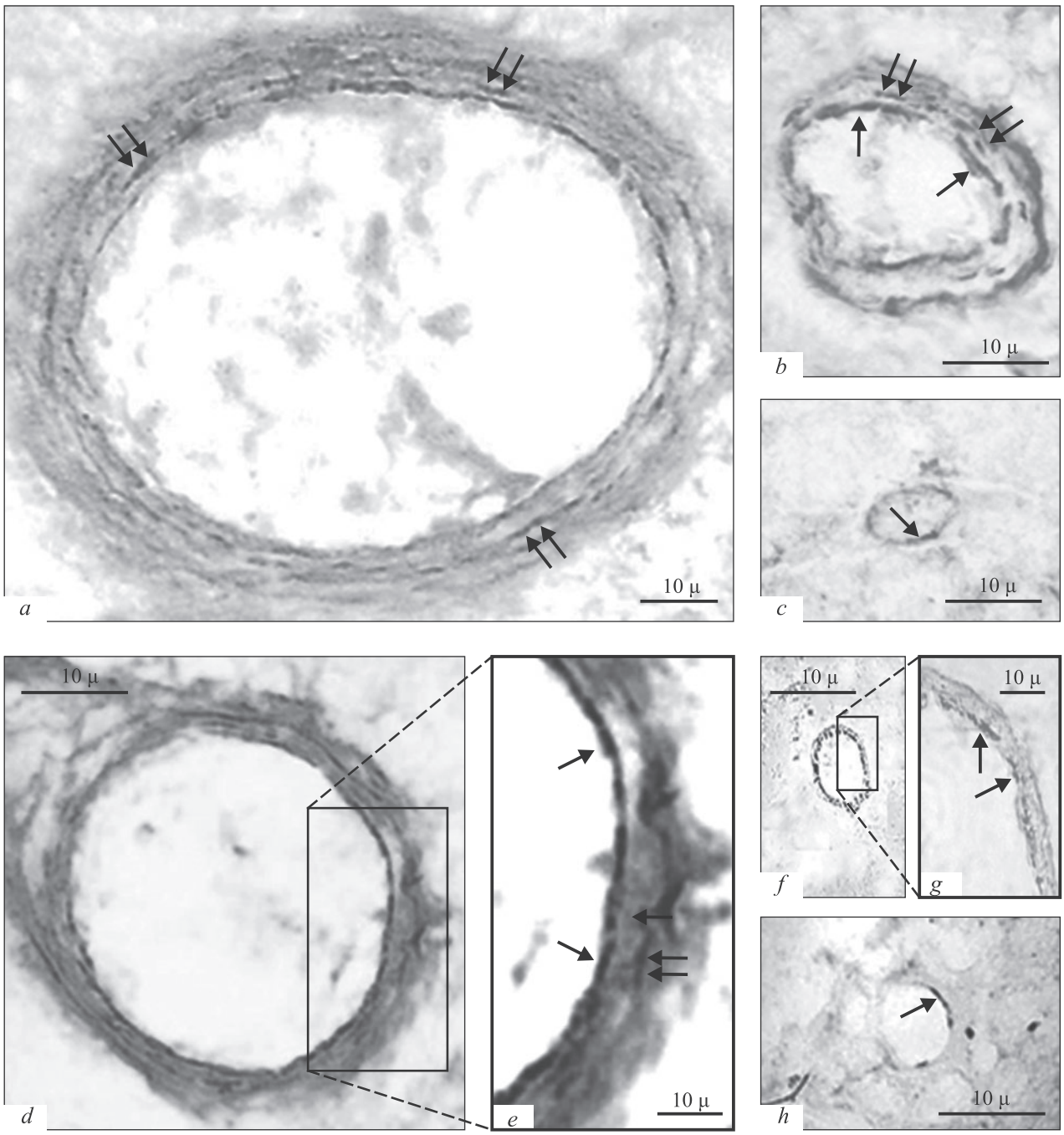

Fig. 1. Immunolocation of HO-2 (*a-c*) and HO-1 (*d-h*) in myocytes (↑↑) and/or endothelium (↑) of pial branches I (*a*), II (*d*, *e*), III (*b*), V (*f*, *g*) and intracerebral arteries (*c*, *h*).

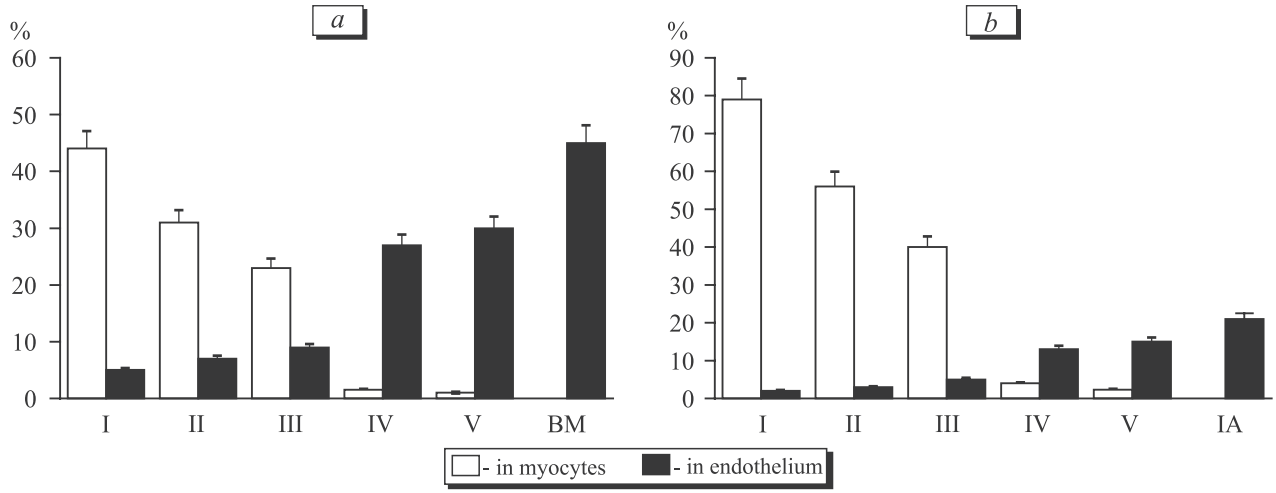

Fig. 2. Numerical density of HO-2+ pial and intracerebral vessels in control rats (*a*) and of HO-1 in response to sodium meta-arsenite (*b*) in myocytes and endothelium of pial branches I-V and intracerebral arteries (IA). The total number of vessels of respective order of branching (diameter) is taken for 100%.

evaluated by staining some sections without first or second antibodies. No immunopositive reaction was detected in control sections.

The numerical density of enzyme-positive vessels of each diameter was calculated in percent of the total number of vessels of the respective size (taken for 100%) by the results of analysis of at least 10 sections. The results were processed by ANOVA. Significance of differences was evaluated by Student's *t* test. The differences were considered significant at $p<0.05$.

RESULTS

In a previous study [2] we measured the diameters of pial branches I-V and intracerebral arteries: 108-86, 74-46, 45-34, 30-12, 10-8, and 11-8 μ, respectively. The muscular tunic of the pial arteries is thinner and the lumen wider than in the vessels located in the majority of other organs. In pial branches I, the diameter of the tunica media consisting of 4-5 layers of smooth myocytes varies from 12.7 to 16.7 μ. In pial branches II, the number of myocyte layers is less — just 3, and the diameter of the muscle tunic is 8.6-9.2 μ. In pial branches III usually consisting of 2 layers of these cells, the tunic diameter varies from 5.8 to 6.3 μ. In smaller pial branches (IV-V) the contractile cells form one layer 2.8-3.2 μ in diameter; in pial branches V, the myocytes are located as a rule at some distance from each other. The muscular tunic of intracerebral arteries is usually 18-25% thinner than in the pial arteries of similar size.

No expression of HO-1 was detected by the immunohistochemical method in the pial and intracerebral arteries of control rats. Studies by Western immunoblotting method failed to detect HO-1 (protein with molecular weight of 32 kDa) in the cerebral vessels, heart, lungs, and aorta of rats and mice [4]. However, in some cases we observed nonspecific staining of the arteriolar wall as a result of diffuse precipitation during immunohistochemical reaction. Presumably, for this reason some scientists speak about the presence of HO-1 in normal vessels [7].

The expression of HO-2 in control rats was detected in pial branches I-V and intracerebral arteries (Fig. 1, *a-c*). Fine granular brown deposition was detected in the structural components of vascular walls, stained various shades of brown, in proportion to the precipitate density. The location and volume of the reaction product largely depended on the vessel diameter and location with respect to the brain surface. In large pial branches (order I) with well-developed tunica media the granular precipitate, indicating the location of HO-2, was detected mainly in myocytes (Fig. 1, *a*). The intensity of the contractile cells staining in the outer and inner layers of the tunica media differed little, presumably due to similar mechanisms of activation of various groups of vascular myocytes in CO-induced vasomotoricity [8,14]. The incidence of myocytes expressing HO-2 in pial branches II-III was 1.5-2 times less (Fig. 2, *a*). The immunopositive contractile cells were just solitary in pial branches IV-V and none were found in intracerebral vessels. The probable targets for CO in vascular myocytes could be cGMP and Ca^{2+}-activated potassium channels [7,15]. Carbon monoxide stimulated K-dependent flow by inducing hyperpolarization of myocytes causing vasodilatation, this leading to moderate, but stable reduction of AP [9,10]. Vasorelaxation induced by CO was arrested by blocking cGMP and Ca^{2+}-activated potassium channels [8,11].

The expression of HO-2 was detected by reverse PCR in myocytes of large arteries but not in the en-

dothelium [5,14]. This result suggested that the endothelium played no appreciable role in CO-induced vasorelaxation. However, according to our data, HO-2 markers were present in the endothelium of small pial branches (III-IV) and intracerebral vessels (Fig. 1, *b*, *c*). Estimations showed that the percentage of vessels with immunopositive endothelium was significantly higher in the brain tissue than in the pia mater, the level of these vessels increased with decreasing their diameter (Fig. 2, *a*). In the brain tissue, the precipitate was also deposited in the walls of some capillaries, in neurons and astrocytes.

According to modern concepts, the endothelium plays the leading role in gas transmitter interactions and initiation of vasomotoricity [2,6,15]. Any kind of manifestation of the activity of at least small arteries is associated with endotheliocyte hyperpolarization, after which the electric signal, generated in these cells, is transmitted, through myoendothelial conjunctions, to vascular myocytes [2,12].

The use of sodium meta-arsenite was inessential for immunolocation and quantitative distribution of HO-2, while the expression of HO-1 in experimental rats was found mainly in myocytes of orders I-II pial branches and in the endothelium of small pial and intracerebral arteries (Fig. 1, *d-h*). In these cases, dark-red dust-like granules labeled the inner lining of vessels along the entire perimeter or its greater part. Comparative analysis showed that the number of vessels with HO-1^+ endothelium in experimental rats was almost 2-fold lower than of HO-2^+ vessels in control rats, while for vessels with HO-1^+ myocytes the relationship was opposite (Fig. 2, *b*).

Hence, our data indicate that normally HO-1 plays a negligible role in the production of CO in myocytes, while exposure to factors, causing expression of the inducible form of the enzyme, causes an increase in the production, particularly so in the vessels with well-developed muscle tunic.

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