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Post-ischemic changes of [^{3}H]glycine binding in the gerbil brain after cerebral ischemia

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Abstract

Sequential changes of [^{3}H]glycine binding in the gerbil were investigated in selectively vulnerable areas 1 h to 7 days after 10 min of cerebral ischemia. A significant reduction in [^{3}H]glycine binding was found in the hippocampus and thalamus from as early as 1 h after ischemia. In contrast, the striatum and frontal cortex showed a significant decline in [^{3}H]glycine binding from 5 h after recirculation. Thereafter, a severe reduction in [^{3}H]glycine binding was observed in all regions 7 days after ischemia. MAP2 (microtubule-associated protein 2) immunoreactivity was unaffected in the hippocampus, frontal cortex and thalamus up to 48 h after ischemia. Thereafter, a severe loss of MAP2-immunoreactive neurons was found in these regions, especially in the hippocampal CA1 sector. However, the striatum showed a severe loss of MAP2 immunoreactivity from 24 h after ischemia. These results demonstrate that transient cerebral ischemia causes severe reduction in [^{3}H]glycine binding throughout the brain, and this reduction precedes the neuronal damage in selectively vulnerable areas. These findings suggest that a neurotransmitter, glycine, may play a key role in the pathogenesis of post-ischemic neurodegeneration in selectively vulnerable areas.

Keywords: Cerebral ischemia; Glycine; MAP2 (microtubule-associated protein 2); Receptor autoradiography; Immunohistochemistry

1. Introduction

It is now well established that transient cerebral ischemia causes necrosis of specific neuronal populations that lie in the hippocampus, striatum, thalamus and certain layers of the cerebral cortex in experimental animals (Kirino, 1982; Pulsinelli et al., 1982; Smith et al., 1984; Araki et al., 1989). The ischemic neuronal damage is thought to be mediated, in part, by a variety of neurotransmitters. Several lines of evidence suggest that excitotoxic mechanisms, triggered by the ischemia-induced release of excitatory amino acid glutamate, may constitute a major factor in determining selective vulnerability (Rothman and Olney, 1987; Benveniste et al., 1989). The increase in extracellular glutamate, however, is rapidly reversed in vulnerable areas following recirculation (Benveniste et al., 1984; Mitani and Kataoka, 1991) and does not fully explain the mechanisms of selective vulnerability.

Glycine, an inhibitory amino acid, has recently been shown to enhance the excitatory response of glutamate at the *N*-methyl-D-aspartate (NMDA) receptor (Johanson and Ascher, 1987). This receptor has an anatomical distribution distinct from that of strychnine-sensitive glycine receptors, but similar to that of the NMDA receptors (Monaghan and Cotman, 1985; Bristow et al., 1986). Therefore, the action of glycine is thought to be mediated by strychnine-insensitive binding sites with an anatomical distribution identical to that for the NMDA receptor. Interestingly, results of recent studies suggested that glycine receptor antagonists can reduce ischemic brain damage (Patel et al., 1989) and prevent neuronal cytotoxicity mediated by NMDA (Patel et al., 1990). These observations suggest that glycine plays a central role in the pathogenesis of ischemic neuronal damage. In the present study, therefore, we focused on

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glycine as a neurotransmitter, and analyzed the post-ischemic alterations of glycine binding sites in selectively vulnerable regions using in vitro receptor autoradiography in the gerbil brain. We also visualized MAP2 (microtubule-associated protein 2) immunohistochemically as a sensitive marker of ischemic neuronal damage.

2. Materials and methods

2.1. Ischemic insult

Male adult Mongolian gerbils weighing 60–80 g were anesthetized with 2% halothane in a mixture of 30% oxygen and 70% nitrous oxide. Bilateral common carotid arteries were exposed and the carotid arteries were occluded with aneurysm clips for 10 min, and then the animals were allowed to survive for 1, 5, 24 and 48 h and 7 days after ischemia. Sham-operated animals were treated in the same manner, except for the clipping of the bilateral carotid arteries. Body temperature was maintained at 37–38° C using a heating pad with a thermostat throughout the experiment.

2.2. Receptor autoradiography

The animals were decapitated at different reperfusion times as indicated above, and the brains were removed quickly, frozen in powdered dry-ice and stored at −80° C until assay. 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 used for histopathology. Autoradiographic localization of glycine binding sites in the brain was performed according to the method of Miyoshi et al. (1990) with minor modifications. Briefly, the sections were preincubated with 0.04% Triton X-100 for 10 min at 2° C to allow dissociation of endogenous inhibitors. After washing, the sections were incubated with 80 nM [^{3}H]glycine (New England Nuclear; 38.8 Ci/mmol) in 50 mM Tris acetate buffer, pH 7.4, for 15 min at 2° C. Then the sections were washed 3 times in ice-cold fresh buffer for 10 s. Non-specific binding was determined using 1 mM glycine (Sigma).

The sections were dried under a cold air stream and were exposed to Hyperfilm-^{3}H (Amersham) for 4 weeks in X-ray cassettes with a set of ^{3}H microscales (Amersham). The optical density of the brain regions was measured with a computer-assisted image analyzer, without the operator knowing the experimental protocol, as described previously (Araki et al., 1992a, Araki et al., 1993a). The relationship between optical density and radioactivity was obtained with reference to the ^{3}H microscales co-exposed with the tissue sections. Binding assays were performed in duplicate. Values were expressed as the means ± S.D. Statistical

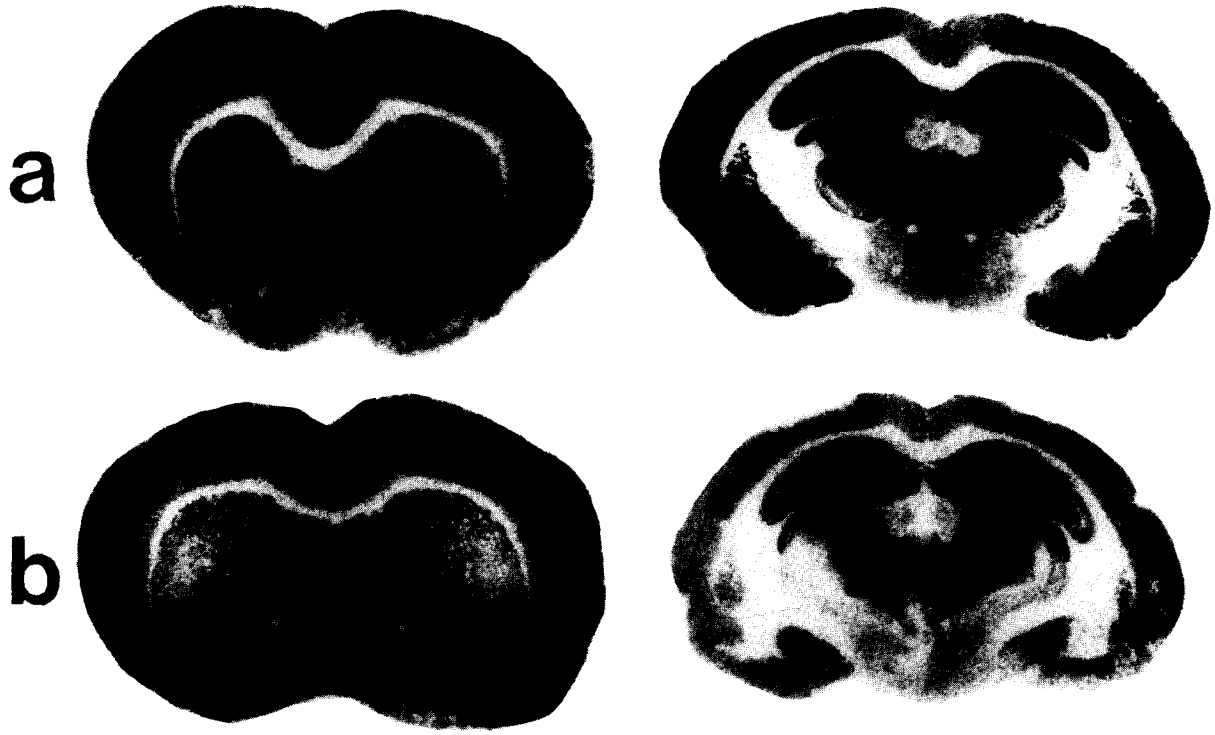

Fig. 1. Autoradiograms of [^{3}H]glycine binding in the gerbil brain following transient cerebral ischemia. (a) Sham-operated. (b) 7 days after ischemia. Right column: striatum. Left column: hippocampus. [^{3}H]Glycine binding was evident in the neocortex, striatum, hippocampus and thalamus in the sham-operated animals. In contrast, marked reduction was noted in these areas 7 days after ischemia.

comparisons were made using an analysis of variance (ANOVA) followed by Dunnett's multiple range test. Each group contained five to eight gerbils.

2.3. MAP2 immunohistochemistry

The animals were anesthetized with sodium pentobarbital at different reperfusion times as indicated above, and the brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) following a heparinized saline flush. The brains were removed 1 h after perfusion fixation and postfixed for 4 h in the same fixative. The brains were immersed in a graded strength of sucrose solutions (10 and 20%) in phosphate buffer over 2 days at 4° C and then frozen in powdered dry-ice. Frozen coronal sections 20 μm in thickness were prepared on a cryostat at −20° C and thaw-mounted onto gelatin-coated slides. The frozen sections were stained with Cresyl violet, and the sections were examined with a light microscope. For immunohistochemical staining, a monoclonal anti-MAP2 antibody (Amersham) and a Vectastain elite ABC kit (Vector Lab., Burlingame, CA) were used as described previously (Araki et al., 1994). The sections were dried and washed for 5 min in 0.01 M phosphate buffered saline (PBS, pH 7.4), followed by 30 min of preincubation with 10% normal horse serum. The brain sections were then incubated with anti-MAP2 antibody (1:500) overnight at 4° C. After 10-min rinse in changes of PBS, the sections were incubated with biotinylated second antibody for 2 h and then with a avidin-biotin peroxidase complex for 30 min at room temperature. Immunoreactions were visualized using 0.05% diaminobenzidine and 0.02% hydrogen peroxide in 0.05% Tris-HCl buffer (pH 7.6). Each group contained five gerbils.

3. Results

3.1. Receptor autoradiography

Representative autoradiographs of [^{3}H]glycine binding sites are shown in Fig. 1. Post-ischemic changes in [^{3}H]glycine binding are summarized in Table 1. In sham-operated gerbils, [^{3}H]glycine binding was greatest in the hippocampus. The neocortex, striatum and thalamus also had a relatively high density of [^{3}H]glycine binding. This finding was consistent with a previous report (Bristow et al., 1986).

In gerbils subjected to ischemia, [^{3}H]glycine binding significantly decreased in the hippocampus and thalamus throughout the recirculation periods. Marked reduction in these areas was observed 7 days after ischemia. On the other hand, the striatum and frontal cortex showed no significant alteration in [^{3}H]glycine binding 1 h after ischemia. Thereafter, [^{3}H]glycine binding was significantly reduced in these regions from 5 h after ischemia. Seven days after ischemia, a marked reduction in [^{3}H]glycine binding was seen in the frontal cortex and striatum.

3.2. MAP2 immunohistochemistry

Representative microphotographs of MAP2 immunostaining in the brain are shown in Fig. 2. In sham-operated gerbils, MAP2-immunoreactive neurons were present in the brain. Reaction products were

Table 1
Post-ischemic alteration in [^{3}H]glycine binding in the gerbil brain

	Sham-operated	Recirculation time				
		1 h	5 h	24 h	48 h	7 days
Frontal cortex	140 ± 24	147 ± 9	99 ± 38 [b]	89 ± 5 [b]	81 ± 10 [b]	57 ± 12 [b]
Striatum						
Lateral	107 ± 15	111 ± 8	80 ± 36 [a]	67 ± 10 [b]	55 ± 9 [b]	41 ± 8 [b]
Medial	129 ± 21	141 ± 17	98 ± 37	90 ± 14 [a]	80 ± 15 [b]	58 ± 14 [b]
Hippocampus						
CA1 sector						
Stratum oriens	416 ± 35	335 ± 53 [a]	335 ± 50 [a]	333 ± 52 [a]	338 ± 62 [a]	111 ± 22 [b]
Stratum radiatum	461 ± 45	374 ± 56 [a]	367 ± 51 [b]	378 ± 46 [a]	384 ± 51 [a]	115 ± 24 [b]
Stratum lacunosum-moleculare	339 ± 39	277 ± 34 [b]	285 ± 34 [a]	281 ± 42 [a]	273 ± 14 [b]	99 ± 23 [b]
CA3 sector	199 ± 26	152 ± 17 [b]	148 ± 24 [b]	154 ± 3[illegible] [b]	135 ± 8 [b]	68 ± 14 [b]
Dentate gyrus	330 ± 43	250 ± 32 [b]	254 ± 36 [b]	244 ± 37 [b]	225 ± 27 [b]	117 ± 45 [b]
Thalamus	149 ± 16	120 ± 10 [b]	124 ± 15 [b]	114 ± 15 [b]	110 ± 9 [b]	78 ± 12 [b]

Optical density was converted to fmol/mg tissue using ^{3}H microscales. Values are expressed as means ± S.D. [a] $P < 0.05$, [b] $P < 0.01$ vs. sham-operated group (Dunnett's multiple range test). Striatum (Lateral): the dorsolateral part of striatum. Striatum (Medial): the ventromedial part of striatum. $n = 5–8$ animals.

visible uniformly in the perikarya and dendrites. Negative control sections incubated without primary antibody showed no immunoreactivity in the brain. In animals subjected to ischemia, MAP2 immunoreactivity in the striatum was unchanged up to 5 h after ischemia, but a loss of MAP2 immunoreactivity was seen in the striatal neurons from 24 h after ischemia. Seven days after ischemia, a severe loss of MAP2-immunoreactive neurons was seen in this region. In contrast, conspicuous MAP2 immunostaining was found in the hippocampus up to 48 h after ischemia. Thereafter, marked loss of MAP2-immunoreactive neurons was found in the hippocampal CA1 and CA3 sectors 7 days after ischemia. The immunoreactivity also decreased in the dentate hilar neurons. Furthermore, MAP2 immunoreactivity increased markedly in the molecular layer of dentate gyrus.

3.3. Histopathology

Representative photographs with Cresyl violet staining in the brain are shown in Fig. 2. Sham-operated gerbils showed no neuronal damage throughout the brain. Gerbils subjected to ischemia revealed no neuronal damage in the hippocampus, neocortex and thalamus up to 48 h after ischemia. In contrast, the striatum showed conspicuous neuronal damage from 24 h after ischemia. Seven days after ischemia, severe neuronal damage was noted in the selectively vulnerable areas. The most frequently affected areas were the hippocampal CA1 sector and striatum, followed by the hippocampal CA3 sector and the third and fifth layers of the frontal cortex. In the thalamus, mild to moderate damage was found. However, no neuronal damage was seen in the dentate gyrus.

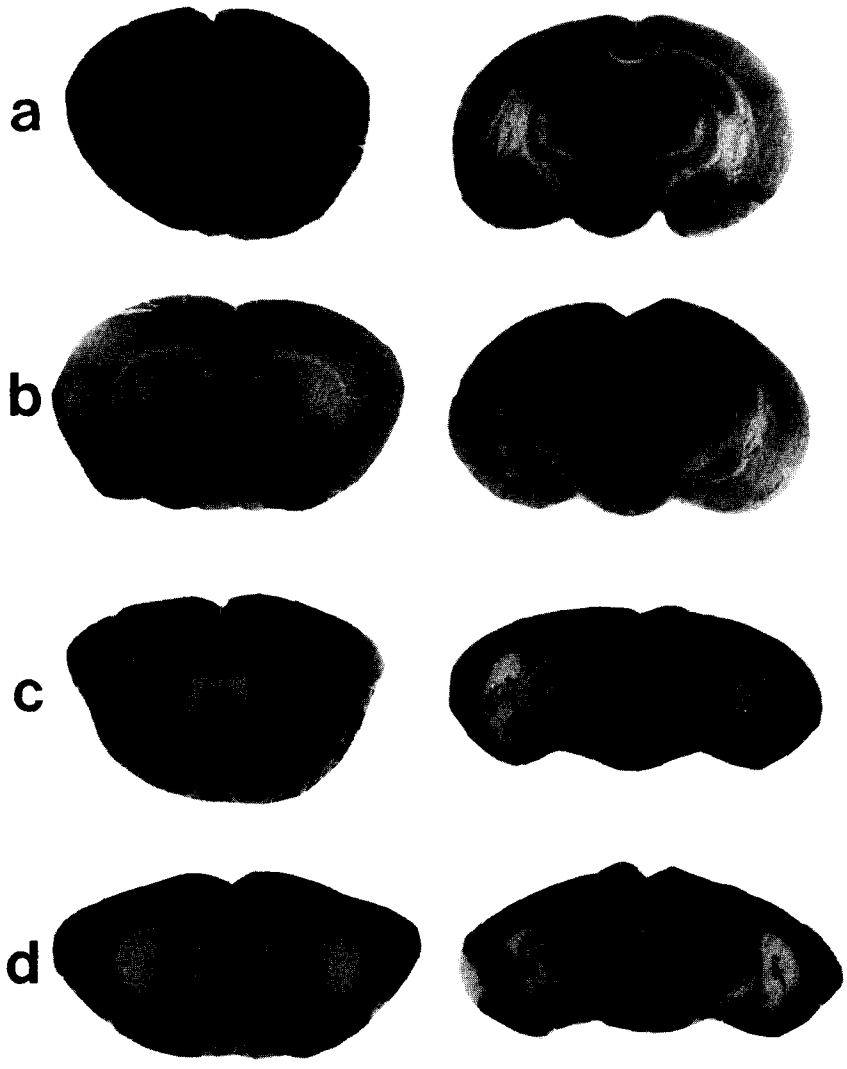

Fig. 2. Representative photographs with Cresyl violet staining (a,b) and MAP2 immunostaining (c,d) in the gerbil brain following transient cerebral ischemia. (a,c) Sham-operated. (b,d) 7 days after ischemia. Sham-operated gerbils showed no conspicuous neuronal damage throughout the brain (a,c). Seven days after ischemia, neuronal damage was found in the frontal cortex, striatum, hippocampal CA1 and CA3 sectors, and thalamus (b). Marked damage in MAP2-immunoreactive neurons was also seen in these areas after 7 days (d).

4. Discussion

The present study provided evidence of post-ischemic reductions of [^{3}H]glycine binding in selectively vulnerable regions. The hippocampus and thalamus showed a significant reduction in [^{3}H]glycine binding from as early as 1 h after ischemia, whereas the striatum and frontal cortex exhibited a significant decline from 5 or 24 h after recirculation. Thus, post-ischemic changes in [^{3}H]glycine binding were rapid in the hippocampus and thalamus, and were relatively delayed in the striatum and frontal cortex. Thereafter, a severe reduction in [^{3}H]glycine binding was observed in the frontal cortex, striatum and hippocampal CA1 and CA3 sectors, where neuronal damage was seen 7 days after ischemia. Interestingly, histopathology of the intact dentate gyrus also revealed a significant decline in [^{3}H]glycine binding. The results suggest that the alteration of [^{3}H]glycine binding precedes ischemic neuronal damage, and this change may reflect the down-regulation of glycine receptors.

Massive release of various neurotransmitters such as glutamate, aspartate, dopamine and γ-aminobutyric acid (GABA) during and/or after cerebral ischemia leads to neuronal injury (Erecinska et al., 1984, Globus et al., 1988). Among them, glutamate is thought to be a major contributor to neuronal cell death in ischemia. Several studies have previously demonstrated that activation of the NMDA receptor is involved in excitotoxic ischemic neuronal damage (Rothman and Olney, 1987; Choi and Rothman, 1990). The high density of NMDA binding sites in the hippocampal CA1 sector can possibly explain the selective vulnerability of this region. However, the dentate gyrus, which is most resistant to ischemia, is also very rich in NMDA receptors, whereas the hilus, which is most vulnerable to ischemia, has only a few NMDA binding sites (Monaghan and Cotman, 1985). We recently reported that NMDA binding was unchanged throughout the early recirculation periods and was relatively resistant to degenerative processes after cerebral ischemia in gerbils (Araki et al., 1993b). These observations suggest that the distribution of the NMDA receptor cannot always explain the pattern of selective vulnerability after ischemia.

Recently, accumulated evidence demonstrated a role for glycine in the pathogenesis of cerebral ischemia. These observations were mainly based on the demonstration that glycine can potentiate the effect of glutamate at the NMDA receptors (Johanson and Ascher, 1987), and glycine antagonists, such as HA-966 (1-hydroxy-3-aminopyrrolidone-2) and 7-CK (7-chlorokyrurenic acid), can reduce ischemic brain damage in gerbils (Patel et al., 1990). Furthermore, recent studies suggest that the glycine concentration increases markedly during and after ischemia in selectively vulnerable regions (Globus et al., 1991; Baker et al., 1991). Therefore, the sustained high levels of extracellular glycine may play a major role in the post-ischemic excitotoxic effect of glutamate, even when extracellular glutamate concentration is rapidly reversed after cerebral ischemia. However, sequential changes in [^{3}H]glycine binding sites after ischemia are not fully understood for selectively vulnerable areas.

The present study showed that transient cerebral ischemia caused a severe reduction in [^{3}H]glycine binding in selectively vulnerable areas early in the recirculation period before neuronal loss. This post-ischemic alteration of [^{3}H]glycine binding showed a significant reduction in most of the selectively vulnerable areas throughout the experiment. This finding is partly consistent with the findings of Jorgensen et al. (1989) who demonstrated a significant reduction in [^{3}H]inositol 1,4,5-trisphosphate (IP_3) binding to the neocortex and hippocampus from rats exposed to cerebral ischemia. For this reason, they also suggested that the lasting decline of [^{3}H]IP_3 binding could be due to down-regulation of the intracellular receptor following excess stimulation with IP_3 or calcium during and after ischemia. We also reported that transient cerebral ischemia in gerbils can cause a severe reduction in [^{3}H]IP_3 binding in most of the selectively vulnerable areas at 1 h to 1 month of recirculation. Therefore, our present results suggest that the lasting decline in [^{3}H]glycine binding to the selectively vulnerable areas may reflect the down-regulation of glycine receptors following excess stimulation with glycine during and after ischemia. This down-regulation of glycine binding sites may be a defense against ischemic neuronal damage. Thus, our finding is of particular interest in relation to the mechanisms of ischemic neuronal damage.

On the other hand, marked reduction in [^{3}H]glycine binding was also seen in the dentate gyrus which was most resistant to ischemia throughout the recirculation periods. Furthermore, our immunohistochemical study showed that MAP2 immunoreactivity increased markedly in the molecular layer of dentate gyrus 7 days after ischemia. The reason for these phenomena is presently unclear. However, we observed previously that transient cerebral ischemia in gerbils can cause a significant increase in [^{3}H]phorbol 12,13-dibutyrate (PDB) and a significant reduction in [^{3}H]IP_3 binding in the dentate gyrus 7 days after recirculation (Araki et al., 1992b). Therefore, our finding in the present study suggests post-ischemic dysfunction of intracellular signal transduction and neurotransmission in the dentate gyrus. Further detailed investigation is required to clarify this finding.

In conclusion, we observed that transient cerebral ischemia causes reductions in [^{3}H]glycine binding throughout the brain. The alteration, in particular, precedes ischemic neuronal damage in the selectively vulnerable areas. This finding suggests that glycine may

be involved in the pathogenesis of post-ischemic neurodegeneration in selectively vulnerable areas.

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