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Research report

Infusion of FK506, a specific inhibitor of calcineurin, induces potent tau hyperphosphorylation in mouse brain

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Abstract

Calcineurin is a Ca^{2+}/calmodulin-dependent protein phosphatase expressed at high levels in brain. Many electrophysiological and pharmacological findings have shown that calcineurin plays an important role in brain function. FK506 is always used as a specific calcineurin inhibitor in these researches. But these reports did not quantify the calcineurin activity in FK506-treated brain. Here we first investigated the inhibitory effect of FK506 injected into the mouse brain ventricle on CN activity. FK506 reduced calcineurin activity in a dose-dependent manner, without affecting its amount. Injection of 12.5 nmol FK506 also significantly enhanced the phosphorylation of tau at Ser-262 (12E8 site), Ser-198, Ser-199, and/or Ser-202 (Tau-1 site) and Ser-396 and/or Ser-404 (PHF-1 site), without affecting total tau. It is suggested that calcineurin plays an important role in tau phosphorylation, dependently of its activity. Compared with the effects of cyclosporin A, another specific inhibitor of CN in our previous study, we first evaluate that such infusion of FK506 is more effective than that of cyclosporin A on calcineurin inhibition and tau phosphorylation.

Keywords: Phosphatase; Brain ventricle; Inhibitor; Calcineurin activity; Tau phosphorylation; Cyclosporin A

1. Introduction

Calcineurin (CN) is a highly abundant phosphatase in the brain and it is the only Ca^{2+}- and calmodulin-dependent protein serine/threonine phosphatase [21,8]. CN is widely distributed among eukaryotes, with an extremely high level of expression in mammalian brain and its structure is conserved from yeast to man. CN has a narrower range of substrates than those of other phosphatases. It dephosphorylates some important neuronal substrates, including cytoskeletal proteins such as tau. Tau is a major neuronal microtubule-associated protein. Its normal function is to promote the assembly of microtubules from tubulin subunits and to stabilize the microtubules. Phosphorylated tau, particularly at sites in or adjacent to microtubule binding domains (e.g. Ser-262), has a reduced affinity for microtubules. Hyperphosphorylation of tau causes itself to lose biological activity, become toxic, and aggregate into paired helical filaments (PHFs), the major component of neurofibrillary tangles (NFT) in Alzheimer's disease (AD) [9,6,10]. Tau phosphorylation is catalysed by tau protein kinases and reversed by tau protein phosphatases, including CN. CN can dephosphorylate hyperphosphorylated tau *in vitro* [7]. Inhibition of CN induces hyperphosphorylation of tau at multiple sites in cells [20]. Moreover, it has been reported that reduction of CN activity in brain by antisense oligonucleotides leads to persistent phosphorylation of tau protein [5].

Because CN is important in cellular signal transduction, it is involved in many biological processes, including immune responses and cardiac hypertrophy [19]. The studies on biological roles of CN have progressed to the important discovery that it is the target of the immunosuppressant drug cyclosporin A (CsA) and FK506, which can also be found as tacrolimus [3,13]. CsA and FK506 inhibit CN activity after forming complexes with cytoplasmic immunophilins, cyclophilins and FKBP [2]. The immunophilin–immunosuppressant complex binds CN and inhibits its function by sterically hindering the access of

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substrates to the catalytic site. Using CsA or FK506 as probes, the physiological roles of CN in brain can be revealed. Most electrophysiological and pharmacological studies including long-term potentiation (LTP), long-term depression (LTD) and behavioural evidence are surveyed in the hippocampal slices including our lab [15,16] and day-old-chick brains [1]. But unlike the studies *in vitro* [4,14], these reports did not quantify CN activity in brain tissues.

This project was designed to investigate the inhibitory effect of injected-FK506 on CN activity and tau phosphorylation. In addition, we focused on a comparison of these effects between FK506 and CsA.

2. Materials and methods

2.1. Materials

RII peptide was obtained from BioMoL Research Laboratories Inc. and [γ-^{32}P] ATP from Beijing Furi Biologic and Medicinal Engineering Co. (Beijing, P.R. China). The catalytic subunit of cAMP-dependent protein kinase was obtained from Promega Chemical Co. FK506 was from E. Merck Co. and okadaic acid (OA) was from Sigma–Aldrich. All other reagents were of standard laboratory grade and the highest quality available from commercial suppliers.

2.2. Surgical procedures for drug infusion

Male Balb/c mice weighing approximately 18 g were supplied by the Experimental Animals Center of Peking University. Animals were group housed under following laboratory conditions: temperature 20 ± 1 °C, humidity 40–60%, 12:12-L/D cycle, lights on at 08:00 h. Mice had free access to food and water. Animals were treated in accordance with the current law and the NIH Guide for Care and Use of Laboratory Animals. Each mouse was first anesthetized with urethane [1.5 g/kg, intraperitoneally (i.p.)] and placed on a stereotactic instrument with the incisor bar set 2 mm below the ear bars (*i.e.* flat skull). A 50-μL Hamilton syringe was stereotactically placed into the left ventricle at the coordinates for bregma and dura of AP-0.6, L-1.6 and V-2.4 (in mm) after the scalp had been incised and retracted. FK506 stock solution was diluted in aCSF (artifical cerebrospinal fluid) containing 140 mM NaCl, 3.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.2 mM Na_2HPO_4, pH 7.4 to the concentrations required for each experiment. Unless otherwise indicated, 5 μl solution per hemisphere of the appropriate concentration was administered into the left ventricle, while the same volume of an equivalent concentration of DMSO diluted in aCSF was used for control purposes. After injection, the scalp was sewn up immediately. All surgical procedures were performed under sterile conditions, and penicillin was injected to prevent infection.

2.3. Preparation of mouse brain extracts

Mice were killed at appropriate time after injection of FK506. The brains were immediately removed and homogenized on ice with a syringe at 4 °C in 50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.2% NP-40, 1.0 mM phenylmethylsulfonyl, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 2 μg/ml pepstatin. The tissue homogenates were centrifuged at 16,000 × *g* and the supernatants were used to assay the phosphatase activity. The protein concentration was determined by the method of Bradford.

2.4. Protein phosphatase assay

RII peptide was ^{32}P-labeled using the catalytic subunit of cAMP-dependent protein kinase as described [17]. The protein phosphatase activity in mouse brain extracts was measured mainly as described [22] except for some modifications. The prepared mouse brain extracts were diluted 1:150 (v/v) in 2× assay buffer (100 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM $MnCl_2$, 1 mM DTT, 0.05% NP-40, 0.25 μM CaM, 200 nM OA) or 2× EGTA buffer (100 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM $MnCl_2$, 1 mM DTT, 0.05% NP-40, 0.32 mM EGTA, 0.25 μM CaM, 200 nM OA). 10 μl of diluted brain extract was mixed with 10 μl of 40 μM ^{32}P-labeled RII peptide dissolved in MilliQ water, and incubated for 10 min at 30 °C. The reaction was terminated by addition of 180 μl of 83.3 mM H_3PO_4. The released inorganic ^{32}P was quantitated by liquid scintillation counting. Phosphatases activities were presented in form of nanomoles of phosphate released/mg of protein/min at 30 °C. CN activity was calculated as the difference in protein phosphatase activity in 2× assay buffer and 2× EGTA buffer. Each sample was determined in triplicate. The levels of all CN activity were expressed as relative to CN activity in control mice.

2.5. Western blot analysis

Phosphorylation of tau at various sites was determined by Western blotting as described [5]. The monoclonal antibodies (mAb) used were site-specific and phosphorylation-dependent tau antibodies, including PHF-1 against PHF-tau phosphorylated at Ser-396 and/or Ser-404, mAb Tau-1 against PHF-tau unphosphorylated at Ser-198, Ser-199, and/or Ser-202, and mAb 12E8 against PHF-tau phosphorylated at Ser-262. Anti-tau and anti-CNA are polyclonal antibodies against total tau and the catalytic subunit A of CN (CNA). The blots were developed with anti-Tau (1:1000), anti-CNA (1:400), Tau-1 (1:500), PHF-1 (1:100), or 12E8 (1:1000) antibodies and visualized with an enhanced chemiluminescence kit (Pierce) followed by exposure to Kodak X-Omat BT film. The immunoreactivity of the tau bands was analyzed quantitatively with BIO-RAD Quantity One software and expressed as the sum of optical density. The levels of total tau,

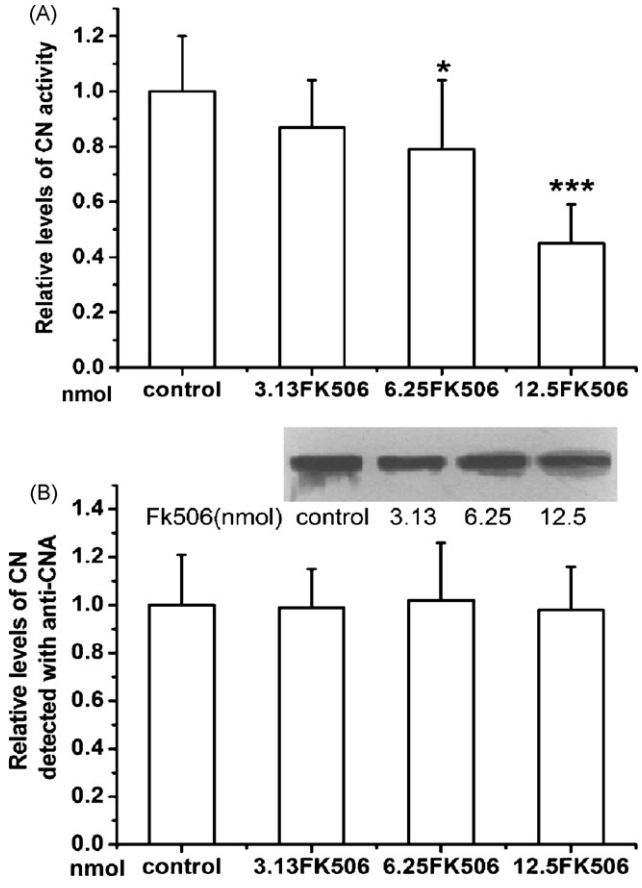

Fig. 1. FK506 inhibits CN without altering the amount of CN in the mouse brains. The activities and amounts of CN in mouse brains collected 24 h after injection with vehicle, various concentrations of FK506 were determined. The phosphatase activity of CN in the mouse brain extracts was measured using ^{32}P-labeled RII peptide substrate. Each sample was determined in triplicate (A). Anti-CNA antibody was used to measure total CN by Western blotting (B). All data are expressed as mean ± S.D. of 10 mice. *$p < 0.05$, ***$p < 0.001$ compared with control.