Alterations of [3H]Forskolin Binding in the Postischemic Rat Brain

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Nagasawa H., Araki T. and Kogure K.

Department of Neurology, Institute of Brain Diseases, Tohoku University School of Medicine

Introduction

Postischemic delayed neuronal damage has been reported in the ipsilateral thalamus and substantia nigra which lay outside ischemic areas of rat brain after middle cerebral artery (MCA) occlusion[1,2]. In these reports, the histological appearance of these remote areas was characterized by degeneration of most neurons with no necrotic changes of neuroglia and blood vessels. The mechanism of such a delayed phenomenon in the exo-focal remote areas is unclear, but it has been speculated that it might be caused by a transsynaptic process neuroanatomically associated with ischemic foci and that intracellular and transsynaptic signal transduction systems might play important roles in this mechanism[3-5].

Cyclic adenosine monophosphate (cAMP) is known to be a second messenger. The diterpene compound forskolin is a potent activator of adenylate cyclase [6]. Forskolin is believed to activate adenylate cyclase at a site on the catalytic subunit[6-9], and the anatomical distribution of the forskolin binding sites has been mapped in the brain by autoradiographic imaging[10,11]. It is thought that autoradiographic analysis of forskolin can delineate neuronal damage and plasticity of the postischemic rat brain[12]. In the present study, we examined chronological changes of forskolin binding sites of the rat brain after 90 min of MCA occlusion and after such occlusion followed by different periods of recirculation in order to clarify the mechanisms of exo-focal postischemic neuronal damage.

Materials and Methods

Ischemia model

Adult male Wistar rats of the SPF strain weighing 280-300 g were allowed free access to food and water before and after all procedures. Six rats were used in each experiment. A detailed description of the surgical procedure has been previously reported[13]. In brief, after induction of anesthesia with a gas mixture of 70% N_2O and 2% halothane (the balance being O_2), the right middle cerebral artery (MCA) was occluded with a silicone rubber cylinder attached to a nylon surgical thread introduced from the bifurcation of the internal carotid artery immediately after ligation of the ipsilateral common and external carotid arteries. The

cylinder was made of 4-0 nylon surgical thread (Nitcho Kogyo Co., Ltd.), 16 mm long. This cylinder was coated with silicone (Xantopren, Bayer Dental) and mixed with a hardener (Elastomer Activator) to increase the thickness of the distal 5 mm to 0.25-0.30 mm. After introducing the embolus, the internal carotid artery was ligated just distal to the point of insertion. The embolus extended from the bifurcation of the internal carotid artery to the proximal portion of the anterior cerebral artery (ACA). The origin of the right MCA and that of the right posterior communicating artery were occluded by the silicone rubber cylinder. In 6 sham-operated control rats, the right internal and external carotid arteries were ligated. Surgery was performed within 15 min with no bleeding. Body temperature was kept at normal limits with a heating pad. Following surgery, anesthesia was discontinued and all rats exhibited neurologic deficits characterized by left hemiparesis with upper extremity dominancy and right Horner's syndrome. After 90 min of MCA occlusion, the 6 rats were decapitated with no recirculation, and in the other rats, recirculation was achieved by pulling the thread out of the internal carotid artery under the same anesthetic conditions as during surgery. Once again, the rats were allowed free access to food and water. Although the ipsilateral common and external carotid arteries had been ligated, the ischemic area could be reperfused via the cerebral arterial circle (circle of Willis) through the contralateral carotid and basilar arteries, and by collateral circulation of the cortical branches of the cerebral arteries. The rats were killed by decapitation 3 h, 6 h, 1 day, 3 days, 1 week, 2 weeks, and 4 weeks after recirculation. After decapitation, the brains were quickly removed and frozen in powdered dry ice, and stored at -80°C until assay. Serial coronal sections 12 μm in thickness were cut on a cryostat and thaw-mounted onto gelatin-coated slides. Adjacent sections were stained with cresyl violet and Luxol fast blue, as well as with hematoxylin and eosin, in order to confirm the ischemic areas.

Autoradiography

The localization and the chronological changes of forskolin binding sites in the brain after 90 min of ischemia were measured by the autoradiographic method of Worley et al.[14] Sections were incubated for 10 min at room temperature in a buffer (50 mM Tris-HCl pH 7.7, 100 mM NaCl, 5 mM $MgCl_2$) containing 10 nM [^{3}H]forskolin (New England Nuclear, spec. act. 38 Ci/mmol). Following incubation, sections were washed twice for 2 min each time at 4°C in the same buffer, briefly rinsed in distilled water at 4°C and dried under a cold stream of air. Non-specific binding was calculated in the presence of unlabelled 10 μM forskolin (Sigma). Autoradiograms were prepared from the sections by exposing them to [^{3}H]sensitive hyperfilm (Amersham) with a tritium standard microscale (Amersham) for 4 weeks in standard X-ray cassettes.

Statistical analysis

Cerebral [^{3}H] tissue concentrations of the autoradiograms were determined by means of a computerized microdensitometric system. Data regarding the forskolin binding sites in each structure of the brain were analyzed using a t-test with $p<0.01$ and $p<0.001$ considered to be statistically significant.

Results

The values of control and chronological alteration of [^{3}H]forskolin binding sites in each structure of rat brain after 90 min of MCA occlusion and after such occlusion followed by different periods of recirculation are shown in Table 1. The [^{3}H]forskolin binding sites were highly concentrated in the caudate putamen and the substantia nigra of the sham-operated control rats as reported by Worley et al.[14] Postischemic alteration of [^{3}H]forskolin binding sites in the ischemic foci was obvious during ischemia (Fig. 1). After 90 min of MCA occlusion with no recirculation, the binding sites decreased to approximately 70% in the anterior neocortex (FrPaSS) and to 30% of each control value in the lateral part of the caudate putamen (CPu-L), both of which were supplied by the occluded MCA. Thereafter, [^{3}H]forskolin binding sites of the ischemic side decreased to approximately 50% in the FrPaSS and to 10% in the CPu-L of each control value 4 weeks after ischemic insult (Table 1, Fig. 2). On the contrary, there was no alteration on day 1, but 3 days after recirculation, marked reduction of [^{3}H]forskolin binding sites was observed in the ipsilateral substantia nigra, which was remote from the precedent ischemic areas (Fig. 3). There were no significant changes of [^{3}H]forskolin binding sites in the thalamus, hippocampus, amygdala, and pons of both hemispheres during and after the ischemia compared with the values of control animals.

Discussion

The present study indicated that two different alterations of the second messenger system took place in postischemic rat brain. In the ischemic foci, the ipsilateral anterior neocortex (FrPaSS) and the lateral part of the caudate putamen (CPu-L), areas directly affected by ischemic insult, [^{3}H]forskolin binding sites rapidly decreased during 90 min of ischemia. On the other hand, in the ipsilateral substantia nigra, which lay outside the ischemic areas, [^{3}H]forskolin binding sites were not significantly changed compared with changes in the control value 1 day after ischemic insult; thereafter, marked reduction of these binding sites was first observed 3 days after the ischemia.

Gehlert et al. reported that direct injection of kainic acid into the caudate putamen of the rat brain reduced [^{3}H]forskolin in the caudate putamen and the ipsilateral substantia nigra, but that direct injection of ibotenic acid into the substantia nigra did not affect [^{3}H]forskolin binding in any part of the structures.[15] This fact indicates that [^{3}H]forskolin binding in the substantia nigra is presynaptic on projections from the caudate putamen and that forskolin-sensitive adenylate cyclase is intrinsic to the striatal-nigral projection.

The present study indicates the following: (1) the rapid reduction of [^{3}H]forskolin binding in the CPu-L is due to the direct damage of neuronal cell bodies or interneurons in these structures induced by ischemic insult; (2) the delayed reduction of [^{3}H]forskolin binding in the substantia nigra is caused by presynaptic lesions of the striatal-nigral projections; (3) the CPu-L is an important constituent of striatal-nigral projections, because the marked reduction of [^{3}H]forskolin binding in the substantia nigra was homogeneously observed though the medial part of the caudate putamen (CPu-M) was not affected even 4 weeks after ischemia.

This delayed change of the second messenger system observed in the substantia nigra was concurrent with the abnormal calcium accumulation detected there in our previous study.[1] Moreover, both phenomena, i.e., the reduction of [^{3}H]forskolin binding sites and abnormal calcium accumulation, in the substantia nigra on the ischemic side preceded the histologic findings of delayed neuronal damages.

To clarify the mechanism of the delayed neuronal damage caused by a transsynaptic process associated with the ischemic foci, chronological changes of some neurotransmitters and their receptors in each structure must be considered. In case of the striatal-nigral pathway, the caudate nucleus of the rat contains high concentrations of several neurotransmitters, such as acetylcholine, dopamine, glutamate, substance-P, and gamma-aminobutyric acid (GABA). The neuroinhibitory transmitter GABA plays an important functional role in the striatalnigral pathway.[16-19] Moreover, dopamine type 1 receptors also have a presynaptic location in the substantia nigra. Direct injections of neurotoxins into the caudate putamen also reduced D_1 dopamine receptors in the caudate putamen and substantia nigra.[18,20] The close relationship between D_1 dopamine receptors and adenylate cyclase in both the striatum and the substantia nigra has also been reported.[21-23] These facts suggest that the reduction of [^{3}H]forskolin binding at the presynaptic sites of the substantia nigra may reflect the alteration of the presynaptic population of D_1 dopamine receptors which may be coupled with forskolin-sensitive adenylate cyclase.

In the present study, we examined the mechanism of exo-focal deayed neuronal damage focusing on the changes in the substantia nigra. In another exo-focal region, the thalamus, further investigation using different markers of the second messenger system and more specific neurotransmitters is required because of the lack of significant changes of [^{3}H]forskolin binding in the thalamus during and after the ischemia. The mechanism of exo-focal postischemic delayed neuronal damage is still far from being understood. However, it is strongly suggested that this delayed phenomenon is caused by a transsynaptic process associated with the ischemic foci.

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Table 1 [^{3}H]forskolin binding in each structure of the rat brain after 90 min of MCA occlusion followed by various periods of recirculation

Structure	Control	90-min ischemia	3 hours	6 hours	1 day
Ischemic side					
FrPaM	47.89 ± 3.95	45.79 ± 1.84	43.42 ± 1.84	48.42 ± 10.26	46.31 ± 8.15
FrPaSS	44.21 ± 6.32	31.89 ± 4.21*	30.36 ± 2.36*	30.63 ± 5.52*	30.52 ± 5.52*
CPu(L)	228.42 ±22.10	62.10 ± 8.15**	63.94 ±11.05**	41.57 ± 2.36**	27.63 ± 3.42**
CPu(M)	183.42 ±16.05	198.15 ±18.15	198.42 ±35.78	234.99 ± 27.63	209.47 ± 23.15
Hippocampus	32.63 ± 3.42	29.63 ± 3.15	30.63 ± 3.94	32.63 ± 3.42	33.94 ± 2.89
Thalamus(VPN)	34.21 ± 3.68	33.21 ± 6.57	32.63 ± 7.36	34.10 ± 2.89	35.26 ± 4.21
Amygdala	40.79 ± 7.11	26.31 ± 3.94	27.36 ± 6.31	32.10 ± 10.52	30.52 ± 6.05
Substantia nigra	75.53 ± 8.68	77.23 ± 5.39	76.39 ± 4.95	77.36 ± 7.36	71.84 ± 13.42
Pons	21.84 ± 1.32	23.24 ± 1.58	22.59 ± 2.02	20.26 ± 1.84	21.84 ± 2.10
Non-ischemic side					
FrPaM	48.68 ± 4.47	48.68 ± 3.42	44.99 ± 4.21	47.89 ± 14.73	47.89 ± 9.99
FrPaSS	42.37 ± 2.89	47.10 ± 1.31	43.68 ± 2.89	48.94 ± 9.21	47.89 ± 8.68
CPu(L)	223.42 ±22.11	231.05 ±21.05	255.26 ±28.15	240.78 ± 34.21	238.15 ± 26.05
CPu(M)	182.63 ±16.31	184.47 ±18.42	207.36 ± 36.84	227.36 ± 31.57	208.94 ± 24.21
Hippocampus	33.68 ± 3.16	30.36 ± 2.63	30.78 ± 6.68	31.84 ± 2.89	34.74 ± 5.78
Thalamus(VPN)	34.74 ± 4.47	33.68 ± 4.21	33.42 ± 6.05	34.73 ± 6.31	33.94 ± 7.10
Amygdala	37.11 ± 4.74	42.36 ± 5.78	42.63 ±11.57	49.47 ± 4.73	46.31 ± 9.26
Substantia nigra	77.68 ± 9.99	79.25 ± 4.98	75.87 ± 6.25	69.73 ± 11.84	71.31 ± 21.57
Pons	21.58 ± 1.58	22.39 ± 1.48	21.25 ± 1.98	18.42 ± 1.31	21.31 ± 1.57

Values are given in mean ± S.D. fmol/mg tissue using six animals.
FrPaM = frontoparietal cortex, motor area, supplied by anterior cerebral artery;
FrPaSS = frontoparietal cortex, somatosensory area, supplied by middle cerebral artery;
CPu(L) = lateral segment of caudate putamen; CPu(M) = medial segment of caudate putamen.
*$p<0.01$; **$p<0.001$, significant difference from control values using a t-test.

Table 1 [^{3}H]forskolin binding in each structure of the rat brain after 90 min of MCA occlusion followed by various periods of recirculation (continued)

Structure	3 days	1 week	2 weeks	4 weeks
Ischemic side				
FrPaM	43.42 ± 3.68	47.10 ± 4.99	48.15 ± 6.31	49.21 ± 2.36
FrPaSS	18.94 ± 1.05**	18.68 ± 2.10**	17.63 ± 1.78**	21.84 ± 2.89**
CPu(L)	20.52 ± 1.31**	20.78 ± 2.36**	22.89 ± 1.98**	22.63 ± 1.84**
CPu(M)	185.78 ± 37.89	196.57 ± 27.63	217.63 ± 17.63	186.58 ± 28.42
Hippocampus	33.94 ± 4.47	36.57 ± 3.42	43.15 ± 3.42	37.37 ± 3.16
Thalamus(VPN)	35.22 ± 3.42	33.78 ± 3.94	33.42 ± 2.89	37.89 ± 6.84
Amygdala	20.21 ± 2.36	21.42 ± 1.84	24.21 ± 3.68	22.16 ± 9.74
Substantia nigra	22.10 ± 0.78**	21.05 ± 1.31**	21.57 ± 0.78**	23.16 ± 3.42**
Pons	22.36 ± 1.31	22.10 ± 1.05	20.78 ± 0.52	23.68 ± 6.05
Non-ischemic side				
FrPaM	54.47 ± 6.05	50.26 ± 6.05	50.78 ± 3.42	49.21 ± 3.42
FrPaSS	51.84 ± 2.89	50.52 ± 6.57	51.84 ± 6.31	52.63 ± 9.21
CPu(L)	243.94 ± 26.05	247.15 ± 27.36	248.42 ± 16.84	237.63 ± 20.78
CPu(M)	207.89 ± 18.68	192.36 ± 17.10	200.52 ± 12.89	176.32 ± 18.68
Hippocampus	43.42 ± 6.05	39.73 ± 3.42	42.89 ± 3.42	38.68 ± 4.47
Thalamus(VPN)	36.31 ± 9.99	42.10 ± 1.31	43.42 ± 3.15	42.00 ± 7.63
Amygdala	48.94 ± 6.31	45.78 ± 5.52	45.26 ± 9.73	47.89 ± 16.58
Substantia nigra	77.63 ± 18.94	69.21 ± 11.05	72.89 ± 8.94	78.95 ± 5.52
Pons	22.89 ± 1.57	22.63 ± 1.57	22.89 ± 2.36	22.37 ± 0.78

Values are given in mean ± S.D. fmol/mg tissue using six animals.FrPaM = frontoparietal cortex, motor area, supplied by anterior cerebral artery; FrPaSS = frontoparietal cortex, somatosensory area, supplied by middle cerebral artery; CPu(L) = lateral segment of caudate putamen; CPu(M) = medial segment of caudate putamen. *p<0.01; **p<0.001, significant difference from control values using a t-test.

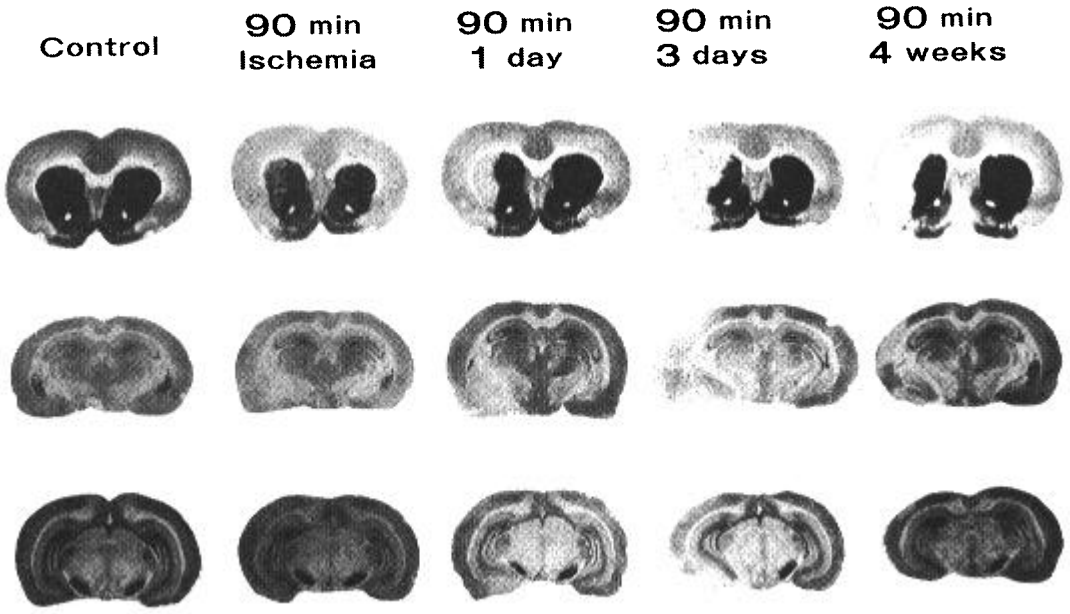

Fig. 1. [^{3}H]forskolin autoradiograms of the brains of sham-operated control rats and those obtained after 90 min of MCA occlusion with no recirculation and followed by 1-day, 3-day, and 4-week recirculation. Representative autoradiograms show coronal sections at the level of the caudate putamen (top), the thalamus (middle), and the substantia nigra (bottom).

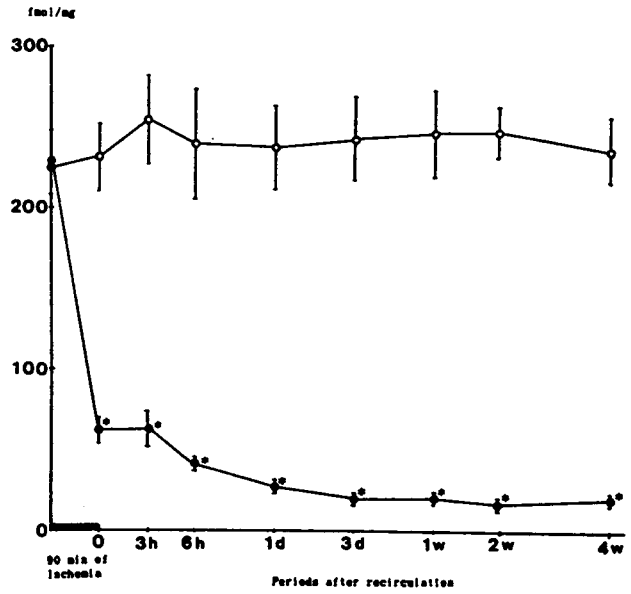

Fig. 2. Time course of [^{3}H]forskolin binding in the caudate putamen of both the ischemic side (●) and the non-ischemic side (○)of rat brain obtained after 90 min of MCA occlusion with no recirculation and followed by various periods of recirculation. Results are expressed as means ± S.D. fmol/mg tissue from 6 animals. *p<0.001, significant difference from control values using a t-test.

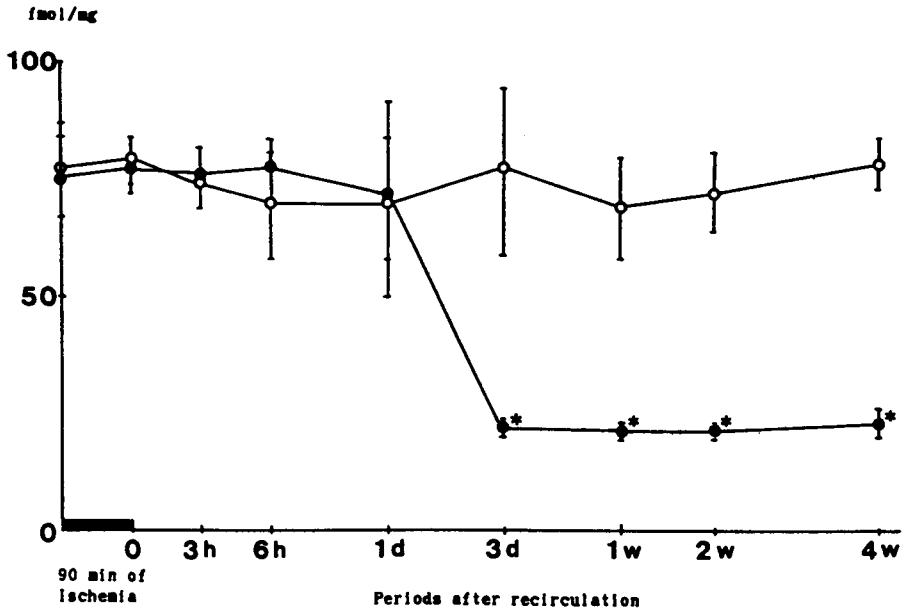

Fig. 3. Time course of [^{3}H]forskolin binding in the substantia nigra of both the ischemic side (●) and the non-ischemic side (○) of rat brain obtained after 90 min of MCA occlusion with no recirculation and followed by various periods of recirculation. Results are expressed as means ± S.D. fmol/mg tissue from 6 animals. *p < 0.001, significant difference from control values using a t-test.