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Increase of Synapsin I, Phosphosynapsin (ser-9), and GAP-43 in the Rat Hippocampus after Middle Cerebral Artery Occlusion

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The loss of neurons and synaptic contacts following cerebral ischemia may lead to a synaptic plastic modification, which may contribute to the functional recovery after a brain lesion. Using synapsin I and GAP-43 as markers, we investigated the neuronal cell death and the synaptic plastic modification in the rat hippocampus of a middle cerebral artery occlusion (MCAO) model. Cresyl violet staining revealed that neuronal cell damage occurred after 2 h of MCAO, which progressed during reperfusion for 2 weeks. The immunoreactivity of synapsin I and GAP-43 was increased in the stratum lucidum in the CA3 subfield as well as in the inner and outer molecular layers of dentate gyrus in the hippocampus at reperfusion for 2 weeks. The immunoreactivity of phosphosynapsin was increased in the stratum lucidum in the CA3 subfield during reperfusion for 1 week. Our data suggest that the increase in the synapsin I and GAP-43 immunoreactivity probably mediates either the functional adaptation of the neurons through reactive synaptogenesis from the pre-existing presynaptic nerve terminals or the structural remodeling of their axonal connections in the areas with ischemic loss of target cells. Furthermore, phosphosynapsin may play some role in the synaptic plastic adaptations before or during reactive synaptogenesis after the MCAO.

Key Words: Hippocampus, MCAO, Synapsin I, Phosphosynapsin, Growth associated protein-43 (GAP-43), Reactive synaptogenesis

INTRODUCTION

Cerebral ischemia is caused by an alteration in the energy metabolism, calcium influx, and ion homeostasis, which give rise to neuronal cell death and the loss of brain function (Schmidt-Kastner et al, 1991; Kitagawa et al, 2001; Butler et al, 2002). The middle cerebral artery occlusion (MCAO) technique has been used as an animal model of cerebral ischemia, and it closely resembles a clinical stroke in humans due to the focal type of the ischemic lesion (Huh et al, 1998; Chopp et al, 1999; Sopala et al, 2000). After the MCAO, direct ischemic damage occurs in the corpus striatum and the cerebral cortex. However, the hippocampus is also susceptible to ischemia and usually delayed damage occurs. The loss of neurons and synaptic contacts may lead to plastic modifications, including synaptic reorganization, which can contribute to the functional recovery after a brain lesion (Hoff, 1986; Dailey et al, 1994). In order to regulate the plastic synaptic modifications, the synaptic vesicular proteins play an important role.

Among the synaptic proteins, synapsin I is specifically associated with the cytoplasmic surface of the synaptic vesicle membrane and regulates the release of neurotransmitters, neurite outgrowth, and new synapse formation during normal development, following various brain lesions such as cerebral ischemia in adults (Dailey et al, 1994; Marti et al, 1999; Bernabeu et al, 2000; Kitagawa et al, 2001). Synapsin I is a phosphoprotein, and the phosphorylation of synapsin is a modulator of the interactions between the actin filaments and the synaptic vesicles (Greengard et al, 1993). It is phosphorylated by the Ca^{2+}-calmodulin dependent protein kinase I or II (CaM kinase I or II), the protein kinase A (PKA), and the mitogen-activated protein kinase (Menegon et al, 2000; Bolay et al, 2002). The growth associated protein-43 (GAP-43) is also associated with neurite outgrowth and plasticity. It is expressed at high levels during neuronal development and regeneration (Schmidt-Kastner et al, 1997; Li et al, 1998; Huang et al, 1999).

Although changes in the synaptic plasticity markers are expected to occur at the later stages of ischemia, it is unclear whether or not these markers may be involved in reactive synaptogenesis after transient focal ischemia. In this study, the alterations in the syanpsin I, phosphosynapsin, and GAP-43 levels were examined in the hippocampus of the MCAO model using an immunohistochemical technique.

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ABBREVIATIONS: MCAO, middle cerebral artery occlusion; GAP-43, Growth associated protein-43; CaMK I, Ca^{2+}-calmodulin dependent kinase I; CaMK II, Ca^{2+}-calmodulin dependent kinase II; PKA, protein kinase A.

METHODS

Animals

Sprague-Dawly male rats, weighing 200~250 g, were used. The animal care was approved by the Experimental Animals Committee of Catholic University.

Middle cerebral artery occlusion (MCAO)

Focal brain ischemia was induced by a MCAO using the intraluminal suture method (Huh et al, 1998), which was modified from that originally described by Koizumi et al (1986).

The animals were anesthetized with 4.0% halothane in a mixture of 70% nitrous oxide and a balance of oxygen. The surgical procedure to occlude the MCA consisted of the insertion of a 4 cm length of 3-0 nylon monofilament, precoated with poly-L-lysine solution (0.1% w/v), via the right external carotid artery into the right internal carotid artery to block the origin of the MCA. Two hours after the MCA occlusion, the rats were tested on a standardized neurobehavioral battery by a blinded investigator to confirm the presence of a neurological deficit. The rats were then re-anesthetized, and the intraluminal suture was carefully removed.

Histology and immunohistochemistry

At different times after focal ischemia (0, 1 week, and 2 weeks), the rats were anesthetized with diethyl ether and perfused by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), via the ascending aorta after the brain circulation was washed out with heparinized saline. The brains were carefully removed and post-fixed at 4°C overnight in fresh 4% paraformaldehyde. The next day, the brains were blocked (bregma −2.8~−4.3 mm), dehydrated and embedded in paraffin, and then coronally cut into 5 μm thick sections after focal ischemia. Coronal sections were analyzed by 1% cresyl violet staining or immunohistochemistry. For immunohistochemistry, the coronal sections were incubated in the presence of mouse monoclonal anti-synapsin I antibody (1 : 100 dilution; oncogene, La Jolla, CA, USA), mouse monoclonal anti-phosphosynapsin antibody (1 : 100, cell signaling, Beverly, MA, USA), or mouse monoclonal anti-GAP43 antibody (1 : 200, chemicon, Temecula, CA, USA) overnight at 4°C. The primary antibody was detected using the avidin-biotin-peroxidase method (Vectastatin Elite ABC kit, Vector Laboratories, CA, USA). The stained sections were dehydrated and mounted.

RESULTS

Neuronal damage during reperfusion after 2 h of MCAO

Cresyl violet staining was used to ascertain the reliability of the ischemic model used in this study and to assess the extent as well as the time course of the ischemic injury. The neuronal cells in the pyramidal cell layer of the hippocampus in the contralateral side were found to maintain a normal morphology at 2 h after MCAO (Fig. 1A and E). However, the neuronal cells in the both CA1 and CA3 subfields of the ipsilateral side began to show damage after 2 h of the MCAO (Fig. 1B and F). After 2 weeks, most of the neuronal cells disappeared in the both the CA1 and CA3 subfields of the ipsilateral side (Fig. 1D and H). There was no obvious difference between the contralateral and the ipsilateral sides of dentate gyrus (data not shown).

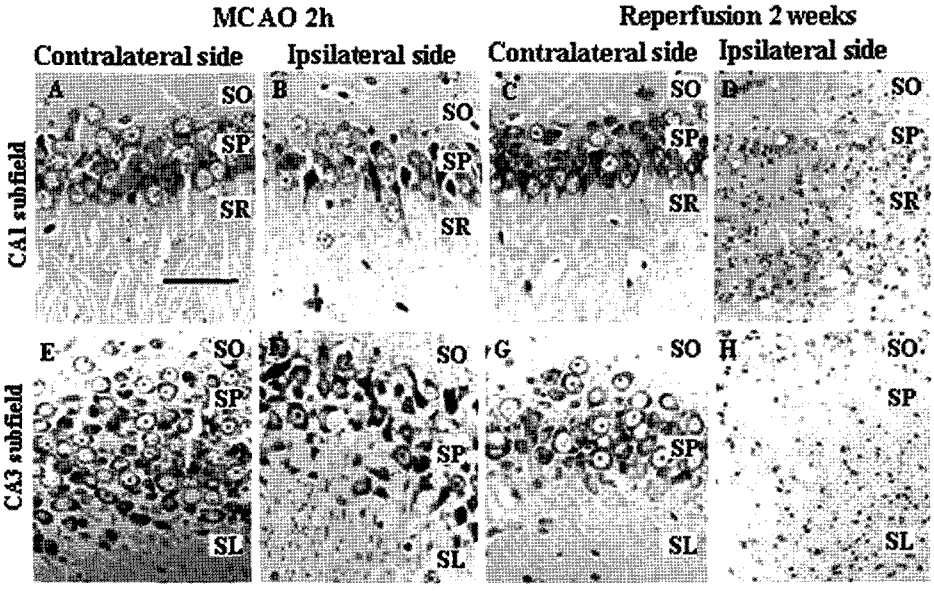

Fig. 1. Photomicrographs of cresyl violet staining in the CA1 and CA3 subfields of hippocampus following 2 h of MCA occlusion (A, B, E, F) and reperfusion for 2 weeks (C, D, G, H). (SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SL, stratum lucidum) scale bar=100 μm.

Changes of the immunoreactivity of Synapsin I, GAP-43 and Phosphosynapsin

We investigated whether synapsin I and GAP-43, which are synaptic plasticity markers, were altered during reperfusion after MCAO. The immunoreactivity of synapsin I and GAP-43 was altered during reperfusion for 2 weeks after MCAO. As shown in Fig. 2A, C, E and G, they stained in a shape of a puncta along the neuronal processes of the contralateral side, but they were absent in the pyramidal cell layer in the hippocampus. During early reperfusion, there were no differences in synapsin I and GAP-43 immunoreactivity between the contralateral side and the ipsilateral side (data not shown). However, synapsin I and GAP-43 immunoreactivity increased strongly in the stratum radiatum of the CA1 subfield and stratum lucidum of the CA3 subfield in the hippocampus of the ipsilateral side (Fig. 2B, D, F and H) at reperfusion for 2 weeks. In the case of dentate gyrus, the molecular layers displayed a trilaminar immunostaining pattern in the contralateral side. The immunoreactivity of synapsin I and GAP-43 was also increased in the inner and outer zones of the molecular layer of the ipsilateral side on reperfusion for 2 weeks (Fig. 2B and F).

The immunoreactivity of phosphosynapsin (ser-9) was increased in the stratum lucidum of the CA3 area and was decreased in the hilus of the dentate gyrus on reperfusion for 1 week (Fig. 3). It was then normalized after reperfusion for 2 weeks (data not shown).

DISCUSSION

In the present study, transient focal ischemia (MCAO) was shown to produce delayed neuronal damage in the hippocampal pyramidal cell layer at the later reperfusion stage. Usually, the cortical and striatal area are known as the MCA territory, and the hippocampus is considered to be remote or in the non ischemic area. However, secondary ischemic neuronal damage, which takes a long time to develop, can be generated outside of the MCA territory such as the hippocampus in the intraluminal MCAO model, because the hippocampus receives an entorhinal cortical input (Sopala et al, 2000).

Although it is widely accepted that the damage to the nerve cells in the adult brain is irreversible, there is increasing evidence to indicate the regeneration of nerve cells, the re-establishment of the cell-to-cell connections, or reactive synaptogenesis in an injured adult brain following cerebral ischemia (Dailey et al, 1994; Chopp et al, 1999; Kitagawa et al, 2001). Synapsin I is a vesicular protein, which regulates neurotransmitter release, neurite development, and the maturation of the synaptic contacts during the normal development and after various lesions in the adult brain (Dailey et al, 1994; Marti et al, 1999; Bernabeu & Sharp, 2000). GAP-43 is a growth-associated, nerve tissue-specific protein, and is synthesized at high levels during axonal growth in neuronal development and axonal regrowth during regeneration in the peripheral and central nervous systems (Li et al, 1998; Stroemer et al, 1998; Gregersen et al, 2001). Therefore, they have been considered to be a marker of axonal sprouting.

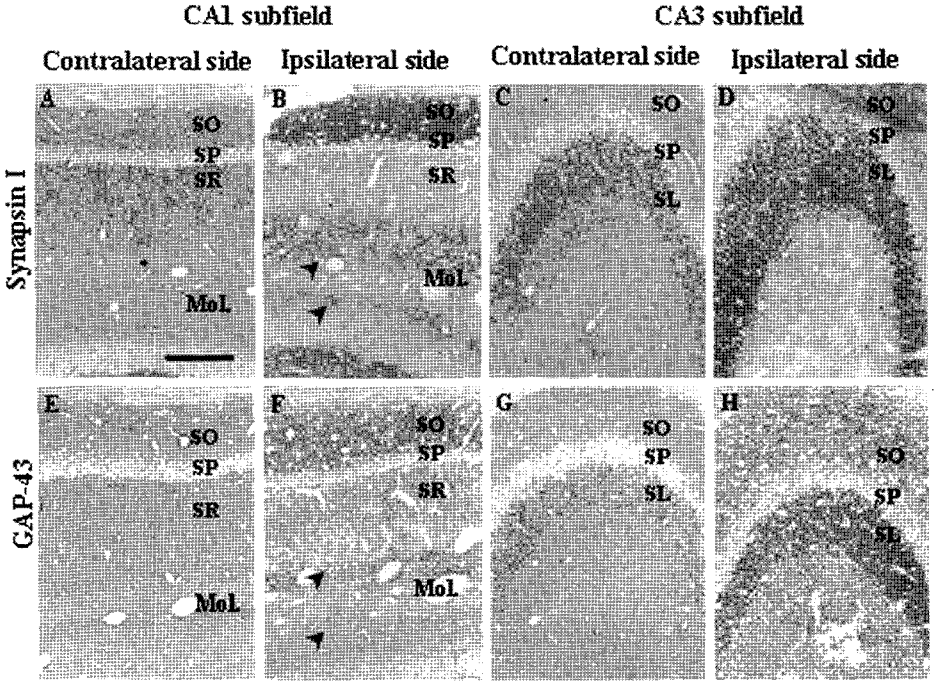

Fig. 2. Immunoreactivity of synapsin I and GAP-43 in the CA1 subfield (A,B, E, F) and the CA3 subfield (C, D, G, H) on reperfusion for 2 weeks. Arrow heads show increased immunoreactivity of synapsin I and GAP-43 in inner and outer molecular layers. (SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SL, stratum lucidum; Mol, molecular layer of dentate gyrus) scale bar=200 μm.

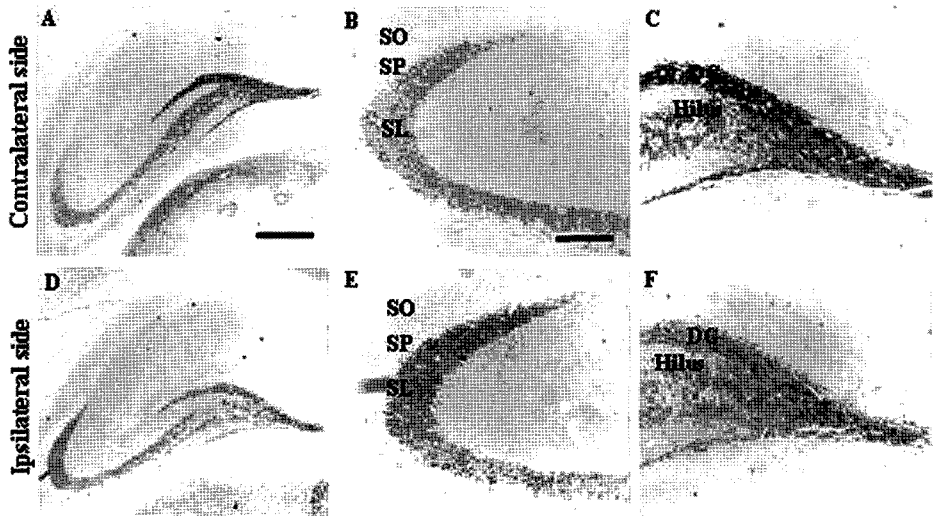

Fig. 3. Immunoreactivity of phosphosynapsin in the contralateral side (A, B, C) and ipsilateral side (D, E, F) on reperfusion for 1 week. (SO, stratum oriens; SP, stratum pyramidale; SL, stratum lucidum) Scale bar=200 μm

There are some reports showing the induction of synapsin I immunoreactivity in the stratum lucidum of the hippocampus after global ischemia in the mongolian gerbil (Marti et al, 1999; Bernabeu & Sharp, 2000). The authors concluded that synapsin I participates in the reactive response of the granule cells of dentate gyrus to the ischemic insult. In addition, the induction of synapsin I expression may be one of the molecular mechanisms by which the plastic adaptations in the hippocampal formation are regulated after ischemia. In the case of GAP-43, it was induced in the penumbra or peri-infarct areas of the rats after MCAO (Li et al, 1998; Chopp et al, 1999; Gregersen et al, 2001) and in the hippocampus after a global ischemia of gerbils (Tagaya et al, 1995) or rats (Schmidt-Kastner et al, 1997). The highest level of GAP-43 was detected during development (Li et al, 1998; Chopp et al, 1999). However, axonal damage after the lesions could stimulate the GAP-43 expression level even in adults. Because neuronal damage is a perquisite for the delayed induction of synapsin I or GAP-43 immunoreactivity, ischemic neurons might have the potential for compensatory sprouting, accompanied by the increased expression of the synaptic plasticity markers, such as synapsin I or GAP-43. Our data indicate that there might be a reactive synaptogenesis when the surviving neurons reorganize their axonal connections in the hippocampus during reperfusion after MCAO. However, little is known regarding the changes in the synapsin I and GAP-43 expression levels in the hippocampus after MCAO.

The increase in the phosphosynapsin immunoreactivity in this study indicates the increase in synapsin phosphorylation by PKA or CaMK I (Menegon et al, 2000; Bolay et al, 2002). Although phosphorylation by CaMK II (phosphosite 2 and 3) is the key step, we did not examine phosphosynapsin at phosphosite 2 and 3 in the present study, because it is not commercially available. Therefore, we used the phoshosynapsin antibody for the phosphosite 1 (ser-9), which also involves the dissociation of the synaptic vesicles. Bolay et al (2002) reported that they could not detect any immunoreactivity with the anti-phosphosynapsin antibody at the phosphosites 2 and 3, possibly because CaMK II mediated phosphorylation is not constitutively present, but emerges transiently after stimulation. In contrary to our data, they also reported a decrease of phosphosynapsin at site I in the frontal penumbral cortex at 1~72 hours of reperfusion after the MCAO. This inconsistency might have been due to the difference in the ischemic time (1 hr vs 2hr), reperfusion time (72 hr vs 1 or 2 wks), the target tissue (frontal cortex vs hippocampus), or the antibodies used. Because the conditions promoting intracellular calcium may induce synapsin phosphorylation by the calcium dependent protein kinases, including PKA or CaMK I, an increase of phosphosynapsin (ser-9) in the rat hippocampus is expected after a MCAO.

In conclusion, delayed neuronal damage was evident in the hippocampus after the MCAO. The increase of the synapsin I and GAP-43 immunoreactivity signifies most likely either the functional adaptation of the neurons through reactive synaptogenesis from the pre-existing presynaptic nerve terminals or the structural remodeling of their axonal connections in the areas with ischemic loss of target cells. In addition, phosphosynapsin may play some role in the synaptic plastic adaptations before or during reactive synaptogenesis after the MCAO.

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REFERENCES

Bernabeu R, Sharp FR. NMDA and AMPA/Kainate glutamate receptors modulate dentate neurogenesis and CA3 synapsin I in

normal and ischemic hippocampus. *J Cerebral Blood Flow Metab* 20: 1669–1680, 2000

Bolay H, Gürsoy-Özdemir Y, Sara Y, Onur R, Can A, Dalkara T. Persistant defect in transmitter release and synapsin phosphorylation in cerebral cortex after transient moderate ischemic injury. *Stroke* 33: 1369–1375, 2002

Butler T, Kassed C, Sanberg PR, Willing AE, Pennypacker KR. Neurodegeneration in the rat hippocampus and striatum after middle cerebral artery occlusion. *Brain Res* 929: 252–260, 2002

Chopp M, Li Y, Jiang N. Increase in apoptosis and concomitant reduction of ischemic lesion volume and evidence for synaptogenesis after transient focal cerebral ischemia in rat treated with staurosporine. *Brain Res* 828:197–201, 1999

Dailey M, Buchanan J, Bergles DE, Smith SJ. Mossy fiber growth and synaptogenesis in rat hippocampal slices in vitro. *J Neurosci* 14: 1060–1078, 1994

Fujie W, Kirino T, Tomukai N, Iwasawa T, Tamura A. Progressive shrinkage of the thalamus following middle cerebral artery occlusion in rats. *Stroke* 21: 1485–1488, 1990

Greengard P, Valtorta F, Czernik AJ, Benfenati F. Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* 259: 780–785, 1993

Gregersen R, Christensen T, Lehrmann E, Diemer NH, Finsen B. Focal cerebral ischemia induces increased myelin basic protein and growth associated protein-43 gene transduction in peri-infarct areas in the rat brain. *Exp Brain Res* 138: 384–392, 2001

Hoff SF. Lesion-induced transneuronal plasticity in the adult rat hippocampus. *Neuroscience* 19: 1227–1232, 1986

Huang KP, Huang FL, Chen HC. Hypoxia/ischemia induces dephosphorylation of rat brain neuromodulin/GAP-43 in vivo. *J Neurochem* 72: 1294–1306, 1999

Huh PW, Belayev L, Zhao W, Busto R, Saul I, Ginsberg MD. The effect of high dose albumin therapy on local cerebral perfusion after transient focal cerebral ischemia in rats. *Brain Res* 804: 105–113, 1998

Kitagawa K, Matsumoto M, Hori M. Protective and regenerative response endogenously induced in the ischemic brain. *Can J Pharmacol* 79: 262–265, 2001

Koizumi J, Yoshida Y, Nazakawa T, Oneda G. Experimental studies of ischemic brain edema, I: a new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. *Jpn J Stroke* 8: 1–8, 1986

Li Y, Jiang N, Powers C, Chopp M. Neuronal damage and plasticity identified by microtubule-associated protein 2, growth-associated protein 43, and cyclin D1 immunoreactivity after focal cerebral ischemia in rats. *Stroke* 29: 1972–1981, 1998

Marti E, Ferrer I, Blasi J. Transient increase of synapsin I immunoreactivity in the mossy fiber layer of the hippocampus after transient forebrain ischemia in the Mongolian gerbil. *Brain Res* 824: 153–160, 1999

Menegon A, Dunlap DD, Castano F, Benfenati F, Czernik AJ, Greengard P, Valtorta F. Use of phosphosynapsin I-specific antibodies for image analysis of signal transduction in single nerve terminals. *J Cell Sci* 113: 3573–3582, 2000

Schmidt-Kastner R, Bedard A, Hakim A. Transient expression of GAP-43 within the hippocampus after global brain ischemia in rat. *Cell Tissue Res* 288: 225–238, 1997

Schmidt-Kastner R, Freund TF. Selective vulnerability of the hippocampus in brain ischemia. *Neuroscience* 40: 599–636, 1991

Sopala M, Frankiewicz T, Parsons C, Danysz W. Middle cerebral artery occlusion produces secondary, remote impairment in hippocampal plasticity of rats-involvement of N-methyl-D-aspartate receptors. *Neurosci Lett* 281: 143–146, 2000

Stroemer RP, Kent TA, Hulsebosch CE. Enhanced neocortical neural sprouting, synaptogenesis, and behavioral recovery with D-amphetamine therapy after neocortical infarction in rats. *Stroke* 29: 2381–2395, 1998

Tagaya M, Matsuyama T, Nakamura H, Hata R, Shimizu S, Kiyama H, Matsumoto M, Sugita M. Increased F1/GAP-43 mRNA accumulation in gerbil hippocampus after brain ischemia. *J Cereb Blood Flow Metab* 15: 1132–1136, 1995