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The involvement of glycogen synthase kinase-3 and protein phosphatase-2A in lactacystin-induced tau accumulation

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Abstract **Here, we demonstrated that lactacystin inhibited proteasome dose-dependently in HEK293 cells stably expressing tau. Simultaneously, it induces accumulation of both non-phosphorylated and hyperphosphorylated tau and decreases the binding of tau to the taxol-stabilized microtubules. Lactacystin activates glycogen synthase kinsase-3 (GSK-3) and decreases the phosphorylation of GSK-3 at serine-9. LiCl inhibits GSK-3 and thus reverses the lactacystin-induced accumulation of the phosphorylated tau. Lactacystin also inhibits protein phosphase-2A (PP-2A) and it significantly increases the level of inhibitor 1 of PP-2A. These results suggest that inhibition of proteasome by lactacystin induces tau accumulation and activation of GSK-3 and inhibition of PP-2A are involved.**

Keywords: Proteasome; Tau; Hyperphosphorylation; Glycogen synthase kinase-3; Protein phosphatase-2A

1. Introduction

The formation of neurofibrillary tangles is one of the recognized hallmark lesions seen in Alzheimer disease (AD) and other tauopathies, and the abnormally hyperphosphorylated tau is the major proteinous component in the tangles [1,2]. Tau is a major microtubule-associated protein. The normal function of tau is to promote microtubule assembly and stabilize the formed microtubules, and thus to establish cellular polarity and maintain the intracellular transport of neurons [3]. When tau is hyperphosphorylated and accumulated in the cells, it becomes incompetent in executing the above biological functions and thus leads to disruption of microtubules.

The upstream factor leading to the accumulation of tau in AD brain is still not fully understood. Previous studies have demonstrated that, among various abnormal posttranslational modifications, hyperphosphorylation accelerates tau accumulation [4]. Tau is a phosphoprotein, and the phosphorylation of tau is regulated by protein kinases and phosphatases. Among various kinases and phosphatases, glycogen synthase kinase-3 (GSK-3) and protein phosphatase-2A (PP-2A) are, respectively, the crucial kinase and phosphatase for the AD-like tau hyperphosphorylation [5–7].

In the neurofibrillary tangles of AD brains, tau is not only hyperphosphorylated but also ubiquitilated [8], suggesting a link between the hyperphosphorylated tau and the proteasome proteolytic system in the accumulation of tau. Proteasome system is responsible for the proteolysis of the majority cellular proteins. However, controversial results have been reported regarding to whether or not tau protein is a substrate of the proteasome [9–12]. We demonstrated recently that the in vitro phosphorylated recombinant tau was proteolyzed as efficiently as normal tau by 20S proteasome [13]. Another recent study also showed that phosphorylation of tau is a signal for its degradation by ubiquitin-proteasome system [14]. As normal proteasome can efficiently degrade tau protein, it is suggested that the accumulation of tau may be caused by an impaired proteasome system, which is indeed observed in AD brain [15,16]. Therefore, in addition to hyperphosphorylation, the damaged proteasome may be another upstream factor responsible for the accumulation of tau in AD brain.

To investigate the role of proteasome in tau accumulation and the underlying mechanisms, we in the present study utilized lactacystin, an irreversible inhibitor of 20S proteasome, to inhibit the proteasome activity in HEK293 cells stably expressing tau441. And then we investigated the effect of proteasome inhibition on tau accumulation and the involvement of GSK-3 and PP-2A. We have demonstrated that lactacystin inhibits proteasome activity dose-dependently with a concomitant accumulation of tau and dissociation of tau from microtubules. Lactacystin also activates GSK-3 and inactivates PP-2A. The activation of GSK-3 involves down regulation of the ser-9-phosphorylated kinase and the inhibition of PP-2A is relevant to the elevation of PP-2A inhibitor 1 (I_1-PP-2A).

2. Materials and methods

2.1. Antibodies and chemicals

Rabbit polyclonal antibodies (pAb) PS214, PS262 and PT231 against tau phosphorylated at Ser-214, Ser-262 and Thr-231 were purchased from Biosource International (Camarillo, CA, USA). pAb against total GSK-3β and phospho-GSK-3β at Ser-9 and pAb against PP2A C subunit were from Cell Signaling Technology, Inc. (Beverly, MA, USA). pAb against I_1PP2A was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). pAb R134d

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to total tau and monoclonal antibody (mAb) PHF-1 against tau phosphorylated at Ser-396/404 were gifts from Dr. Inge Grundke-Iqbal (NYS Institute for Basic Research, SI, NY, USA) and Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY, USA). mAb Tau-1 against tau non-phosphorylated at Ser198/199/202 was from Chemicon International Inc. (Temecula, CA, USA). mAb Tau-5 against total tau was from Lab Vision Corp. (Fremont, CA, USA). mAb DM1A to α-tubulin were from Sigma (St. Louis, MO, USA). Phospho-GS peptide, a specific GSK-3 substrate, was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA). LiCl, a widely used inhibitor of GSK-3, and other chemicals were from Sigma Chemical Co. (St. Louis). [γ-^{32}P]ATP was from Beijing Yahui Biologic and Medicinal Engineering Co. (Beijing, China). Cell culture media were from Gibico (Grand Island, NY, USA). Lactacystin and cycloheximide was from CalBiochem (San Diego, CA, USA), and it was dissolved in dimethyl sulfoxide (DMSO, 0.1% final). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

Wild type human embryonic kidney (HEK293) cells were cultured in DMEM supplemented with 10% fetal bovine serum and maintained in 37 °C and 5% CO_2. The cells were stably transfected with the longest human tau (tau 441) using Lipofectamine 2000 transfection kit according to the manufacturer's instructions. Single cell clones were generated by selection with G418 (250 μg/ml), and the transfection and expression of the target genes were identified by Western blot.

2.3. Assay of proteasome activity

The proteasome activity was measured as described previously [17]. Briefly, the cells were homogenized with buffer containing 12.5 mM KCl, 135 mM Tris–acetate (pH 7.5), 80 mM EGTA, 6.25 mM 2-mercaptoethanol and 0.17% octyl-β-D-glucopyranoside, and centrifuged at $12\,500 \times g$ for 15 min at room temperature. Then, the supernatant fraction was collected and protein concentration was estimated by Bradford method [18]. To determine chymotrypsin-like activity of proteasome, 100 μg of proteins were incubated for 30 min at 37 °C with 100 μl of 100 mM Hepes–HCl (pH 7.5) containing 50 μM of substrate (Ala-Ala-Phe-7-amido-4-methyl coumarin), the reaction was stopped by addition of 100 μl of 220 mM sodium acetate buffer and placed for 30 min at 4 °C. The precipitated non-degraded protein was separated at $400 \times g$ for 30 min at 4 °C and 200 μl aliquot of the supernatant was mixed with 2 ml of distilled water. Fluorescence of the samples was measured with F 4500 Spectrofluorometer (Sigma) at 370 nm excitation and 430 nm emission wavelengths.

2.4. Assay of the phosphorylated tau

The phosphorylation of tau at various sites was determined by Western blotting. In briefly, cells were homogenized at the desired time points in 80 μl RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% (v/v) NP-40, 0.1% (w/v) SDS, 100 μM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride and 1 μg/ml each of leupeptin, aprotinin and pepstatin A. The cell lysates were boiled, sonicated briefly and centrifuged for 15 min at $12\,500 \times g$. Protein concentrations in the supernatant were determined using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Cheshire, UK) with the bovine serum albumin as standard. Equal amount of proteins was isolated on 10% SDS polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Hybond C-Super, Amersham Pharmacia Biotech, Amersham, UK). The blots were blocked in 5% (w/v) non-fat milk powder in 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.02% (v/v) Tween-20 (TTBS) and probed with PS214 (1:1,000), or PS262 (1:500), or PT231(1:1000) or Tau-1 (1:30,000), or PHF-1 (1:500), or Tau-5 (1:200), or R134d (1:5000),or DM1A (1:1000), or I_1PP2A (1:600) or PP2A C subunit (1:1000) overnight at 4 °C. The blots were developed with horseradish peroxidase-conjugated secondary antibodies (1:5000) and visualized by enhanced chemiluminescent substrate kit and exposure to CL-XPosure film (Pierce). The immunoreactivity of tau was quantitatively analyzed by Kodak Digital Science 1D software (Eastman Kodak Co., New Haven, CT, USA) and expressed as mean optical density. The level of total tau was expressed as relative level of the mean optical density against control or normalized against β-actin as indicated in the figure legends, and the level of the phosphorylated tau was normalized to total tau.

2.5. Assay of microtubule-binding activity of tau

Microtubule-binding activity of tau was measured by the method described previously [19,20]. In brief, the cells were harvested in a high-salt reassembly buffer (100 mM Tris, 0.5 mM $MgSO_4$, 1 mM EGTA, 2 mM dithiothreitol, and 750 mM NaCl, pH 6.8) supplemented with 0.1% Triton X-100, 20 μM taxol, 2 mM GTP, and a mixture of protease inhibitors (2 mM phenylmethylsulfonyl fluoride, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanon, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-hydrochloride, leupeptin, pepstatin A, and soy bean trypsin inhibitor; 1 μg/ml each) at 37 °C. Cell lysates were homogenized with 15 strokes in a warm Dounce homogenizer and then immediately centrifuged at $50\,000 \times g$ for 20 min (25 °C). The supernatant (S) containing unbound tau was removed and the remaining pellet (P) was resuspended in sample buffer. After measurement of the protein concentration, the samples were subjected to Western blot analysis as above. The ratio of tau bound to microtubules (P) versus unbound (S) was assessed by comparing the immunoreactivity of tau in these two fractions.

2.6. Assays for kinase and phosphatase activities

Cells were lysed with 100 ml buffer containing 50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 10 mM β-ME, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, 2 mM benzamidine, and 1 μg/ml each of leupeptin, pepstatin A and aprotinin at 4 °C for 10 min. The lysates were then centrifuged at $16\,000 \times g$ for 15 min. Equal volume of phosphatase inhibitor cocktail (1 mM Na_3VO4 and 10 mM NaF, pH 7.0) was added to the supernatants for assay of GSK-3 activity as described [21–23]. For the assay of PP-2A activity, phosphorylase-b (2 mg/ml) was phosphorylated into phosphorylase-a by incubating it for 10 min at 30 °C in 40 mM Tris–HCl (pH 8.5), 20 mM β-ME, 0.2 mM $CaCl_2$, 15 mM $MgCl_2$, 0.5 mM γ-^{32}P-ATP, and 10 mg/ml phosphorylase kinase. The product of γ-^{32}P-phosphorylase was separated from free ATP on a Sephadex G-50 column, and the protein-containing fractions were collected. The activity of PP-2A toward γ-^{32}P-phosphorylase-a was assayed by the release of γ-^{32}P as described previously [24,25]. The reaction was carried out in a 20 μl reaction mixture containing 50 mM Tris (pH 7.0), 10 mM β-ME, 0.1 mM EDTA, 7.5 mM caffeine, 7.5 ng/ml γ-^{32}P-phosphorylase-a, 0.2 μg/μl inhibitor 1 (PP-1 specific inhibitor), and 0.06 mg/ml cell lysates. The reaction was started by adding γ-^{32}P-phosphorylase-a, after incubation for 30 min at 30 °C, the reaction mixture (5 μl) was spotted on a chromatography paper already spotted with 10 μl stop solution, i.e., 4 mM cold ATP in 20% trichloroacetic acid (TCA). The released γ-^{32}P was separated from the substrate by ascending chromatography in 5% TCA in 200 mM NaCl, and the radioactivity was counted by liquid scintillation counter (A-5082, TECAN, Austria).

2.7. Statistical analysis

Data were analyzed with SPSS statistical software. The one-way analysis of variance procedure followed by least significant difference post hoc tests was used to determine the statistical significance of differences of the means.

3. Results

3.1. Effect of proteasome inhibition on total tau and its biological activity

HEK293 stably expressing human tau441 (HEK293/tau441) was utilized for the study. After treatment of the cells with 2.5, 5 and 10 μM lactacystin for 24 h, we observed that the activity of proteasome decreased in a dose-dependent manner (Fig. 1A). This result confirms the inhibitory effect of lactacystin in the experimental system. Then we measured the level of total tau after lactacystin treatment. It was shown