Immunohistochemical expression of phospholipase C in global and focal ischemic encephalopathy in Gerbil : Relationship with morphological changes

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Phospholipase C(PLC) and related enzymes in signal transduction system are closely linked to cellular damage in ischemic encephalopathy. This study was undertaken to elucidate the time sequential changes of PLC isoenzymes(β and γ) in vulnerable areas of hippocampus in global ischemia and infarcted area in focal infarction. Mongolian gerbils were used because of their susceptibility to ischemic encephalopathy and divided into the following groups: the bilateral ischemia with various reperfusion periods group, unilateral progressive ischemia group, and focal ischemia group induced by infusion of iron particles through the femoral artery. The changes of PLC isoenzymes were observed immunohistochemically and matched with morphological changes. In the global ischemia with reperfusion group, the changes were most significant in hippocampus. Sequential changes of neurons such as red neurons at an early stage progressed to pyknotic neurons at a later stage were noted with typical delayed neuronal damage in the cornu ammonis (CA)1 subfield of hippocampus. Red neurons and pyknotic neurons as well as intracytoplasmic inclusion in 3 to 24 hours of reperfusion showed loss of PLC isoenzymes as well as tubulin. The changes of PLC expression were corresponding to the degeneration of neurons with no discernible time sequential changes in remaining neurons. In the unilateral hemispheric progressive ischemia group, ischemic damage was far more marked and extensive with no selective injury pattern according to time and location. At 1 day, there was diffuse vacuolization and necrosis of neuropil with a loss of neuron. Admixed surviving neurons and vacuolated neuropil showed increased reaction to anti-PLC antibodies, which could be either an evidence of protein synthesis responding to ischemic insult or an artifactual change. Focal ischemia group showed time sequential changes of blood vessels and white blood cells with necrosis of surrounding tissue. Degenerating hippocampal neurons in infarction also showed a strong positive reaction to anti-PLC antibody, which was most likely due to condensation of cytoplasm rather than increased synthesis. This study showed different changes of PLC expression in global ischemic encephalopathy with reperfusion, progressive ischemia, and focal infarction, which suggested different pathophysiologic mechanism between these conditions.

Key Words: Ischemic encephalopathy, Infarction, Phospholipase C

INTRODUCTION

Selective vulnerability of neurons in different areas of the central nervous system has been an object of great interest and experimental studies. Neurons in the hippocampus show a different pattern of reaction or degeneration according to the location as well as type of injury. The cornu ammonis(CA)1 subfield is especially well known for the selective vulnerability of its neurons to ischemic insult and its slowly progressing neuronal death after transient forebrain ischemia(delayed neuronal death)(Kirino, 1982 ; Pulsinelli et al., 1982 ; Kirino et al., 1984 ; Wieloch, 1985). Within the CA1 region, subiculum-CA1 and medial CA1 showed different immunohistochemical reaction, which suggested differences in cellular vulnerability between these subfields(Hatakeyama et al., 1988). The neurons in CA3 subfield are well known to show intraneuronal eosinophilic inclusion bodies during reperfusion after transient ischemia(Ito et al., 1975). The pathophysiologic mechanism of these different vulnerability or reactions of neurons remains unclear.

An elevated intracellular Ca^{2+} level has been considered as one of the important pathophysiologic factors in ischemic delayed neuronal damage. There are several mechanisms of intracellular Ca^{2+} influx during ischemia : through NMDA receptor-gated voltage-sensitive and agonist-operated calcium channels, 1,4,5-inositol triphosphate(IP_3)-mediated intracellular release of Ca^{2+} from the endoplasmic reticulum, reversed $\text{Na}^+/\text{Ca}^{2+}$ antiporter, and a nonspecific Ca^{2+} channel(Choi, 1988 ; Siesjö and Bengtsson, 1989 ; Choi, 1990). Phospholipase C(PLC) is an enzyme that catalyzes the phosphodiesteratic cleavage of phosphatidylinositol(PI). A total of 8 mammalian PLC isoenzymes have been described(PLC β 1-4, γ 1-2, δ 1-2) with molecular weight ranging from 85 to 150 Kda. The γ type enzymes are unique in that they contain SH2 and SH3 domains and are subject to activation by a number of protein tyrosine kinases which associate

with the SH2 domains and induce their activation by phosphorylation(Meisenhelder et al., 1989 ; Nishebe et al., 1989 ; Rana and Hokin, 1990). In contrast, activation of PLC β 1-4 is mediated by the G protein(Wange et al., 1991 ; Wu et al., 1992). PLC β is present in neurons in striatum, hippocampus, and dentate gyrus as well as in certain thalamic nuclei and PLC γ is ubiquitously present in all neurons of the central nervous system(Suh et al., 1988 ; Rhee et al., 1989). Several neurotransmitters including glutamate released from synaptosomes by ischemia activate PLC(Fisher and Agranoff, 1987). Activated PLC yields two intracellular second messengers, IP_3 and 1,2-diacylglycerol(DG), from phosphatidylinositol 4,5-bisphosphate(PIP_2), that control a variety of cellular functions by the ability of IP_3 to mobilize Ca^{2+} from intracellular stores and by DG to activate protein kinase C(PKC). In addition, the activation of PLC further influences the release of fatty acids by phospholipase A_2 (PLA_2) in subsequent course, which is the important step in ischemic damage of the cells(Prasad et al., 1991).

The purpose of this study was to see if there were any significant time sequential changes of PLC isoenzymes in vulnerable areas of hippocampus and ischemic areas under different experimental conditions. The time sequential morphological changes of neurons and expression of PLC isoenzymes(β and γ) of hippocampal neurons were examined in two models of experimental global ischemic encephalopathy induced by bilateral 5 min common carotid artery(CCA) ligation with reperfusion for various time and an unilateral ligation of CCA without reperfusion in Mongolian gerbils. The changes were also examined at various times in focal ischemia induced by infusion of iron powder through the femoral vein, which has a different proposed pathophysiologic mechanism for lesion. The changes of immunohistochemical reaction to anti-PLC β and γ antibodies were compared with the changes seen by anti-tubulin antibody as well as hematoxylin-eosin(H-E) and cresyl violet(CV) stain. Significance of various morphological changes and expression of PLC isoenzymes in global and focal cerebral ischemia were discussed with a review of previous reports.

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MATERIALS AND METHODS

Production of global ischemia and focal infarction of the brain

Mongolian gerbils (*Meriones unguiculatus*) of both sexes weighing 50~70 gm were used for the present study. Each gerbil was allowed free access to food and water before and after surgery. Under ketamine (10 mg/ml) anesthesia (intraperitoneal injection, 0.09 ml/kg), both CCAs of each gerbil were exposed through a midline incision in the neck and were occluded using miniature Mayfield aneurysm clips. For the evaluation of various postischemic periods, the clips were released after 5 minutes' ischemia and gerbils were followed for 0 or 30 minutes, 1, 2, 3, 6, or 12 hours, and 1, 2, or 7 days. Restoration of carotid blood flow was visually verified and the skin incision was sutured. At least one sham-operated gerbil (no arterial occlusion took place) as a control was included with each group of 3 to 7 occluded gerbils. For the investigation of progressive ischemia, the unilateral CCA was occluded for 3, 5, 10, 15, 30, or 60 minutes, and 1, 2, 3, or 7 days (2 gerbils each).

Focal ischemia was induced in 14 gerbils as follows. Under ketamine anesthesia, incision of scalp skin was made and a round magnet of 3 mm thickness and 7 mm diameter (surface magnetic power of 2,000~2,400 Gauss) was attached on right parietal bone by glue. The right femoral vein was exposed and iron particles (γ - Fe_2O_3 , $0.2 \times 0.02 \mu\text{m}$) mixed in saline solution were infused through a rubber catheter (0.67 mg/100 gm of body weight). Two gerbils were examined after 1, 2, or 6 hours and 1, 2, 3, or 7 days. The rectal temperature of the gerbils was maintained at 36°C using a heating pad and a heating lamp during the procedure.

Tissue preparation and immunohistochemical method

At the scheduled time, each gerbil was anesthetized and the brain was fixed with a transcardiac infusion of 4% paraformaldehyde following perfusion with isotonic saline to remove blood from the cerebral vasculature. The brains were removed and fixed in the same solution for a further 24 hours. Coronal sections of the supratentorial portion of each brain were taken and embedded in paraffin.

The immunohistochemical reaction was accom-

plished using the peroxidase-antiperoxidase method as described with some modification (Hsu and Raine, 1984). Briefly, each deparaffinized 5- μm coronal section was reacted with a primary antiserum (see below) for 60 min before reaction with the peroxidase-antiperoxidase complex by LSAB kit from DAKO (Santa Barbara, CA, USA). The peroxidase reaction was carried by incubation with link antibody and streptavidin for 20 min, respectively, and subsequently with AEC (3-aminoethyl 9-carbasol). The sections were counterstained with Meyer's hematoxylin to visualize cell nuclei. For comparison, adjacent sections were stained with H-E and CV as well as immunohistochemical study with anti-tubulin antibody. A coronal section was incubated with nonimmunized serum from the same species used to raise each primary antiserum. No control section showed a positive reaction. The antiserum for PLC β and γ from bovine brain, a generous gift from Prof. Pann-Ghill Suh at Pohang University of Science, was raised in a Balb/C mice and the culture soup was diluted to 1:100 and 1:200, respectively. The antiserum for tubulin from rat brain, raised in a mouse, was purchased from Zymed (San Francisco, CA) and diluted to 1:50.

RESULTS

Normal hippocampal neurons and common morphological changes of neurons in ischemic conditions

The hippocampal neurons of normal gerbil showed a difference in size and amount of Nissl substance according to the region. The neurons in the CA3 and CA4 region had the largest amount of cytoplasm and Nissl substance, which was well documented by CV stain. Anti-tubulin antibody showed strong positive reaction in nerve cell bodies and dendrites. Anti-PLC β antibody showed finely dispersed granules covering the nucleus and cytoplasm of neurons and diffuse background stain of neuropil. Anti-PLC γ antibody showed the strongest reaction at the margin of cytoplasm. Reactivity was similar in subfields of hippocampus.

The changes were mainly evaluated in hippocampus in global ischemia with reperfusion and progressive ischemia groups and in infarcted areas in the focal infarction group. The changes seen in ischemic lesion by H-E stain were neurons with hyperchromatic condensed nucleus and expanded perineuronal

spaces(HC cells) and neurons with eosinophilic cytoplasm(red neurons)(Fig. 1a & b) in early stage and pknotic neurons with condensed eosinophilic cytoplasm and pknotic nucleus at later stage in the affected hippocampal and cortical areas. In addition, pyramidal cells in CA3 region showed intracytoplasmic eosinophilic inclusion(EI) bodies(Fig. 1c & d). Cresyl violet stain was helpful to identify HC cells with squeezed nucleus and condensed cytoplasm. Red neuron and Intracytoplasmic eosinophilic inclusion bodies showed faint blue cytoplasm with loss of Nissl substance by CV stain(Fig. 1d). Pknotic neurons showed bluish homogenous cytoplasm with dot-like nucleus by CV stain.

Loss of reaction for tubulin in nerve cell bodies and/or dendrites and background stain in neuropil were used as the criterion for lesions. Loss of reaction and increased reaction in neuronal cell bodies or background stain in neuropil were considered as an abnormal reaction for anti-PLC β and γ antibodies. HC cells as well as red and pknotic neurons showed negative reaction in cell body and dendrites with anti-tubulin antibody(Fig. 1b). Anti-PLC β and γ

antibodies showed negative reaction in cell body of these cells. EI bodies showed loss of reaction to anti-tubulin and anti-PLC β and γ antibodies.

Bilateral ischemia for 5 minutes with various recirculation periods

At 5 min after the bilateral CCA ligation, 1 out of 4 gerbils showed HC neurons in CA1 and CA4 of hippocampus. At 30 min after reperfusion, 1 gerbil showed scattered HC neurons and the other 1 gerbil showed HC cells in unilateral CA1 and CA3-4 areas. The remaining 2 showed multifocal edema in hippocampus and cortex with no neuronal changes. At 1 hour after restoration of blood flow, all 3 gerbils showed HC cells at subiculum-CA1 and CA2 areas. At 2 hours after reperfusion, all 3 gerbils showed no changes. One of the 2 sham-operated gerbils showed HC neurons in dentate gyrus and CA4. At 3 hours after restoration of blood flow, 1 gerbil showed degenerating neurons in CA1 and CA2 areas with loss of reaction by anti-tubulin and anti-PLC β and γ antibodies. The other 2 showed occasional EI

Fig. 1. Neuronal changes seen in 5 min bilateral global ischemia with reperfusion for 6 hours(a & b) and 12 hours(c & d). a : Neurons in subiculum-CA1 showing red neurons with dark eosinophilic cytoplasm(X100, H-E). b : Loss of tubulin in cell bodies and dendrites in subiculum-CA1(X50, ABC). c : Neurons in CA3 showing eosinophilic inclusion(EI) bodies(arrows)(X100, H-E). d : Pale blue homogenous stain of EI bodies with cresyl violet stain(X50, cresyl violet).

bodies in unilateral CA3, which were noticed by H-E stain. Anti-tubulin and anti-PLC β and γ antibodies did not show discernible changes. At 6 hours after reperfusion, all 3 showed EI bodies in CA3 and 2 showed red neurons in subiculum-CA1 (Fig. 1a & b).

The 2 sham-operated gerbils showed edema and scattered HC neurons in 1 and groups of HC in 1 gerbil. At 12 hours after reperfusion, 3 out of 4 gerbils showed EI bodies in CA3 (Fig. 1c & d) and 2 showed neurons with eosinophilic cytoplasm in the subiculum-CA1 region. Cytoplasmic eosinophilia was more intense in EI bodies than the red neurons in subiculum-CA1 region. Two of them showed scattered neurons with negative reaction with anti-tubulin and anti-PLC β and γ antibodies in lateral CA1 and CA2 regions. One gerbil did not show any changes. At 24 hours after reperfusion, 1 out of 3 gerbils showed red neurons in subiculum-CA1 and CA2 regions and EI bodies in CA3. Anti-tubulin and anti-PLC β and γ antibodies showed scattered negative neurons in lateral CA1. The other 1 showed edema and HC neurons. One gerbil did not show any changes. The 2 sham-operated gerbils showed scattered HC neurons. At 2 days after restoration of blood flow, all 7 gerbils showed many pknotic

neurons in subiculum-CA1 and CA2. Degenerating neurons were also noted in the lateral CA1 region. One showed increased reaction to anti-PLC β antibody in remaining neurons. The 2 sham-operated gerbils did not show any changes. At 1 week after reperfusion, all 6 gerbils showed diffuse loss of neurons and eosinophilic cell ghosts in CA1 with loss of reaction to anti-tubulin and anti-PLC β and γ antibodies (Fig. 2a-d). There were scattered surviving nerve cell bodies and dendrites. Several red neurons were remaining in CA2 region. The 2 sham-operated gerbils did not show any changes.

Progressive ischemia

No changes were identified at 3 and 5 min after ligation of left CCA. At 10 and 15 min, 1 each out of 2 gerbils showed edema with no neuronal changes. The neurons showed increased reaction to anti-PLC β antibody in those gerbils. At 30 min, 1 out of 2 gerbils showed HC neurons in CA2 and CA3. The other one showed edema with no neuronal changes. At 60 min, both gerbils showed diffuse neuronal changes (HC cells) in CA1 and CA2 region. In addition, the neurons in the thalamus and the fronto-parietal cortex showed similar changes. At 1 day, 2

Fig. 2. Neuronal changes of hippocampal CA1 in 5 min bilateral global ischemia with reperfusion for 7 days. a: Diffuse loss of neurons with pyknotic neurons (X50, H-E). b: Remaining neurons showing tubulin in cell bodies and dendrites (X50, ABC). c-d: Remaining neurons with PLC β (c) and γ (d) expression in cell body and background neuropil (X50, ABC).

Fig. 3. Changes of cerebral cortex and hippocampus in unilateral progressive ischemia for 1 day. **a:** Multifocal degeneration of neuropil with loss of neuron in cerebral cortex and numerous pyknotic neurons in CA1, CA4, and dentate gyrus in left hemisphere (X10, H-E). **b:** Increased PLC- β expression in remaining neurons and background neuropil in left hemisphere (X33, ABC).

gerbils showed diffuse vacuolization and necrosis of neuropil with loss of neurons in the left hemisphere involving cerebral cortex and thalamus (Fig. 3a). Hippocampal neurons were mostly pyknotic in all subfields. Anti-PLC β and γ antibodies showed increased background reaction with increased reaction in remaining neurons in cerebral cortex and condensed strong reaction in pyknotic hippocampal neurons (Fig. 3b). Two and 1 gerbils examined at 3 and 7 days after ligation, respectively, showed increased background reaction by anti-PLC β and γ antibodies with no changes in hippocampi.

Focal ischemia

Infusion of iron powder through femoral vein showed plugging of powder in small blood vessels in meninges as well as in brain parenchyme with resultant focal infarction, which was well documented in our previous studies (data not shown). Although the size and location of infarcts were quite variable in this experimental model, changes in and around the infarction were similar depending on the time after the injury.

At 1 hour after infusion of iron powder, a localized pale area was noted in neuropil. At 2 hours after infusion, a localized pale area was noted in neuropil with perineuronal edema. Anti-tubulin and anti-PLC

β and γ antibodies showed loss of reaction in neuronal cell body with diffuse background reaction at the center of the lesion. The neurons at the periphery showed strong positive reaction with anti-PLC antibodies. At 6 hours after infusion, the center of the infarction showed thrombosed capillaries with margination of white blood cells (WBCs) and eosinophilic neurons with pyknotic nuclei (Fig. 4a). Anti-PLC β antibody showed neurons with condensed granules at the periphery of the infarct and anti-PLC γ antibody showed increased background reaction (Fig. 4b). The 1-day-old infarction showed loss of neurons and fragmentation of neuropil with increased background reaction by anti-tubulin and anti-PLC β antibodies. Anti-PLC γ antibody showed faint positive reaction. Necrotic neurons in infarction occurred at the hippocampus showed condensed positive granules by anti-PLC β antibody. Anti-tubulin antibody showed loss of reaction in neuronal cell bodies as well as background in the same area. The 2-day-old infarction showed thrombosed capillaries with necrosis of the vascular wall and extravasated WBCs. Anti-PLC β and γ antibodies showed loss of reaction and background stain in infarction, respectively. The 3-day-old infarction showed fragmented neuropil with infiltration of macrophages. Extravasated WBCs were frequently noted in necrotic area (Fig. 4c). Anti-tubulin

Fig. 4. Focal infarction by infusion of iron particles for 6 hours(a & b) and 3 days(c & d). a: Thrombosed capillaries with margination of white blood cell(WBC)s and pyknotic neurons in the center of infarct($\times 50$, H-E). b: Loss of PLC- β in center of infarct and increased expression in neurons in surrounding area($\times 50$, ABC). c: Blood vessel with necrotic wall with extravasation of WBCs and macrophages in perivascular necrotic area and iron particles in vascular lumen(arrow)($\times 40$, H-E). d: Necrotic hippocampal neurons showing condensed increased PLC β expression(arrows) and macrophages with strong positive reaction in infarction involving thalamus($\times 33$, ABC).

antibody showed faint background stain with loss of reaction in hippocampus. Anti-PLC β antibody showed strong reaction in background and macrophages with strongly positive granules(Fig. 4d). Necrotic neurons in hippocampus also showed condensed increased reaction by anti-PLC β antibody. Anti-PLC γ antibody showed similar but weaker reaction than anti-PLC β antibody. The 7-day-old infarction showed markedly proliferated blood vessels admixed with many macrophages.

DISCUSSION

Because of poor development of circle of Willis, unilateral and bilateral cerebral ischemia can be produced in gerbils by occluding one or both CCAs. Bilateral CCA occlusion in gerbils results in residual blood flow even below 5.0 ml/100 gm/min in wide areas as early as 3 minutes and showed typical sequential damages in hippocampal neurons depending on duration of ischemia, with or without reperfusion, and anatomical regions(global cerebral

ischemia)(Suzuki et al., 1983; Yoshimine et al., 1985; Hatakeyama et al., 1988). Hatakeyama et al(1988) showed that immunohistochemical investigation for tubulin and creatine kinase(CK) BB-isoenzyme was the best method for detecting neuronal changes in early stage. In their study, subiculum-CA1 region just medial to CA1 region was the most vulnerable sub-region of hippocampus and showed positive reaction in 100 % by anti-tubulin and anti-CK antibodies at 0 min recirculation after 5 min ischemia. Focal loss of reaction in dendrites was the first change in their studies but it was not evident in the present study, which could be explained by the difference in antibody and fixatives used. The changes detected by H-E stain were discernible at a later time than immunostaining.

The earliest change seen in this study was scattered HC cells at 5 min after bilateral CCA ligation. They showed loss of tubulin in cell bodies and dendrites similar to previous reports, but their distribution was different in this study. They were found in haphazard pattern in early stage of ischemia(up to 1 day) as well as in sham-operated control animals,

which suggests the nonspecific or reversible nature of the changes. With H-E stain, intraneuronal eosinophilic inclusion bodies in CA3 region were first detected at 3 hours after recirculation in this study compare to previously reported 12 hour and 1 day's reperfusion after 4 or 5 minutes' ischemia. These neurons with inclusion bodies were observed between 3 hours and 24 hours after reperfusion and no inclusion bodies were noted at 2 days or 1 week after reperfusion or in progressive ischemia up to 1 day. There were degenerated neurons with pyknotic nuclei and condensed red cytoplasm at CA2 next to CA3, which suggested the progression of neuronal damage from cells with EI. These findings confirmed conditionally reversible nature of these inclusion bodies, which could recover or degenerate further according to duration of ischemic period. Neurons with eosinophilic cytoplasm (red neurons) were first noted in subiculum-CA1 at 6 hours by H-E stain and increased in numbers at 12 hours. They were noted in CA2 at 24 hours. These neuronal changes were progressed to typical pyknotic neurons and increased in number in subiculum-CA1 and CA2 regions at 24 hours and noted in lateral CA1 at 48 hours and full blown at 1 week. Those findings were similar to previous reports. Loss of Nissl substance with bluish homogeneous appearance of neuronal perikarya of EI and red neurons by CV corresponded to negative reaction by antibodies, which were consistent with damages in the cytoskeletal system.

Previous studies on progressive global cerebral ischemia by ligation of bilateral CCAs showed immunohistochemical lesion in subiculum-CA1 and CA2 in 50~75 % of gerbils by 3 minutes of ischemia and 100 % by 5 minutes of ischemia. The changes were much less and later in this study, which showed diffuse neuronal damages in CA1 and CA2 regions after 60 minutes and diffuse vacuolization and necrosis of neuropil in left hemisphere at 1 day. Only the left CCA was ligated in this study because of high mortality rate of bilateral CCA ligation more than 10 minutes, which could explain the difference between the present study and previous reports. The changes were more generalized and did not show sequential changes according to duration in this group compared to reperfusion group.

In global ischemia with or without reperfusion, studies on pathophysiologic mechanisms were focused on various changes in protein synthesis (Thilmann et al., 1986; Araki et al., 1990) and break down

of membrane phospholipid with accumulation of products were observed during early stage of ischemia. Those findings suggested important roles of participating enzymes in signal transduction system. During the early stage of ischemia arachidonic acid (AA) increased due to the breakdown of PI by PLC and DG lipase system (Ikeda et al., 1986; Abe et al., 1987). Abe et al showed rapid recovery of the levels of PI in the cortex during reperfusion periods contrast to no recovery in the CA1 subfield. In addition, the amount of AA continued to increase up to 1 day of reperfusion in the CA1 subfield, which suggests that the activation of PLC and breakdown of PI by PLC continues even after the reperfusion period in this region and plays an important role in postischemic delayed neuronal death. Since the levels of neurotransmitters that activate PLC decrease after reperfusion, this continuous breakdown of PI suggested the possibility of other mechanisms, which was not well understood. Kempinski et al (1987) showed site-, time-, and PG-specific changes in postischemic production of AA metabolites (eicosanoid) in ischemia with subsequent recirculation in gerbil brain. Biochemical study on PLA₂ and PLC in complete brain ischemia model showed early action of PLC during the first 2 minutes of ischemia and PLA₂ acting predominantly after 2 minutes of ischemia (Umemura et al., 1992a). Their study showed phenylmethylsulfonyl fluoride (PMSF), a PI-specific PLC inhibitor, inhibited the increase of arachidonic and stearic acid during the early period of ischemia in rat neocortex. They also reported that PMSF pretreatment significantly ameliorated ischemic delayed neuronal damage in the rat hippocampal CA1 subfield (Umemura et al., 1992b), which confirmed the role of PLC in ischemic damage of the brain. This pathophysiologic mechanism has been considered especially in areas with scarce blood vessels such as CA1 of hippocampus.

In this study, the changes of PLC isoenzymes in global ischemia with reperfusion corresponded to degenerating neurons similar to loss of reaction to anti-tubulin antibody and no discernible time sequential relationship between the ischemic lesion and level of PLC isoenzyme was detected by immunohistochemical reaction. Slightly increased reaction to anti-PLC β antibody in remaining neuron at 2 days could suggest a defensive reaction to ischemia, similar to stress protein synthesis following transient ischemia (Nowak, 1985; Liu et al., 1993). Double immunostaining with other antibodies to stress protein will show

the relationship between these. Previous study showed that global ischemia by decapitation had no effect on basal PLC activity or the amounts of PLC β_1 in membrane fractions from ischemic hippocampi (Nishida et al., 1994). The changes seen in this study could not be compared with theirs due to the difference in experimental models and method of examination. Further study with chemical quantitation in different areas of the hippocampus probably will show different patterns of changes and in situ hybridization study will show changes of protein synthesis in subfields of hippocampus during early stage.

Compared to ischemia with reperfusion, the progressive ischemic lesion showed extensive damage in the hippocampus as well as in the cortex and the thalamus. The lesion was further advanced at 1 day, showing diffuse break down of brain tissue with markedly increased background stain by anti-PLC antibodies. In addition, the necrotic neurons in the hippocampus also showed increased immunohistochemical reaction. The significance of those increased reactions was not clear in this study, although they were more likely artifactual changes in condensed degenerating neurons. The surviving neurons at the periphery of the infarcts showed strong positive reaction with no neuronal damage. Similar cells were also observed in previous studies. These strong positive reaction could also be the result of production of more protein due to ischemic insult.

Focal ischemia induced by iron particle infusion through the femoral vein showed well documented typical time sequential changes of ischemic infarction. Palor of neuropil with loss of cells and decreased reaction to anti-tubulin and anti-PLC antibodies occurred after 1 hour. The changes of blood vessels and WBCs such as margination (or adhesion) were noticed after 6 hours and necrosis of vascular wall and extravasation of WBCs at 2 days. Background reaction to anti-PLC antibodies was increased after 1 day.

The pathophysiologic mechanism in focal ischemia induced by air embolism or vascular injury with u-v light was considered to be related to the changes of small blood vessels and WBCs (Hallenbeck et al., 1986; Kochanek and Hallenbeck, 1992). The changes occurring in early stage of inflammation have been considered as important steps in this type of ischemia. Observation of changes of PLC isoenzyme expression in this model could simply show the resultant expression of these enzymes rather than

expression of pathophysiologic mechanism in ischemic lesion induced by emboli. Increased reaction to anti-PLC antibodies in background and necrotic neurons was noted after 6 hours, which were similar to progressive global ischemia. In addition, the macrophages specific in focal ischemia showed strong positive reaction to these antibodies. The significance of those positive reaction was not clear in this study.

Studies were performed on other enzymes of the signal transduction system in ischemic encephalopathy, because of sequential activation and the close relationship of these enzymes. Protein phosphorylation by PKC with consequent secretion and degranulation of lysosomal enzymes, and activation of the oxidative burst is critically involved in the function of the nervous system. Protein kinase A, C, and Ca^{2+} /calmodulin-dependent protein kinase II phosphorylate several proteins involved in neurosecretion and cytoskeletal organization (Hemmings et al., 1989). Inhibition of protein kinase C has been shown to protect the CA1 neurons of the hippocampus from ischemic injury (Yoshidomi et al., 1989; Hara et al., 1990). Inhibitors of tyrosine kinase also blocked tyrosine phosphorylation and prevented delayed neuronal death in the hippocampus (Campos-Gonzalez and Kindy, 1992; Kindy 1993). The changes of PKC isoenzymes were examined in experimental ischemia of rat neocortex (Louis et al., 1988; Louis et al., 1991; Shim et al., 1994). The redistribution of PKC to the cell membranes was observed during and following ischemia, which could be due to ischemia induced receptor activation and subsequent changes (Cardell et al., 1990). Louis et al. (1988) showed a rapid (5 min) ischemia-dependent translocation of the enzyme from the cytosol to particulate membrane fraction with complete reverse upon reperfusion. After 30 min of ischemia, there were substantial losses in PKC activity and content as measured by [^3H]phorbol dibutyrate binding. Shim et al. (1994) showed 10 min acute ischemia with reperfusion inhibited activity of PKC isoenzymes and prolonged ischemia longer than 2 hours induced the expression of 3 PKC isoenzymes. The sequential activation and close relationship between PLC and PKC enzymes suggested possible similar reaction of these two and study of PLC enzyme on same focal ischemia induced by middle cerebral artery clipping would be helpful to see the relationship.

In conclusion, the changes of PLC of the hippocampal neurons in 5 min global ischemia with re-

perfusion for 0 min to 7 days manifested degeneration of neuronal cytoskeletal system, and increased expression of PLC in remaining neurons were most likely reactive changes to ischemic stress. Increased PLC expression of pknotic neurons and background of necrotic areas of global progressive ischemic lesion at 1 day was thought to be an artifactual changes due to cytoplasmic condensation and break down of neuropil, respectively. Similar findings of PLC expression was noted in focal ischemia at 2 hours, and both focal ischemia and progressive global ischemia also showed increased expression of PLC in surviving neurons around the necrotic areas. These findings suggested similar pathophysiologic mechanisms between those two, and different mechanism between them and global ischemia with reperfusion.

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