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PROCESSING OF β /A4 AMYLOID PRECURSOR PROTEIN IS ALTERED IN THE HIPPOCAMPUS OF RESERPINIZED RAT BRAIN

Hiroshi Komachi[1], Yasufumi Shirasaki[2], Tadashi Miyatake[1] and Katsuhiko Yanagisawa[1]*

[1]Department of Neurology, Tokyo Medical and Dental University, Tokyo, Japan

[2]Exploratory Research Laboratories II, Daiichi Seiyaku Research Co. Ltd., Tokyo, Japan

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Summary: Immunoreactivities of β/A4 amyloid precursor protein (APP) and soluble derivative of APP (APPs) were determined in the hippocampus and the striatum of reserpinized rat brains. Significant decrease of APPs immunoreactivity was observed in the cytosolic fraction of hippocampus, but not of striatum, whereas APP immunoreactivity was found to be increased in the membrane fraction of hippocampus. These findings suggest that processing of APP in hippocampus may be regulated through the monoaminergic signal transduction system.

One of the pathological hallmarks in Alzheimer's disease (AD) brain is a deposition of β / A 4 amyloid protein (Aβ), a 4 kD cleaved product of the 110 to 130 kD glycosylated, membrane-spanning precursor protein (amyloid precursor protein, APP) (1). Since point mutations in the APP gene were discovered in familial AD (2, 3), alteration of APP processing seems to be closely related to the primary cause of AD. However, little is known of the mechanism of regulation of APP processing. Recently it was reported that the activation of protein kinase C (PKC) caused an alteration of APP metabolism in a way that the release of soluble derivative of APP (APPs) was increased (4-7) with a corresponding decrease of Aβ production (8, 9). Previously we reported the reduced membrane-associated PKC in the hippocampus of reserpinized rat brain (10, 11), which is similar to the changes in AD brains (12, 13). Since morphological studies also indicate monoaminergic dysregulation in AD brains (14, 15), reserpinized animals seem to be useful model for the study on AD

*Corresponding author. Present address: Department of Neuropathology, Institute for Brain Research, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan. Fax:81-3-5800-6852.

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pathophysiology. The purpose of the present study is to determine the effect of reserpinization on the APP processing and to see whether alteration in APP metabolism is topologically correlated with the redistribution of PKC, which was observed in reserpinized rat brain (10, 11).

Materials and Methods

Reserpinization . In the first experiment, Wistar male rats weighing 200g were reserpinized by intraperitoneal injection, as previously reported (10). Reserpine was solubilized in acetic acid and diluted in phosphate-buffered saline containing 7% propylene glycol. Rats were injected with reserpine (5 mg/kg of body weight) and sacrificed 8 hours later by decapitation under weak anesthesia with halothane. Control rats received vehicle injections. In the second experiment, rats had free access to food containing 100 mg reserpine per kilogram (F2, Funabashi Nojo, Japan). Control rats had free access to the diet without reserpine. After four weeks of peroral administration, rats were sacrificed in the same manner as in the first experiment.

Sample preparation . The hippocampus and the striatum were dissected, and stored at -80°C until use. Forty milligrams of wet weight of dissected tissue was homogenized in 500 μ l of ice-cold phosphate-buffered saline (pH 7.4) with a Teflon-glass, motor-driven homogenizer. The homogenates were centrifuged at 100,000 g for 30 minutes at 4 °C to obtain membrane and cytosolic fractions. To avoid nonspecific binding of antibodies against APP, APP and soluble derivative of APP (APPs) were extracted using the following procedures. The resulting pellets were rehomogenized in phosphate-buffered saline containing 0.5% Triton X-100 with a Teflon-glass homogenizer. The homogenates were centrifuged at 100,000 g for 30 minutes at 4°C, supernatants containing 200 μ g of protein were applied to DEAE ion-exchange chromatography columns (Pharmacia, Sweden), following removal of Triton X-100 by Bio-Beads SM2 column. The APP was eluted with 0.5M NaCl, and sedimented in ice-cold 10% trichloroacetic acid by centrifugation at 10,000g for 5 minutes. For the study of APPs in the cytosolic fractions, samples were applied to the DEAE ion-exchange chromatography column without prior Triton X-100 treatment.

Gel electrophoresis and Western blotting . The protein concentration in the samples were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, U.S.A.). Twenty micrograms of protein from each sample was separated by 7.5% SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (16), and electrophoretically blotted onto nitrocellulose sheets by the method of Towbin et al. (17). The blots were immersed in 5% skim-milk in Tris-buffered saline (pH 8.3) containing 0.1% Tween 20 (TBS-T) overnight at 4°C. After washing with TBS-T, the blots were incubated with polyclonal antibodies against synthetic peptide APP_{45-62} (NAPP) or $APP_{666-695}$ (CAPP) for 1 hour at room temperature. After a second wash with TBS-T, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, Buckinghamshire, UK) for 1 hour at room temperature. After a third wash, immunoreactive proteins were visualized with an enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK). The films were quantitatively analyzed with a scanning densitometer (Shimazu, CS-9000) at a wavelength of 540 nm.

Results

The specificities of immunoreaction of APP and APPs were examined in cytosolic and membrane fractions of the homogenate of hippocampus. In the cytosolic fraction, a band with an apparent molecular weight of 105 kD was immunolabeled with NAPP, but not with CAPP. From this result, it was suggested that C-terminal truncated derivative of APP, but not full-length APP, was the species present in the cytosolic fraction (Fig. 1a). In the membrane fraction, both of NAPP and CAPP immunolabeled bands with the same molecular weight, indicating that these molecules were full-length APP (Fig. 1b).

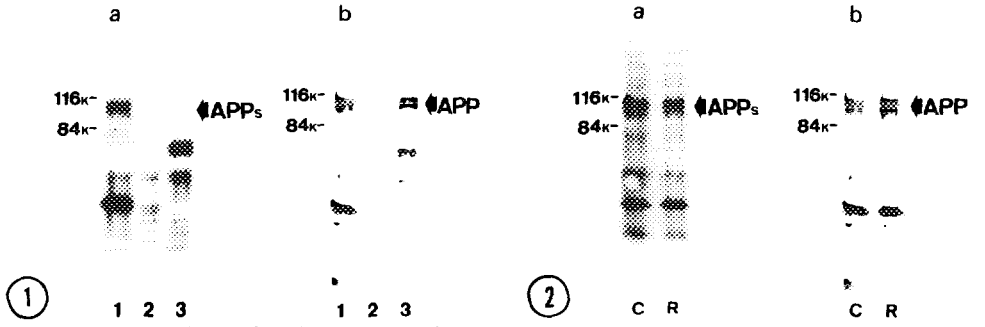

Fig. 1. Immunoblots of the homogenate of hippocampus from control rat brain.
panel a; cytosolic fraction, b; membrane fraction, immunolabeled with NAPP (lane 1), preabsorbed NAPP (lane 2) and CAPP (lane 3)

Fig. 2. Immunoblots of the homogenates of hippocampus from control and reserpinized rat brains, labeled with NAPP.
panel a; cytosolic fraction, b; membrane fraction, C; control rat, R; reserpinized rat

Figure 2 shows immunoblots of the cytosolic and the membrane fractions separated from the combined hippocampus homogenates of five control and five reserpinized rat brains. In the cytosolic fraction, the intensity of APPs immunoreactivity appeared to be decreased in the reserpinized rats. Conversely, the intensity of APP immunoreactivity in the membrane fraction from the same homogenate was increased. In the whole homogenate, no differences in immunoreactivities of NAPP were observed between control and reserpinized rat brains (data not shown). To investigate the data statistically, another series of animals were sacrificed to obtain hippocampus tissues, which were examined individually. For the study of APP in the cytosolic fractions, Western blottings were performed in the same manner as for the first series of animals; however, it was impossible to obtain enough amount of membrane-bound APP by ion-exchange chromatography from the individual hippocampus samples due to the relatively small volume of tissue. Therefore, statistical analysis was carried out only for the study of APPs in the cytosolic fractions. In quantitative analysis of the blots, the decrease of APPs immunoreactivities in the hippocampus was statistically significant ($p<0.05$) (Fig. 3 A). To determine whether the alteration in the APP processing was topologically different, the striatum was also dissected and studied. There was no significant change of APPs immunoreactivity in the striatum (Fig. 3 A). In the study of chronic reserpinization by peroral administration, decrease in the immunoreactivity of APPs was also found to be statistically significant ($p<0.05$) (Fig.3 B).

Discussion

An invariant feature in AD brain is an abnormal accumulation of Aβ; however, the mechanism of aberrant processing of APP which leads to β-amyloidogenesis is unclear. Successful treatments for the regulation of APP processing invariably involve activation of

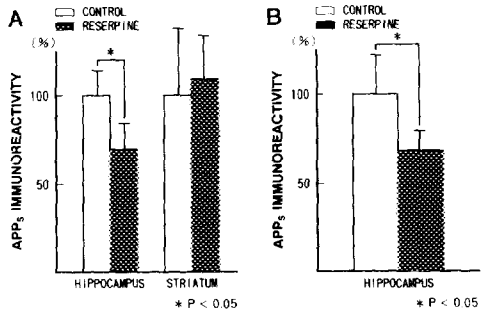

Fig. 3. Quantitative analysis of immunoblots for APPs in the cytosolic fractions. Rate were reserpinized by single intraperitoneal injection (A) or peroral administration for 4 weeks (B). Significant decrease of APPs immunoreactivity was observed in the cytosolic fraction of the hippocampus both experiment (A) and (B). Conversely, no significant change was observed in the striatum.

PKC in cultured cells by agents including phorbol dibutyrate (4, 6), phorbol ester (5), phorbol 12-myristate 13-acetate (7) and acetylcholine receptor agonist (18). The regulation of APP processing by PKC activation seems to be important from the viewpoint of pathophysiology of AD because of the following. First, the increase of the secretion of APPs by PKC activation is coincident with a decrease of Aβ production (8, 9). Second, the redistribution of PKC was observed in the hippocampus and cerebral cortices of AD brains, which are preferential sites for the deposition of Aβ (12, 13).

Having noticed that PKC activation leads to the changes of APP processing and Aβ production and that PKC distribution is altered in AD brains, the question then arises as to the mechanism of PKC redistribution in AD brains. In 1990, Kalberg and Sumners found that adrenergic receptor agonist induces the translocation of PKC from cytosol to membrane in cultured cells (20). The pivotal role of the monoaminergic system in the regulation of PKC distribution was also confirmed in our previous studies (10, 11) which demonstrated PKC redistribution similar to that in AD brains, by reserpinization, which was an effective procedure for the deprivation of monoamines (21, 22). Together with morphological observations which showed remarkable neuronal loss in the locus caeruleus, the origin of monoaminergic projections, in AD brains (14, 15), one could speculate that monoaminergic dysregulation may be the cause of the abnormal redistribution of PKC in AD brains.

The present study is the first report, to our knowledge, of successful regulation of APP processing in animals. The molecular relationship between the monoamine deprivation and the alteration of APP processing remains to be determined; however, the topological correlation between the redistributioin of PKC (10, 11) and the alteration of APP processing in reserpinized rat brain strongly suggests that PKC is involved in the regulation of APP processing. Recently, it was reported that the alteration of APP processing by PKC activation is not due to the direct phosphorylation of APP by PKC because the release of phosphorylation-site truncated molecule is also enhanced by PKC activation (9, 19). It was also reported that

phosphorylation of APP which was induced by the activation of PKC occurred on its ectodomain (9), indicating the involvement of ecto-protein kinase, but not of PKC. Based on this evidence, it is postulated that activation of PKC causes the phosphorylation of another molecule which is involved in the regulation of APP processing (9).

Previous *in vitro* studies have shown that APP is processed via the secretory pathway or lysosomal-endsomal pathway through reinternalization from cell-surface (23-25); however, it is still unclear which pathway is involved in the abnormal generation of Aβ in AD brains. Since, in the present study, the decrease of APPs in the cytosolic fraction occurred with a corresponding increase of APP in the membrane fraction, it is suggested that reserpinization may lead to a decrease in APPs generation through a shift in APP trafficking or an alteration in α-secretase activity.

It remains to be determined whether generation of Aβ is accelerated by reserpinization; however, our model is useful for the study of APP processing which may be topologically different in brains.

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References

1. Kang, J. , Lemaire, H. G. , Unterbeck, A. , Salbaum, J. M. , Masters, C. L. , Grzeschik, K. H. , Multhup, G. , Beyreuther, K. and Muller-Hill, B. (1987) Nature 325, 733-736
2. Goate, A. , Chartier-Harlin, M. C. , Mullan, M. , Brown, J. , Crawford, F. , Fidani, L. , Giuffra, L. , Haynes, A. , Irving, N. , James, L. , Mant, R. , Newton, P. , Rooke, K. , Roques, P. , Talbot, C. , Pericak-Vinces, M. , Roses, A. , Williamson, R. , Rossor, M. , Owen, M. and Hardy, J. (1991) Nature 349, 704-706
3. Mullan, M. , Crawford, F. , Axelman, K. , Houlden, H. , Lilius, L. , Winblad, B. and Lannfelt. L. (1992) Nature genetics 1, 345-347
4. Buxbaum, J. D. , Gandy, S. E. , Cicchetti, P. , Ehrlich, M. E. , Czernik, A. J. , Fracasso, R. P. , Ramabhardran, T. V. , Unterbech, A. J. and Greengard, P . (1990) Proc Natl Acad Sci USA 87, 6003-6006
5. Caporaso, G. L. , Gandy, S. M. , Buxbaum, J. D. , Ramabhardran, T. V. and Greengard, P. (1992) Proc Natl Acad Sci USA 89, 3055-3059
6. Gillespie, S. L. , Golde, T. E. and Younkin, S. G. (1992) Biochem Biophys Res Commun 187, 1285-1290
7. Fukushima, D. , Konishi, M. , Maruyama, K. , Miyamoto, T. , Ishiura, S. and Suzuki, K. (1993) Biochem Biophys Res Commun 194, 202-207
8. Gabuzda, D. , Busciglio, J. and Yankner, B. A. (1993) J Neurochem 61, 2326-2329
9. Hung, A. Y. and Selkoe, D. J. (1994) EMBO J 13, 534-542
10. Komachi, H. , Yanagisawa, K. , Shirasaki, Y. and Miyatake, T. (1993) Neurosci Let 149, 6-8
11. Komachi, H. , Yanagisawa, K. , Shirasaki, Y. and Miyatake, T. (1994) Brain Res 634, 127-130
12. Masliah, E. , Cole, G. , Shinohama, S. , Hansen, L. , DeTeresa R. , Terry, R. D. and Saitoh, T. (1990) J Neurosci 10, 2113-2124

13. Masliah, E. , Cole, G. M. , Hansen, L. A. , Mallory, M. , Terry, R. D. and Saitoh, T. (1991) J Neurosci 11, 2759-2767
14. German, D. C. , Manaye, K. F. , White, C. L. , Woodward, D. J. , McIntire, D. D. , Smith, W. K. , Kalaria, R. N. and Mann, D. M. A. (1992) Ann Neurol 32, 667-676
15. Mann, D. M. A. , Yates, P. O. and Marcyniuk, B. (1984) J Neurol Neurosurg Psychiat 47, 201-203
16. Laemmli, U. K. (1970) Nature 277, 680-685
17. Towbin, H. , Staehelin, T. and Gordon, J . (1979) Proc Natl Acad Sci USA 76, 4350-4354
18. Nitsch, R. M. , Slack, S. S. , Wurtman, R. J. and Growdon, J. H. (1992) Science 258, 304-307
19. Silva, O. A. B. C. , Iverfeldt, K. , Oltersdorf, T. , Sinha, S. , Liberburg, I. , Ramabhadran, T. V. , Suzuki, T. , Sisodia, S. S. , Gandy, S. and Greengard, P. (1993) Neuroscience 57, 873-877
20. Kalberg, C. J. and Sumners, C. (1990) Biochem Biophys Res Commun 166, 22-28
21. Fabrazzo, M. , Costa, E. and Mocchetti, I. (1991) Mol Pharmacol 39, 144-149
22. Sharma,V. K. , Harik, S. I. , Ganapathi, M. , Busto, R. and Banerjee, S. P. Exp Neurol 65, 685-690
23. Cole, G. , Huynh, T. V. and Saitoh, T. (1989) Neurochem Res 14, 933-939
24. Golde, T. E. , Estus, S. , Younkin, L. H. , Selkoe, D. J. and Younkin, S. G. (1992) Science 255, 728-730
25. Haass, C. , Koo, E. H. , Mellon, A. , Hung, A. Y. and Selkoe, D. J. (1992) Nature 357, 500-502