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LOVASTATIN SUPPRESSES THE ABERRANT TAU PHOSPHORYLATION FROM FTDP-17 MUTATION AND OKADAIC ACID-INDUCTION IN RAT PRIMARY NEURONS

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Abstract—Statins are a class of cholesterol-lowering drugs and have been suggested therapeutic use for neurodegenerative diseases including Alzheimer's disease (AD). Our recent studies revealed a neuronal protective effect of lovastatin (LOV) from N-methyl-D-aspartic acid (NMDA) excitotoxicity. The neuroprotective mechanism of statins, however, is far unknown. Here we demonstrated that LOV suppressed the aberrant tau phosphorylation both from frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) mutation and okadaic acid (OA) induction in cultured rat primary neurons. The protective effect of LOV occurred at multiple pathological sites of tau protein, including Tyr181, Tyr231 Ser202/Tyr205, Tyr212/Ser214 and Ser396/Ser404. Further analysis revealed that the potential mechanism of the suppressive effect of LOV resulted from two aspects, activating OA-inhibited protein phosphatase 2A (PP2A) activity and attenuating OA-induced activity of tau kinases CDK5/P25 and CDK2/4, but not glycogen synthase kinase 3β (GSK3β). These findings give new insights into the molecular mechanism of LOV-mediated neuroprotective effect and provide experimental evidence for its therapeutic use in AD.

Key words: lovastatin, okadaic acid, tau phosphorylation, Alzheimer's disease, PP2A, CDK.

INTRODUCTION

Cholesterol dyshomeostasis is associated with the pathophysiology of sporadic Alzheimer's disease (AD) (Chen and Fernandez, 2001; Burns and Duff, 2002; Wolozin, 2002; Solomon et al., 2009; Di Paolo and Kim, 2011), which is pathologically characterized by extracellular senile plaques consisting of aggregated amyloid-β (Aβ) peptides and intracellular neurofibrillary tangles (NFTs) containing hyperphosphorylated tau proteins. Statins, HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitors, are a class of cholesterol-lowering drugs and show a beneficial effect in preventing the progress of AD-included neurodegenerative diseases (McGuinness et al., 2010; Roy and Pahan, 2011; Shepardson et al., 2011; Wang et al., 2011). Statins exert their neuroprotective effects in both cholesterol-dependent and -independent pathways (Van der Most et al., 2009). Statin treatment increased secreted amyloid precursor protein (sAPP)α production (Ma et al., 2009) and reduced Aβ generation (Wolozin et al., 2006; Höglund and Blennow, 2007), which might result from a statin-induced protein isoprenylation (Ostrowski et al., 2007) or/and the altered activity of secretases (Parsons et al., 2007; Postina, 2012). Recently, statins were reported to reduce the NFT burden in a mouse model of tauopathy and modulate the phosphorylation of its major constituent tau as well (Boimel et al., 2009). However, the underlined mechanism is unclear.

Abnormal hyperphosphorylation of tau protein has been implicated in the pathogenesis of AD and other tauopathies, such as Pick's disease and frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) (Lee et al., 2001 and Pei et al., 1997). The aberrant phosphorylation of tau may result from the mutation of tau itself or the imbalanced activities of tau kinases and phosphatases. It has been reported that the R406W mutation reduced the phosphorylation of Tau at Ser396 and Ser404, and resulted in less solubility and less capability of binding to microtubules than wild type tau (Pérez et al., 2000; Vogelsberg-Ragaglia et al., 2000). On the other hand, tau phosphorylation is normally well regulated by tau kinases and phosphatases. The former includes glycogen synthase kinase (GSK-3)-α and -β, mitogen-activated protein kinase (MAPK) family, CDK2 and CDK5, among which GSK-3β and CDK5 can phosphorylate tau at most of the known AD sites (Gong et al., 2006; Wang et al., 2013). For tau phosphatases, protein phosphatase 2A (PP2A) accounts for the major *in vivo* tau phosphatase activity in the human brain (Liu et al., 2005) and its expression and activity are reduced in selected areas of Alzheimer's brains (Gong et al.,

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Abbreviations: AAV, adeno-associated virus; ACAT, acyl-CoA: cholesterol acyltransferase; AD, Alzheimer's disease; CDK, cyclin-dependent kinase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FTDP-17, frontotemporal dementia and Parkinsonism linked to chromosome 17; GSK3, glycogen synthase kinase 3; LDH, lactate dehydrogenase; LOV, lovastatin; NFTs, neurofibrillary tangles; OA, okadaic acid; PP2A, protein phosphatase 2A; sAPP, secreted amyloid precursor protein.

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1993). The balance might be also broken by some chemicals, such as okadaic acid (OA), an inhibitor of PP2A and PP-1. OA has been extensively used to induce the phosphorylation of tau in cultured mammalian cells (Ho et al., 1997; Zhang and Simpkins, 2010). OA is also involved in cholesterol metabolism by stimulating acyl-CoA:cholesterol acyltransferase (ACAT) in isolated rat hepatocytes (Hernández et al., 1997).

In the present study, we examined the effect of lovastatin (LOV) on the aberrant tau phosphorylation from FTDG-17 mutant tau T40R406W and T40V337M and OA-induced hyperphosphorylation in cultured rat primary neurons, and investigated the underlying mechanism using OA-induction model, which provide more evidence to support the therapeutic use of statins in AD.

EXPERIMENTAL PROCEDURES

Cell culture, agent treatment and infection with adeno-associated virus (AAV)

Rat primary neurons were isolated from embryos of Sprague–Dawley rats at embryonic day 17 as described previously (Ma et al., 2009). Isolated neurons were plated into precoated 6-well plates with poly-D-lysine (100 μg/ml) for immunoblotting assay or 96-well plates for lactate dehydrogenase (LDH) assay. The cultured neurons at 14-day *in vitro* were used for experiment assay. LOV (Sigma) was added at 500 nM and incubated for 4 days as show no toxicity (Ma et al., 2009). OA (Sigma) treatment was performed at dose 30 nM for 1 h or as indicated in specific experiment. The solvent of 0.1% DMSO was used as treatment control.

AAV expressing FTDP-17 mutant tau, T40R406W and T40V337M, were gifts from Dr. Francesca-Fang Liao (University of Tennessee Health Science Center, Memphis, TN). For infection of rat primary neurons, 10-day-old cultures were used. The infection was perform at four multiplicity of infection in a one-third-volume reduced medium for 1 h, and then the medium was rendered back to its original volume. Neurons were further cultured up to 2 days *in vitro* when the experiments were performed.

Immunoblotting and antibodies

Protein extracts were prepared in ice cold lysis buffer (10 mM Tris/Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 5 mM DTT, 1% NP-40, and protease inhibitors cocktail). 20-μg total proteins quantified with Lowry methods were analyzed on Novex 4–20% Tris–glycine gel (Invitrogen) and transferred to PVDF membrane. In Western blot, primary antibodies used were: Tau Y5 (1:1000; Biosource), PHF-1 (Ser396/Ser404; 1:1000; Biosource), AT270 (Tyr181; 1:1000; Innogenetics), AT180 (Tyr231, 1:1000; Pierce Endogen), AT8 (Ser202/Tyr205, 1:1000; Pierce Endogen), AT100 (Tyr212/Ser214, 1:1000; Pierce Endogen), GSK3α/β and phosphor-GSK3α/β(Ser21/9) (1:1000; Cell Signaling), phosphor-GSK3α/β (Tyr279/216) (1:1000; ECM Bioscience), Cyclin D1(DCS6) (1:1000; Cell Signaling), β-actin (1:5000; Sigma), other antibodies PP2A, phosphor-PP2A, CDK5, P35/P25, P27, Cyclin B1 and Cyclin E (1:1000, Santa Cruz).

LDH activity assay

LDH activity assay was performed with Cytotoxicity Detection Kit (LDH, Roche-Applied Science) according to the procedure of manufacture. 1% Triton X-100-treated samples were used as positive control.

PP2A enzyme activity assay

PP2A activity assay was performed as described (Gong et al., 1993). PP2A activities were measured in 50 mM Tris–HCl buffer, pH 7.0, containing 1.0 mM EGTA, 0.03% Brij35, 2.0 mM $MnCl_2$, 10 mM $MgCl_2$, 200 μM trifluoperazine, 14 mM β-mercaptoethanol, and 1.0 μM [^{32}P]phosphorylase kinase. The reaction was started at 30 °C by the addition of ^{32}P substrate. After 20 min, the reaction was terminated by the addition of one-third volume of stop solution containing 240 mM Tris–HCl, pH 6.8, 12% sodium dodecyl sulfate (SDS), 20% β-mercaptoethanol, 40% glycerol, and 0.2% bromophenol blue. The reaction mixture was then separated by 7.0% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was stained, destained, and then β-subunit of phosphorylase kinase was excised from the gel and the ^{32}P radioactivity of the subunit was determined by Cerenkov counting. The activities of PP2A were calculated from the counts released from the α-subunit of phosphorylase kinase.

GSK-3 activity assay

GSK-3 activity was analyzed with TAU [pS396] human ELISA kit (Lifetechnologies) following the procedure provided by the manufacturer. Briefly, neuronal lysates in 100-μl standard diluent buffer was incubated in microtiter at room temperature for 2 h. To detect GSK-3 activity, 100-μl HuTau [pS396] detection antibody solution was added and incubated for 1 h at room temperature. After the incubation with anti-Rabbit IgG HRP working solution and stabilized with chromogen, the microtiter plate was read at 450 nm.

Statistics

The immunoblotting results from three to five experiments were measured with Image J software (NIH). Data were analyzed with software GraphPad Prism 4.0, and presented as mean ± SE. For comparison, the Bonferroni test was used; results were reported as significant only when $p < 0.05$.

RESULTS

LOV suppresses the phosphorylation of FTDP-17 mutant tau

To examine the effect of statins on the aberrant phosphorylation from tau mutation, we overexpressed FTDP-17 mutant tau, both T40R406W and T40V337M, in primary neurons by infection with adenovirus.