Changes in the Concentrations of Cerebral Proteins Following Occlusion of the Middle Cerebral Artery in Rats

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Using an immunoblotting technique, we investigated changes in the concentrations of microtubule-associated protein 2, 200-kDa neurofilament, tubulin, myelin-associated glycoprotein, and 2':3'-cyclic nucleotide 3'-phosphodiesterase in the brains of 40 rats following occlusion of the left middle cerebral artery or sham operation. Compared with those 4 hours after surgery, concentrations of all proteins decreased significantly in the left hemisphere 3 days after surgery (p<0.01). Microtubule-associated protein 2 was the most susceptible to ischemia, and its mean±SEM concentration decreased to 23±9.4% of that in concurrent sham-operated controls. Degradation products of microtubule-associated protein 2 and myelin-associated glycoprotein were detected on the blots. Furthermore, in the contralateral hemisphere (where calpain might be activated), concentrations of these two proteins decreased to 57±12.0% and 83±4.3% of those in concurrent sham-operated controls, respectively, 3 days after surgery. Changes in the concentrations of cerebral proteins in the contralateral hemisphere are important for understanding clinical symptoms not attributable solely to the ipsilateral lesion following a focal cerebral stroke. (Stroke 1990;21:917-922)

We have reported that endogenous myelin protease is activated in myelin purified from brains of persons with multiple sclerosis. This protease has been characterized as calpain, which degrades myelin proteins such as myelin basic protein and myelin-associated glycoprotein (MAG). In cerebrospinal fluid we have detected protease activity that is inhibited by serine protease inhibitors such as camostat mesilate (FOY305). This protease activity increases in cerebrospinal fluid from patients in an acute phase of multiple sclerosis and those with viral meningoencephalitis. Cerebral proteins have been thought to be the targets of proteolysis by proteases under conditions of increasing calcium influx and/or lowered pH in ischemic lesions. Recently, it has been demonstrated that transient cerebral ischemia markedly decreases the concentrations of microtubule-associated protein 2 (MAP2) and calpsectin in gerbil brain. An immunohistochemical experiment revealed a decrease in the concentration of tubulin in the CA1 area of the gerbil hippocampus following transient ischemia.

To investigate the relation between tissue damage and protease activities after ischemia, using an immunoblotting technique we examined changes in the concentrations of several cerebral proteins in each hemisphere following occlusion of the left middle cerebral artery (MCA) of rats. We studied MAP2, MAG, and a 200-kDa neurofilament (200kdNF), which are good substrates of calpain, and tubulin and 2':3'-cyclic nucleotide 3'-phosphodiesterase (CNP). We analyzed the entire left hemisphere to examine not only the core of the ischemic zone but also the penumbra. The entire right hemisphere was analyzed for any contralateral effects of the focal ischemic lesion.

Materials and Methods

We anesthetized 40 male Sprague-Dawley rats weighting 340–440 g using inhaled 2% halothane in 30% O₂ and 70% N₂. In 20 rats the proximal portion of the left MCA was permanently occluded using a microsurgical technique that was modified from our original method; using a microbipolar unit (Keisei-
Ika, Tokyo, Japan) the exposed MCA was electrocauterized just medial to the olfactory tract and severed. In the 20 sham-operated rats, the surgical procedure was carried out in a similar manner, except the exposed MCA was not electrocauterized nor severed. After closure of their surgical wounds, the rats were returned to their cages and permitted free access to food and water. Five MCA-occluded rats and five sham-operated rats were decapitated 4 hours or 1, 3, or 7 days after surgery and the brains were quickly removed. After the hemispheres were separated and weighed, each was immediately frozen and stored at −70°C until assay.

Each hemisphere was homogenized in 10 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid, 1 mM N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-3-methyl-butyramine (E-64-c), and 1 mM FOY305. Appropriate aliquots were taken for the assay. Electrophoresis was carried out on polyacrylamide gel slabs according to Laemmli and Favre12 after delipidation of freeze-dried aliquots of the homogenate with 3:2 (vol:vol) diethyl ether: ethanol. The running gels were 5% polyacrylamide for MAP2 and 200kdNF and 11% polyacrylamide for MAG, tubulin, and CNP. On each side, for each time, the same amount of total protein in aliquots from the five pairs of an MCA-occluded and a sham-operated rat were applied to each lane. To assure the validity of our quantitative analysis of each protein, preliminary immunoblot experiments over a wide range of protein concentrations were carried out to determine the linear range of this method (Figure 1). The amount of total protein applied was 50 µg for MAP2, 20 µg for 200kdNF, and 10 µg for MAG, tubulin, and CNP to obtain a linear response to decreasing amounts of these proteins in the total protein. The protein concentration of homogenates was determined by the method of Lowry et al.15 Some gels were stained with Coomassie blue to determine the albumin content in the sample.

Mouse monoclonal antibody raised in response to bovine MAP2 was prepared by a standard hybridoma technique.14 Rabbit polyclonal antibody directed against rat MAG of the central nervous system was prepared according to Quarles et al.15 Mouse monoclonal antibody to rat 200kdNF was obtained from Enzo Biochemicals (New York, New York). Rabbit polyclonal antibody to tubulin was obtained from Advance Co. (Tokyo, Japan). The preparation of rabbit polyclonal antibody directed against bovine CNP has been described.16 Biotinylated anti-mouse or anti-rabbit IgG and avidin–biotin complex standard kits were purchased from Vector Laboratories, Inc. (Burlingame, California). Each antibody was diluted in 3% bovine serum albumin, anti-MAP2 antibody at 1:400, anti-MAG antibody at 1:200, anti-200kdNF antibody at 1:500, anti-tubulin antibody at 1:400, anti-CNP antibody at 1:1000, and biotinylated anti-mouse or anti-rabbit IgG at 1:200.

According to Towbin et al,17 the blots were immunostained using an avidin–biotin complex peroxidase procedure with antisera, and the color was developed using 4-chloro-1-naphthol. The blots were then scanned with a spectrophotometer (CS-910, Shimadzu, Tokyo, Japan) operating at 560 nm for the semiquantification of proteins. Blots of each pair of aliquots were immunostained and scanned individually to avoid uneven staining intensity within a blot and to overcome the differences in staining intensity among blots. The change in concentration of each
In the right hemisphere, concentrations of MAP2 and MAG also decreased significantly, to 57±12.0% (p<0.01) and 83±4.3% (p<0.05), respectively, 3 days after surgery (Figure 4).

As shown in Table 1, wet weight of the left hemisphere in MCA-occluded rats was significantly higher 1 day (p<0.01) and significantly lower 7 days (p<0.01) after surgery than that in the concurrent sham-operated rats. However, there was no significant difference in wet weight of the right hemisphere at any time.

As shown in Figure 5, bands of albumin were prominent 1, 3, and 7 days after surgery in the left hemisphere of MCA-occluded rats. Densitometry showed that 3 days after surgery albumin comprised 8% of the total protein in the left hemisphere of MCA-occluded rats but only 4% of that in concurrent sham-operated rats. No remarkable difference in albumin was detected in the right hemisphere at any time (data not shown).

Discussion
A major cytoskeletal protein in the brain, MAP2 is present at high concentrations in dendrites. One of the triplets of neurofilaments, 200kdNF is abundant

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FIGURE 3. Representative immunoblots stained for microtubule-associated protein 2 (MAP2) and myelin-associated glycoprotein (MAG) in left hemisphere of rats at each time after surgery. Lane O, rat with left middle cerebral artery occlusion; lane S, rat with sham operation.

FIGURE 4. Graphs of mean ± SEM changes in percentage of protein remaining intact in right hemisphere of five rats 4 hours and 1, 3, and 7 days after left middle cerebral artery occlusion. Percentage change relative to protein concentration in five corresponding sham-operated rats. MAP2, microtubule-associated protein 2; MAG, myelin-associated glycoprotein.

in axons. Tubulin is a component of microtubules and is distributed in both axons and dendrites. These proteins play an important role in regulating neuronal morphogenesis. MAG is a membrane protein of the central and peripheral nervous system myelin sheaths and may play a role in axon-myelinating cell interactions because of its particular localization in the periaxonal area. CNP has been widely used as a marker for myelin-oligodendrocytes in the central nervous system, but its function remains unknown. Cerebral proteins have been thought to be the targets of proteolysis in ischemic lesions.

Concentrations of neuronal cytoskeletal proteins such as MAP2, calspectin, and tubulin decrease following transient ischemia in animals. Our study on rats with permanent left MCA occlusion also revealed decreases in concentrations of the neuronal cytoskeletal proteins MAP2, 200kdNF, and tubulin in the left hemisphere 3 days after surgery; concentrations of MAG and CNP (proteins rich in myelin and found in oligodendrocytes) also decreased. This reduction in concentrations of these two proteins indicates white matter damage. Among the proteins examined, MAP2 was the most susceptible to ischemia. The significant increase in the weight of the left hemisphere 1 day after MCA occlusion suggested that brain edema occurred. Albumin levels in samples from the left hemisphere increased 1 day after MCA occlusion (probably due to damage to the blood-brain barrier); this increase in albumin content may be partially responsible for the decreases in concentrations of the five proteins as percentages of total protein. However, the decreases in concentrations of these five proteins were greater than those expected due only to the effect of an increase in albumin content. Furthermore, the presence of degradation products of MAP2 and MAG on the blots suggests that the decreases in concentrations of these two proteins was mainly due to their degradation.

The concentration of no protein was decreased 4 hours after surgery, which might have been due to dilution of the aliquot with unaffected brain regions not in the MCA territory. The decrease in the concentration of MAP2 in the left hemisphere 3 days after surgery was greater than that expected from contributions of only brain regions in the MCA territory. Our sampling of the entire hemisphere may have limited our ability to observe early minor changes in the MCA territory, but our sampling

| TABLE 1. Wet Weight of Cerebral Hemisphere of Rats After Surgery |
|-------------------|-------------------|-------------------|-------------------|
|                   | Left              |                   | Right             |
|                   | Oclusion          | Sham operation    | Oclusion          | Sham operation    |
| Time              |                   |                   |                   |                   |
| 4 hours           | 797±10            | 754±12            | 718±7             | 728±12            |
| 1 day             | 817±14*           | 742±16            | 734±12            | 725±27            |
| 3 days            | 779±27            | 748±30            | 731±28            | 736±7             |
| 7 days            | 646±16*           | 712±10            | 713±21            | 710±8             |

Values are mean ± SEM in mg for five rats in each group. Left hemisphere was operated side. *p<0.05 different from sham-operated rats by Wilcoxon's nonparametric U test.
techniques allows us to detect total change, including that in the penumbra.

The decreases in protein concentrations 7 days after MCA occlusion were not as marked as those at 3 days. This might be partially due to loss of ischemic tissue, which is suggested by the decrease seen in the wet weight of the left hemisphere. Despite the lack of a significant decrease in wet weight of the right hemisphere, MAP2 and MAG levels recovered by 7 days, suggesting regeneration of these two proteins. Since calcium accumulates in the ischemic MCA territory after MCA occlusion, calpain may be activated in this area and may degrade substrates such as MAP2, MAG, and 200kdNF. Tubulin and CNP, which are not good substrates for calpain, may be degraded by other proteases that are activated by mechanisms other than calcium influx, such as lowered pH.

Proteases secreted by cells that infiltrate ischemic areas may also degrade these five proteins. Significant histopathologic changes were not detected by light microscopy in the contralateral hemisphere. However, our study revealed significant decreases in the concentrations of MAP2 (p<0.01) and MAG (p<0.05) in the contralateral hemisphere 3 days after MCA occlusion. This decrease could not have been modified by an albumin shift because no such shift was detected in the right hemisphere. As both MAP2 and MAG are substrates of calpain, calpain might be activated contralateral to an MCA occlusion. The mechanisms of calpain activation in the contralateral hemisphere need to be clarified.

A decrease in cerebral blood flow contralateral to an MCA occlusion has been reported. Edema fluid might spread along transcallosal fibers from the focus of ischemia. The uncontrolled release of transmitters occurs in transcallosal fibers originating from damaged cells, and in the case of glutamate these transmitters open a receptor-operated calcium ion gate. These factors may be related to the activation of calpain contralateral to an MCA occlusion. Nonetheless, transient changes in the concentrations of cerebral proteins in the hemisphere contralateral to a focal ischemic lesion may be important in understanding the clinical symptoms that are not caused solely by the ipsilateral lesion after a focal cerebral stroke.

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