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Enhanced Rotamase Activity of FK506-Binding Proteins within the Postischemic Brain

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ABSTRACT

Despite immunophilins being abundant in the brain, their functional role there remains unclear. Using rat models of transient focal cerebral ischemia, I investigated the effect of ischemic stress on the rotamase activity of immunophilins in the brain and examined the possible role of immunophilins in postischemic neuroprotection. I found that the rotamase activity of the FK506 binding protein class of immunophilins increases in response to ischemia and may play a key role in regulating the cascade of intracellular events after cerebral ischemia. This study provides direct evidence that the FK506 binding protein family of immunophilins mediate the neuroprotective effects of the immunosuppressant FK506 (tacrolimus) in the ischemic brain and suggests that a neuroprotective signal transduction system involving immunophilins could be used to treat ischemic stroke. (Jikeikai Med J 2003 ; 50 : 9–14)

Key words : brain ischemia, immunophilins, immunosuppressive agents, neuroprotective agents, peptidylprolyl isomerase

Introduction

Recent studies have shown that FK506 (tacrolimus), an immunosuppressant used to prevent allograft tissue rejection, has neuroprotective effects in animal models of cerebral ischemia[1–3]. In immunosuppression, FK506 forms a complex with an immunophilin, FK506 binding protein (FKBP), in T-lymphocytes and inhibits the initiation of signal transduction leading to T-lymphocyte activation[4,5]. Although the biomolecular mechanisms underlying the neuroprotective effects of FK506 remain unclear, studies suggest that immunophilins are involved in the initiation of this response[6]. Immunophilins are a family of ubiquitous, highly conserved proteins with numerous intracellular functions[7,8]. Immunophilins can be divided into two subfamilies according to their affinity to immunosuppressants[9] : the cyclophilin family, which is specific to cyclosporin-A, and the FKBP family, which has an affinity for FK506 and rapamycin. In addition to mediating the immunosuppressive cell response to immunosuppressants, immunophilins function as an enzyme, peptidyl prolyl *cis-trans* isomerase (PPIase), and exhibit rotamase activity[10–12]. PPIases catalyze the *cis-trans* isomerization of the proline imidic peptide bonds in nascent or denatured proteins and play an important role in the regulation of protein folding during protein synthesis[9,10,13].

Hyperthermia can induce mRNA encoding immunophilins in several strains of yeast[14]. Presumably, this response aids other molecular chaperones in the repair of cellular integrity. Because considerable molecular changes occur in the brain after ischemic insult[15], I investigated the general effects of cerebral ischemia on the PPIase activity of immunophilins in

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the rat brain. Furthermore, I studied the role of immunophilins in the brain and investigated their involvement in the mechanism of neuroprotection by FK506 in the postischemic brain.

Methods

Animal model of focal cerebral ischemia

Female Wistar rats weighing 200 to 230 g were used in this experiment (n=30). Transient focal cerebral ischemia was induced with the intraluminal suture method[21]. Briefly, the rats were anesthetized with halothane (4% at induction and 1% to 2% during surgery) in a gas mixture of NO_2/O_2 (70 : 30). The body temperature was maintained at 37±1°C with a thermostatically controlled heating pad connected to a rectal thermometer. A ventral, midline incision was made in the neck, and the right common carotid artery, the external carotid artery, and the internal carotid artery were identified and carefully isolated. A 4-0 nylon monofilament with its tip thinly coated with silicone rubber was inserted through the external carotid artery and passed into the internal carotid artery to occlude the origin of the right middle cerebral artery (MCA). After 30 minutes of MCA occlusion, the monofilament was removed to allow reperfusion of the ischemic region. A left-deviated gait, which characterizes left-sided hemiparesis, indicated successful focal ischemic attack. Anesthetized rats that received midline incisions without intraluminal intervention were also prepared and used as controls.

Preparation of the cytoplasmic extracts

After being reanesthetized with 4% halothane in a gas mixture of NO_2/O_2 (70 : 30), rats were killed on days 1 (n=5), 4 (n=5), and 7 (n=5) after reperfusion. To eliminate blood from the tissues, intracardiac perfusion with heparinized 0.9% saline was performed before extraction of the brain. After dissection, the right and left cerebral hemispheres of each sample were separately sonificated in a double weight of 1 M Tris-HCl solution (pH 7.4). The homogenates were centrifuged at 30,000 rpm (approximately 60,000 g) for 15 minutes at 4°C, after which supernatants were collected to analyze PPIase enzyme activity.

Analysis of PPIase enzyme activity

The method[11,16] used to measure the PPIase enzyme activity uses the reaction between a test peptide, N-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide, coupled with a protease, α-chymotrypsin. In brief, 75 μl of each prepared sample was mixed with 75 μl of the test peptide in 3.0-ml aliquots of 35 mM HEPES buffer solution (pH 7.8) at 10°C, and 10 μl of α-chymotrypsin was added to start the initial reaction for assay analysis. α-Chymotrypsin selectively cleaves peptides that are in the *trans* conformation. At equilibrium, 90% of the test peptide is present in the *trans* conformation and are instantaneously cleaved upon addition of the chymotrypsin, releasing nitroanilide, which can be measured spectrophotometrically at 390 nm. The remaining 10% of the peptide is in the *cis* form and is likewise cleaved upon enzymatic conversion to the *trans* form by the PPIase within the samples. The rate of spectrophotometric change thus reflects the relative activity of the PPIase enzyme of the immunophilins within the samples.

The value of the first-order rate constant of the PPIase enzyme activity of each sample was calculated from the acquired data using the KORE program for analyzing kinetic data[22].

Inhibition of PPIase activity by immunosuppressants

To determine which family of immunophilins are involved in the enhancement of the PPIase activity after cerebral ischemia, aliquots of samples (75 μl each) from the right (ischemic) cerebral hemisphere of rat models killed 1 day after reperfusion (n=5) were exposed to the immunosuppressants FK506, rapamycin, and cyclosporin-A in excess (0.1 μg per aliquot) and allowed to react for 5 minutes at 10°C before PPIase activity was assayed.

FK506 dose dependance

The effects of different doses of FK506 on the samples were investigated. Various doses of FK506 (0.025, 0.05, 0.1, and 1.0 μg) were applied to aliquots of samples from the ischemic hemispheres of rats killed 1 day after reperfusion (n=5). The PPIase enzyme activity of each sample was then analyzed.

In vivo application of FK506

To investigate the effects of FK506 on FKBPs in the brain, rats ($n=10$) received intravenous injections of FK506 (1 mg/kg body weight) and were separated into two groups. Rats in the MCA occlusion group ($n=5$) underwent 30 minutes of right MCA and were killed after 1 day of reperfusion, and control rats ($n=5$) underwent sham operation and were killed the following day. Samples of the brain extracts were prepared for PPIase activity assay as described earlier.

Results

Increased PPIase activity

The PPIase activity in the ischemic (right) hemisphere of the brain 1 day after reperfusion ($k_{right}=0.0497\pm0.022$) was significantly higher than control ($k_{control}=0.02858\pm0.0015$; Fig. 1). In contrast, PPIase activity in the nonischemic (left) hemisphere 1 day after reperfusion showed no increase ($k_{left}=0.0278\pm0.0013$). The enhanced PPIase activity in the ischemic hemisphere gradually decreased over time (day 4 : $k_{right}=0.0458\pm0.0023$) but was still significantly higher than control 7 days after reperfusion ($k_{right}=0.0400\pm0.0019$). In contrast, PPIase activity in the nonischemic hemisphere was lower than control 4 days ($k_{left}=0.0258\pm0.0009$) and 7 days ($k_{left}=0.0208\pm0.0006$) after reperfusion.

Inhibition of PPIase activity by immunosuppressants

The PPIase enzyme activity of the immunophilins in samples from the ischemic hemisphere 1 day after reperfusion was greatly inhibited by FK506 ($k_{FK506}=0.0370\pm0.0021$) and was mildly inhibited by rapamycin ($k_{Rap}=0.0444\pm0.0020$; Fig. 2). Cyclosporin-A, however, had no affect on PPIase activity ($k_{CsA}=0.0522\pm0.0026$).

FK506 dose dependance

FK506 inhibited PPIase activity in a dose-dependent manner ($k_{0.025}=0.0485\pm0.0030$, $k_{0.05}=0.4312\pm0.0027$, $k_{1.0}=0.02647\pm0.0012$; Fig. 3). The highest dose of FK506 (1.0 μg) reduced PPIase enzyme activity to control levels.

In vivo application of FK506

FK506 injection significantly decreased PPIase activity to levels lower than control ($k_{control}=0.02858\pm0.0015$; Fig. 4) and inhibited responses to ischemic insult ($k_{control}=0.00951\pm0.0005$, $k_{right}=0.00982\pm0.0005$, $k_{left}=0.00968\pm0.0006$).

Discussion

Immunosuppression and neuroprotection have

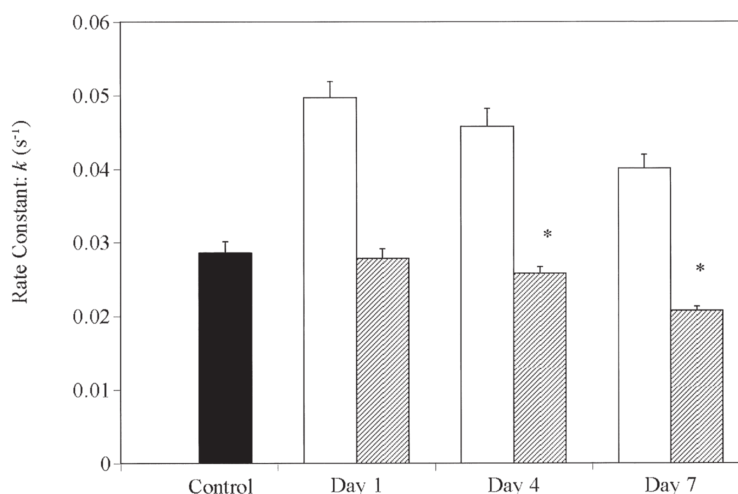

Fig. 1. PPIase activity of immunophilins in the brain increased after cerebral ischemia. Open bars represent the ischemic (right) hemisphere ; shadowed bars represent the non-ischemic (left) hemisphere. Note the gradual decrease in PPIase activity over time.

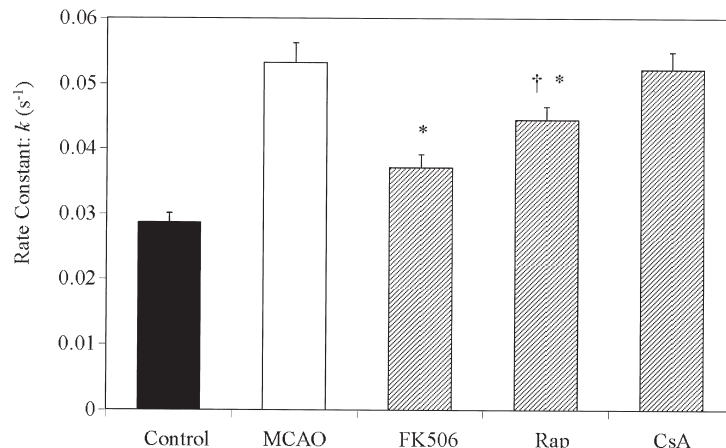

Fig. 2. PPIase activity was inhibited by FK506 and rapamycin, but not by cyclosporin-A. Immunosuppressants were applied to aliquots of samples from the ischemic hemisphere of rats killed after 1 day after transient MCA occlusion.

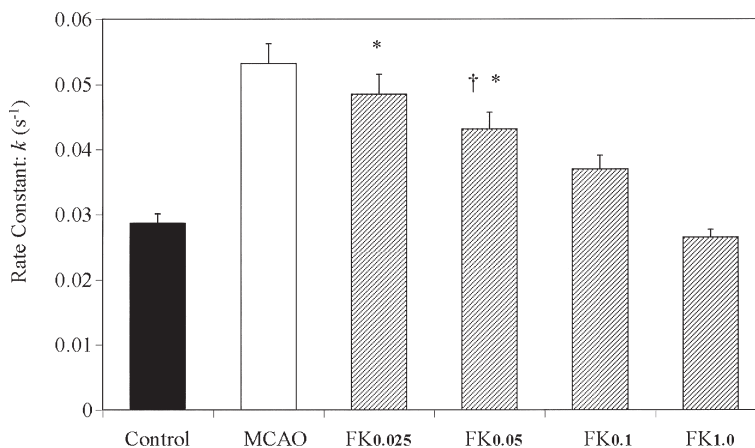

Fig. 3. PPIase activity was inhibited by FK506 in a dose-dependent manner. PPIase activity was decreased to control levels by a high dose (1.0 μg) of FK506.

become new subjects of investigation in the treatment of cerebral ischemia[15,17]. Although recent studies have shown that the immunosuppression with FK506 can ameliorate damage caused by cerebral ischemia, the mechanisms underlying this form of neuroprotection remain unclear[1-3].

Immunoactivation and inflammation can cause devastating secondary damage to brain cells during long-term recovery from ischemic insult[18]. In peripheral areas, scavenging of damaged tissues facilitates repair by allowing normal cells to proliferate. However, in the brain, where cell proliferation cannot be anticipated, such response can expand the boundaries of damage.

Activation of T-lymphocytes is triggered by a series of interactions between intercellular components, such as calcium, calmodulin, and calcineurin[4,5]. The interaction activates calcineurin, a serine threonine phosphatase, which dephosphorylates cytosolic factors and leads to the induction of interleukin-2, a strong activator of the immune system. The immunosuppressants cyclosporin-A and FK506 bind the immunophilins cyclophilin and FKBP, respectively, and form complexes that react with calcineurin and inhibit its phosphatase activity. Thus, by preventing production of interleukin-2, these drugs promote immunosuppression. In contrast, rapamycin forms a complex with the 12-kDa immunophilin FKBP12 and binds to the FKBP-rapamycin associating protein, which inhibits the interleukin-2-depen-

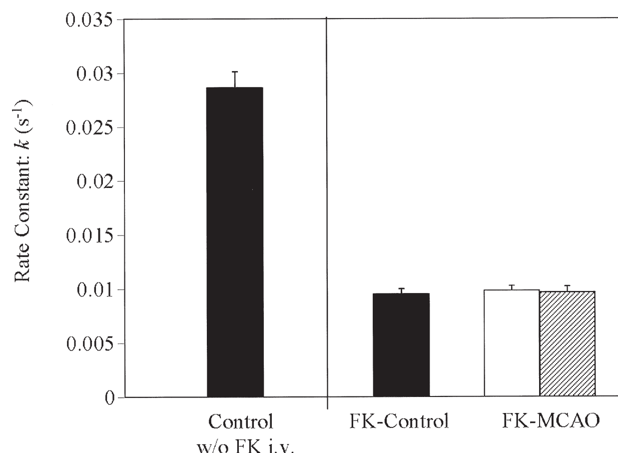

Fig. 4. *In vivo* application of FK506 inhibited PPIase activity in the brain. The closed bar to the left represents the control group without FK506 application; the closed bar to the right represents the control group with in vivo FK506 application; the open bar represents the ischemic (right) hemisphere with transient MCA occlusion and pretreatment with FK506; and the shadowed bar represents the nonischemic (left) hemisphere.

dent proliferation of the T-cell clones[9]. Whether the neuroprotective effects of FK506 are due to the inhibition of T-cell activation in general or to a more direct effect upon the afflicted brain cells is unknown.

The present study has shown that the PPIase enzyme activity of immunophilins increases in a rat model of cerebral ischemia. Because immunophilins regulate protein folding during protein synthesis and repair[10], the enhanced PPIase activity has been suggested to assist various molecular chaperones in the reconstruction of denatured proteins. However, the present study has shown that PPIase activity in the ischemic hemisphere is still enhanced 7 days after the initial ictus. Because molecular chaperones, such as HSP70, are induced soon after transient ischemia[19], the increased PPIase activity is likely to have functions in addition to assisting molecular repair.

The present study found that the enhanced PPIase activity is suppressed by application of FK506 or rapamycin, but not by cyclosporin-A. This finding suggests that the immunophilin responsible for the postischemic increase in PPIase activity belongs to the FKBP family. Furthermore, the moderate inhibition of PPIase activity by rapamycin suggests that FKBP12 is only partially responsible for this postischemic response. The dose-dependent inhibition of PPIase activity by FK506, in which high doses of FK506 reduced PPIase activity to control levels, further supports the possibility that the postischemic increase of PPIase activity in the brain is due to the FKBP immunophilin family and not to cyclophilins. The present findings are consistent with unpublished findings (personal communication: Tsutomu Araki, Department of Neurology, Tohoku University School of Medicine, Sendai) that show an increase in FK506 binding in the brain after transient cerebral ischemia in gerbils and rats.

The general distribution of immunophilins in the tissues of higher animals is not homogenous, and the level of immunophilins in the brain may be up to 40 times that in other organs[20]. This heterogeneity may provide further clues to the role of immunophilins in the normal brain. Also supporting the functional importance of immunophilin-calcineurin interaction in the normal brain is the colocalization in the brain of calcineurin and the FKBP family[6]. Because the brain comprises mostly nonproliferating cells, the presence of a signal transduction system involving the interaction of calcium, calmodulin, calcineurin, and the FKBP family of immunophilins might regulate the cascade of intracellular events triggering the initial response leading to immunoactivation and inflammation in the brain after cerebral ischemia. This possibility is further supported by results of in vivo application of FK506, which presents direct evidence that the neuroprotection by FK506 involves binding to the FKBP family. Furthermore, such mechanisms of neuroprotective immune-regulation in the brain may involve an unidentified internal factor, possibly analogous to the immunosuppressants, which, if confirmed, may help establish a new and highly effective means of preventing neuronal damage after cerebral ischemia.

References

1. Sharkey J, Butcher P. Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischemia. Nature 1994; 371: 336-9.
2. Tokime T, Nozaki K, Kikuchi H. Neuroprotective

effect of FK506, an immunosuppressant, on transient global ischemia in gerbil. Neurosci Lett 1996 ; 206 : 81–4.

3. Drake M, Friberg H, Boris–Moller F, Sakata K, Wieloch T. The immunosuppressant FK506 ameliorates ischaemic damage in the rat brain. Acta Physiol Scand 1996 ; 158 : 155–9.
4. Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP–FK506 complexes. Cell 1991 ; 66 : 807–15.
5. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T–lymphocyte activation. Nature 1992 ; 357 : 695–7.
6. Steiner JP, Dawson TM, Fotuhi M, Glatt CE, Snowman AM, Cohen N, et al. High brain densities of the immunophilin FKBP colocalized with Calcineurin. Nature 1992 ; 358 : 584–7.
7. Koletsky AJ, Harding MW, Handschumacher RE. Cyclophilin : distribution and variant properties in normal and neoplastic tissues. J Immunol 1986 ; 137 : 1054–9.
8. Siekierka JJ, Wiederrecht G, Greulich H, Boulton D, Hung SH, Cryan J, et al. The cytosolic–binding protein for the immunosuppressant FK–506 is both a ubiquitous and highly conserved peptidyl–prolyl cis–trans isomerase. J Biol Chem 1990 ; 265 : 21011–5.
9. Galat A. Peptidylproline cis–trans–isomerases : immunophilins. Eur J Biochem 1993 ; 216 : 689–707.
10. Lang K, Schmid FX, Fischer G. Catalysis of protein folding by prolyl isomerase. Nature 1987 ; 329 : 268–70.
11. Takahashi N, Hayano T, Suzuki M. Peptidyl–prolyl cis trans isomerase is the cyclosporin A binding protein cyclophilin. Nature 1989 ; 337 : 473–6.
12. Harding MW, Galat A, Uehling DE, Schreiber SL. A receptor for the immunosuppressant FK506 is a cis–trans peptidyl–prolyl isomerase. Nature 1989 ; 341 : 758–60.
13. Fruman DA, Burakoff SJ, Bierer BE. Immunophilins in protein folding and immunosuppression. FASEB J 1994 ; 8 : 391–400.
14. Sykes K, Gething M, Sambrook J. Proline isomerase function during heat shock. Proc Natl Acad Sci USA 1993 ; 90 : 5853–7.
15. Kogure T, Kogure K. Molecular and biochemical events within the brain subjected to cerebral ischemia (targets for therapeutical intervention). Clin Neurosci 1997 ; 4 : 179–83.
16. Fischer G, Bang H, Mech C. Determination of enzymatic catalysis for the cis–trans–isomerization of peptide binding in proline–containing peptides (in German). Biomed Biochim Acta 1984 ; 43 : 1101–11.
17. Shiga Y, Onodera H, Matsumoto Y, Kogure K. Cyclosporin A protects against ischemia reperfusion injury in the rat brain. Brain Res 1992 ; 595 : 145–8.
18. Giulian D, Vaca K, Corpuz M. Brain glia release factors with opposing actions upon neuronal survival. J Neurosci 1993 ; 13 : 29–37.
19. Chen J, Graham SH, Zhu RL, Simon RP. Stress proteins and tolerance to focal cerebral ischemia. J Cereb Blood Flow Metab 1996 ; 16 : 566–77.
20. Snyder SH, Sabatini DM. Immunophilins and the nervous system. Nature Med 1995 ; 1 : 32–7.
21. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989 ; 20 : 84–91.
22. Swain CG, Swain MS, Berg LF. Calculation of unbiased first–order rate constants. Acceleration by overrelaxation with a responsive overrelaxation factor. J Chem Inf Comput Sci 1980 ; 20 : 47–51.