Effect of Ischemia on Hydroxylase Cofactor (Tetrahydrobiopterin) and Monoamine Neurotransmitters in Rat Brain

SETSU IJJIMA, M.D., KENJI HARA, M.D., HIROFUMI SUGA, M.D., SHIGENOBU NAKAMURA, M.D., AND MASAKUNI KAMEYAMA, M.D.

SUMMARY Hydroxylase cofactor, monoamine neurotransmitters and their metabolites were measured in ischemic rat brain produced by four-vessel occlusion for 30 and 60 min periods. Slight reduction of hydroxylase cofactor activity was observed in the ischemic cortex after 60 min. Dopamine increased in the brainstem, and its metabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid, increased throughout the brain. Decrease in norepinephrine was observed in the whole brain. Decrease in serotonin and increase in 5-hydroxyindoleacetic acid, a metabolite of serotonin, was observed in the ischemic cerebral cortex. The present study has revealed that there appears to be no significant relationship between hydroxylase cofactor activity and monoamine levels in the ischemic brain. Thus, the hydroxylase cofactor does not play a main role in regulating monoamine synthesis in the acute phase of brain ischemia.

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TYROSINE hydroxylase and tryptophan hydroxylase, the initial and rate-limiting enzymes in the biosynthesis of catecholamines and serotonin, require reduced pteridines as cofactors. Tetrahydrobiopterin (BH4) is thought to be the natural cofactor. Lack of BH4 leads to a severe mental and neurological retardation or malignant hyperphenylalanemia, and defective BH4 metabolism has been suggested in Parkinson's disease, senile dementia, and dystonia. Although BH4 plays such important roles in the brain, little is known about the effect of ischemia on BH4.

It has been suggested that various changes occur on monoamine neurotransmitters in the ischemic brain, which might be responsible for the brain dysfunction. There are, however, many discrepancies among findings presented by previous workers because they used experimental animals of different species or various ischemic periods, or because the distribution of ischemia was not reliably reproducible. At present, however, the rat model of four-vessel occlusion, which has recently been developed by Pulsinelli et al., yields a bilateral forebrain ischemia with a relatively high reproducibility. During four-vessel occlusion, blood flow was severely reduced, to less than 3% of control values, in neocortex, striatum and hippocampus, and blood flow to the brainstem was reduced to 26%.

In the present study, we have measured hydroxylase cofactor activity and concentrations of dopamine (DA), norepinephrine (NE), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in the brains of rats subjected to four-vessel occlusion, in order to evaluate the effect of ischemia on monoamine neurotransmitters and hydroxylase cofactor, and to assess the role of cofactor in ischemic changes of neurotransmitters.

Methods

Male Wistar rats weighing 250–300 gm were subjected to four-vessel occlusion as described by Pulsinelli et al. with some modifications. The rats were anesthetized with sodium pentobarbital (25 mg/kg), and the bilateral vertebral arteries were permanently occluded by electrocautery at the first cervical vertebrae. At the same time, strings were placed loosely around each common carotid artery without interrupting carotid blood flow. After 48 hours, by which time the animals had recovered from anesthesia completely, the exposed common carotid arteries were occluded with clips. Animals that did not become unresponsive within 30 to 60 seconds following carotid artery occlusion and remained responsive during ischemic periods were excluded from the study. Animals which were convulsed during the ischemic period were also excluded, because seizure activity may induce a disturbance in monoamine metabolism. About 25% of the carotid-occluded animals were excluded from the present series. Animals were sacrificed by decapitation following 30 and 60 min periods of occlusion. The brains were quickly removed within 30 seconds, cooled in an ice bath and separated into three regions; cerebral cortex (including hippocampus), deep grey matter (striatum-hypothalamus-midbrain) and brainstem (medulla oblongata), essentially as described by Glowinski et al. The cerebellum was excluded from the analysis. Sham-operated animals were subjected to the same procedures, except for the carotid occlusion.

Hydroxylase cofactor activity was determined by a radioenzymatic assay as described by Levine et al. Dissected parts of the brain were homogenized in 4 vol of ice cold 1.0 N HCl containing ascorbic acid (0.1 mg/ml) and centrifuged for 30 min at 30,000 g at 2°C. Duplicate aliquots (20–40 μl) of supernatant were immediately lyophilized. The hydroxylation reaction was initiated with the addition of 70 μl of the reaction mixture, containing phenylalanine hydroxylase purified from rat liver in excess, quinonoid dihydropterin...
reductase purified from rat brain in excess, 10 μmoles of potassium phosphate buffer (pH 6.8), 0.01 μmole of [4-3H] phenylalanine (30 Ci/mole), 400 units of catalase, and 0.85 μmole of NADH. After 45 min of incubation at 30°C, the reaction was terminated by the addition of 50 μl of 1.2 M sodium acetate buffer (pH 5.5), and the tubes were cooled to 0°C. Twenty-five μl of a solution of iodosuccinimide (50 mg/ml in dimethylsulfoxide) were added to each tube. The alkylation was terminated by the addition of 50 μl of 30% trichloroacetic acid. The tritiated water was separated from other components of the reaction mixture by a small column of Dowex-50 and Dowex-1 acetate. The radioactivity of the eluate was determined by a liquid scintillation counter. Hydroxylase cofactor content in the tissue was calculated from a standard curve of 6-methyltetrahydropterin (6MPH4), a synthetic analog of BH4. The hydroxylase cofactor activity is expressed in terms of 6MPH4 equivalents.

Concentrations of DA, NE, 5-HT, DOPAC, HVA and 5-HIAA were analyzed with a high pressure liquid chromatography coupled with an electrochemical detection (HPLC-ECD), according to the method of Zaczek and Coyle.15 The HPLC-ECD system included an ODS-1262 column (Erma Optical Works), a model 6000-A pump (Waters) and a VMD-101 electrochemical detector (Yanagimoto; potential maintained at +0.80 V; run at sensitivities of 1 to 2 nA). The mobile phase contained 0.01 M sodium acetate, heptane sulfonate (500–700 mg/L), disodium EDTA (100 mg/L) and 6.5% of acetyl nitrite in deionized and distilled water, and pH was adjusted to 4.0. Each sample for HPLC analysis was homogenized in 20 vol (wt/vol) of ice-cold 0.1 N perchloric acid and centrifuged for 15 min at 30,000 g at 2°C to remove denatured protein. Ten to 30 μl aliquots of supernatant were injected into the HPLC system.

For each experiment, animals with a sham operation were used as the controls in order to avoid the influence on monoamines contents from the external environment. Each pair of animals, one for ischemic groups and another for controls, were fed in the same cage and in the same manner, subjected to the same operation except for carotid occlusion and killed at the same time (between 10.00 and 11.00 a.m.). The significance of comparisons between ischemic and control groups was analyzed by the Wilcoxon’s range test for the estimation of differences in the level of significance.

**Results**

The activity of hydroxylase cofactor in the three brain regions of ischemic rats is shown in figure 1, as relative values to controls. The activity of the hydroxylase cofactor decreased in the cortex to 82% of the control value (p < 0.02) after 60 min of ischemia, but remained unchanged in the deep grey and the brainstem, and no alteration was found in cofactor activity in the whole brain after 30 min of ischemia.

The concentration of dopamine in three brain regions of ischemic rats are shown in figure 2-A, as relative values to controls. After 30 and 60 min the concentration of dopamine increased significantly in the brainstem of ischemic rats to 168% (p < 0.05) and 196% (p < 0.02) of the control values respectively, but remained unchanged in other regions of the brain.

The concentration of DOPAC increased in the cortex of ischemic brains to 135% (p < 0.01) and 176% (p < 0.01) of the control values after 30 and 60 min respectively (fig. 2-B). The DOPAC concentration in the deep grey matter increased to 135% (p < 0.02) and 185% (p < 0.01), and in the brainstem to 173% (p < 0.05) and 274% (p < 0.02) after 30 and 60 min respectively.

The concentration of HVA in the cortex increased to 137% (p < 0.01) and 178% (p < 0.01) of control values after 30 and 60 min of ischemia respectively (fig. 2-C). In the deep grey matter the HVA concentration increased to 152% (p < 0.01) and 187% (p < 0.01), and in the brainstem to 169% (p < 0.05) and 250% (p < 0.02) after 30 and 60 min of ischemia respectively. Similar changes in HVA were observed for that of DOPAC.

The NE concentration reduced in the cortex to 83% (p < 0.01) of control values, in the deep grey to 89% (p < 0.02), and in the brainstem to 86% (p < 0.05) after 30 min of ischemia (fig. 3). The concentration of NE reduced more prominently to 73% (p < 0.01), 81% (p < 0.01), and 87% (p < 0.05) of control values respectively, after 60 min of ischemia.

The concentration of 5-HT reduced only in the cerebral cortex of ischemic rats to 71% (p < 0.02) and 72% (p < 0.01) after 30 and 60 min respectively, but remained unchanged in the deep grey matter and the brainstem (fig. 4-A).

The concentration of 5-HIAA in the ischemic brain increased in the cortex to 132% (p < 0.02) and in the deep grey matter to 115% (p < 0.02), and was unchanged in the brainstem after 30 min (fig. 4-B). The concentration of 5-HIAA increased slightly only in the cortex to 113% (p < 0.01) after 60 min.
Discussion

There have been numerous experimental and clinical reports, suggesting that cerebral ischemia causes disturbances in the metabolism of brain monoamines and other putative neurotransmitters. Various controversies exist, however, concerning results obtained from experimental brain ischemia. The main reason may be attributable to the lack of a small-animal model with a predictable distribution of ischemic lesions. The rat model of four-vessel occlusion, which has been developed by Pulsinelli et al., exhibits severe bilateral forebrain ischemia with a good reproducibility. Regional cerebral blood flow and distribution of neuronal damage in this model have been established by the original authors. Although there may exist variations in CBF among individual animals, in the current study over 70% of rats subjected to four-vessel occlusion became clinically unresponsive in the same manner. Thus it appears that this model is suitable for the investigation of the abnormality of neurotransmitters in ischemic brain.

Recent studies indicate that the hydroxylase cofactor, BH4, is below saturating concentrations in cate-
cholaminergic neurons, and thus may play an important regulatory role in the monoamine synthesis.4,5 The present study has revealed, however, that there appears to be no significant relationship between the cofactor activity and monoamine levels in the ischemic brain. While the levels of monoamines changed significantly throughout the brain, slight reduction of the cofactor was observed only in the cortex after 60-min ischemia, and the change in cofactor levels was not prominent. Thus the cofactor does not seem to play a consequential role in the regulation of monoamine synthesis in the acute phase of ischemia. However, further studies are necessary on more prolonged ischemia or on post-ischemic recirculated brain, since the reduction of the cofactor was observed only in rats with ischemia for 60 min, not for 30 min.

DA increased significantly in the brainstem and remained unchanged in other regions. The levels of DOPAC and HVA, metabolites of DA, elevated significantly in the whole brain. These results indicate enhanced DA synthesis and turnover rate, especially in the brainstem where the reduction of blood flow is relatively mild.11,17

Since tyrosine hydroxylase, the rate-limiting enzyme for DA synthesis, requires molecular oxygen, a complete ischemia would reduce the synthesis of DA. The high affinity of tyrosine hydroxylase for oxygen,4,8 however, may leave the brain afield from the lack of the substrate, molecular oxygen. In addition, the increase in cyclic AMP observed in ischemic brain19-20 would activate tyrosine hydroxylase through cAMP-dependent protein-kinase.21 Kogure et al have demonstrated simultaneous increase in cyclic AMP and DA in the embolized brain of rats.22 The influx of CA++ into neurons23 also activates tyrosine hydroxylase through calmodulin-dependent protein kinase.24 The decrease in NE, one of the end-product inhibitors of tyrosine hydroxylase,1 may result in decreased inhibition of tyrosine hydroxylase. Sufficient hydroxylase cofactor could be supplied in the deep grey matter and the brainstem. Considering stimulatory factors mentioned above, the enhancement of the tyrosine hydroxylase activity is likely the case at least in areas with incomplete ischemia.

DOPAC and HVA increased simultaneously throughout the brain. The elevation was most prominent in the brainstem where DA increased significantly and less in the cortex where the DA level was unchanged. The elevation of DOPAC and HVA could result from either increased synthesis, release and degradation of DA, or decreased clearance of metabolites from the brain. Decreased clearance of the tissue does not seem to be a major cause, because 5-HIAA increased much less than HVA which is removed by a similar mechanism to 5-HIAA. Augmented release of DA from nerve terminals alone does not seem to induce the accumulation of metabolites, because DA concentration did not decrease and the majority of DOPAC is formed in the neurons.25 The most plausible interpretation of our results is that elevated DOPAC and HVA levels can be attributed mainly to the increased synthesis and degradation of DA and in part to the augmented release of DA and the decreased clearance of HVA.

A decrease in NE was observed in the whole brain. In accordance with our observations, reduction in NE in the experimental ischemic brain was observed by previous workers, though they used different models of various species.19,26-28 The reduction in NE probably resulted from increased release, decreased reuptake or depressed synthesis. Decrease in uptake of [3H]NE and increase in release were observed in a synaptosome preparation obtained from the ischemic brain of the gerbil by Mršulja et al.30 Dopamine-β-hydroxylase, the enzyme responsible for NE synthesis, shows a much lower affinity for oxygen31 than tyrosine hydroxylase, which leads NE synthesis to be more susceptible to an ischemic insult.

The level of 5-HT decreased and that of 5-HIAA, the metabolite of 5-HT, increased in the cortex of ischemic animals. These results suggest reduced 5-HT synthesis, extensive 5-HT release and decreased clearance of 5-HIAA from the brain. In the ischemic cerebral cortex of gerbils, Mršulja et al demonstrated a decreased rate of 5-HT synthesis, an increased release of 5-HT, and a decreased rate of 5-HIAA removal from the brain,28 in accordance with our results. Tryptophan hydroxylase, the initial and rate limiting enzyme of the biosynthesis of 5-HT, requires molecular oxygen and a pteridine cofactor, BH4,2 like tyrosine hydroxylase. However, cerebral tryptophan hydroxylase shows a poor affinity for oxygen22 and therefore would be easily affected by ischemia. In contrast, tyrosine hydroxylase exhibits a higher affinity for oxygen,1 which may explain the difference between the level of 5-HT and that of DA. Weinberger et al have demonstrated that the catecholamine nerve terminals utilizing NE and DA as transmitters are more susceptible to damage by cerebral ischemia than nerve terminals utilizing 5-HT.24 This may account for the smaller extent of reduction in 5-HT compared with NE which decreased significantly throughout the brain.

The present study has provided results, suggesting a dissociated change in biogenic amines of rat brains after ischemia, which cannot be explained by the change in hydroxylase cofactor. These subtle alterations in biogenic amines would manifest various phenomena such as brain edema or vasospasm. At the same time, the disorder in neurotransmitters may serve as a compensatory mechanism. Thus, findings obtained with experimental ischemia may provide a useful tool for the therapy of acute brain ischemia.

References
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