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Changes in p35/cdk5 System and Mitotic Phosphoepitopes in Okadaic Acid-induced Neurodegeneration: Relevance to Alzheimer's Disease

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ABSTRACT

Hyperphosphorylation of tau is a characteristic feature of the neurodegenerative pathology in Alzheimer's disease (AD), and glycogen synthase kinase-3β (GSK-3β) and the cdk5/p35 system are thought to play important roles in tau hyperphosphorylation. Okadaic acid, which increases tau phosphorylation and neuronal death, has been used as a research model of AD. We previously showed that GSK-3β was inactivated during okadaic acid-induced neurodegeneration. To extend this observation, we have assayed the involvement of the p35/cdk5 system in okadaic acid-induced neurodegeneration. We observed a significant increase in p35 cleavage during okadaic acid-induced neuron degeneration, indicative of cdk5 activation. We also observed sequestration of cdk5 in the nucleus of the neuron, indicating that cdk5 may contribute to neuronal dysfunction rather than to cytoskeleton disruption in the cytosol. We also observed enhanced MPM2 immunoreactivity in the cell bodies of degenerating neurons. These findings provide evidence for the aberrant expression of mitotic cell cycle proteins during the pathogenesis of neuronal degeneration processes, such as those occurring in AD.

Key words: Alzheimer's disease, okadaic acid, p35, Cdk5, tau phosphorylation

INTRODUCTION

Alzheimer's disease (AD) is characterized by the extensive loss of neurons accompanying neurofibrillary tangles (NFTs) and senile plaques (Gomez-Isla et al., 1997). NFTs are found in neuronal cell bodies and are composed of abnormally phosphorylated tau protein. The numbers of NFTs and dystrophic neurites are thought to correlate closely with the degree of dementia (McKee et al., 1991; Arriagada et al., 1992; Terry et al., 1994). During the development of AD, the formation of NFTs appears to undergo progressive sequential changes (Braak et al., 1994; Su et al., 1994; Delacourte et al., 1999), but the mechanisms involved in the formation of NFTs in AD are not clearly defined.

Tau is a microtubule-associated protein that promotes the polymerization of microtubules and stabi-

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lizes neurites. Phosphorylation of normal tau protein, however, inhibits microtubule assembly, and the consequent compromised axoplasmic and dendroplasmic transports are believed to be responsible for AD pathology (Alonso et al., 1996). Aberrant phosphorylation of tau is therefore thought to be a critical step in the formation of NFTs.

Although abnormal kinase activity or decreased phosphatase activity is commonly regarded as the mechanism underlying tau hyperphosphorylation (Gong et al., 1995; Lovestone and Reynolds, 1997), the identity of the enzyme(s) that perform this function in vivo and the mechanisms leading to tau hyperphosphorylation in AD are both not known. Among the kinases involved in tau hyperphosphorylation, the most important are glycogen synthase kinase-3 β (GSK-3β) and the cdk5/p35 system. We and others have shown, however, that GSK-3β was inactivated at sites at which tau was actively phosphorylated (Ferrer et al., 2002; Swatton et al., 2004; Yoon et al., 2005). Cdk5 is a Ser/Thr protein kinase that is highly enriched in neurons, colocalizes to the cytoskeleton and contributes to the phosphorylation of tau (Kobayashi et al., 1993; Lew et al., 1994). Recently, p25, a truncated form of p35, was reported to accumulate 20～40 fold in the AD brain, and the conversion of p35 to p25 was found to cause prolonged activation of cdk5, suggesting that cdk5 may play a specific role in this process (Patrick et al., 1999; Swatton et al., 2004). Contradictory findings, however, have cast doubt on the involvement of p25 in neurodegenerative conditions such as AD and Down's syndrome (Yoo and Lubec, 2001; Tandon et al., 2003). We therefore assayed the involvement of cdk5 in tau phosphorylation using the okadaic acid-induced neurodegeneration model, and in AD.

MATERIALS AND METHODS

Neuron culture

Primary cultures of rat cortical neurons were prepared from the brains of pups at embryonic-day 16. Briefly, the cerebral cortices were dissected in calcium-and magnesium-free Hank's balanced salt solution and incubated with a 0.125% trypsin solution for 10 min at 37°C. Trypsin was inactivated with Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum, and the cortical tissue was further dissociated by serial trituration using Pasteur pipettes. The resulting cell suspensions were diluted in neurobasal medium supplemented with B27 components (GibcoBRL, Grand Island, NY) and plated onto poly- D-lysine- (Sigma, 50μg/ml) and laminin- (1μg/ml, GibcoBRL, Grand Island, NY) coated 48-well plates at a density of 5×10^4 cells per well. Neurons were maintained at 37°C in a 5% CO_2 atmosphere for 12 days prior to the addition of okadaic acid. Okadaic acid (1μM, Boehringer Mannheim, Germany) was dissolved in 0.1% dimethyl sulfoxide (DMSO).

Western blot analysis

Cultures were solubilized directly in sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 2.5% glycerol, and 0.5% 2-β-mercaptoethanol), boiled at 100°C for 5 min, and stored at -20°C until use. Equal amounts of protein were resolved by SDS-PAGE at a constant voltage (130 V) and subsequently transferred to a polyvinylidene difluoride membrane (pore size, 0.2μm; Biorad) at 200 mA for 2 h. After incubation for 1 h in blocking TTBS buffer (10 mM Tris, pH 7.4, 100 mM NaCl, and 0.1% Tween-20) containing 2% bovine serum albumin (BSA) with 5% nonfat milk, the blots were incubated with the primary antibodies overnight at 4°C. Since changes in cdk5 activity can be determined by comparing the ratio of p25 to p35, we used p35 polyclonal C-19 antibody (Santa Cruz Biotechnology). The blots were subsequently washed in TTBS buffer and incubated with secondary antibody coupled to horseradish peroxidase for 1 h at room temperature. Bands were visualized using enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL) and x-ray film.

Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min, and membranes were permeabilized by incubation in 0.05 M Tris-Buffer containing 0.1% Triton X-100 and 2% horse serum for 30 minutes. The cultures were incubated overnight at 4°C with monoclonal antibody against cdk5 (1 : 100, Santa Cruz Biotechnology) or MPM2 (1 : 200, Upstate), washed and incubated with biotinylated secondary antiserum (1 : 200), streptavidine-

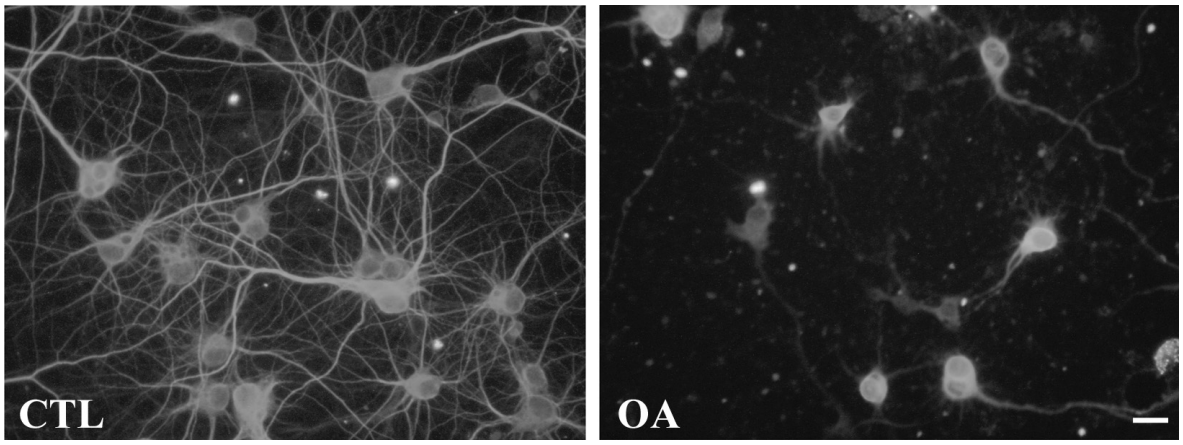


Fig. 1. MAP-2 immunostaining of neuronal cultures 24 h after treatment with okadaic acid (OA). CTL (Control neuron), Scale bar, 20μm.

peroxidase (1 : 300, Vector Labs, CA), and 0.05% diaminobenzidine (DAB) in 0.01% H_2O_2.

RESULTS

Treatment of cultured rat neurons with 10 nM of okadaic acid for 24 hr induced marked neuronal changes, including degeneration of neurites, followed by detachment and death (Fig. 1). To determine the protein levels of p35 and cdk5, whole cell lysates of neurons cultured for 4, 8, 16, 24, and 48 hr with okadaic acid were prepared and assayed by Western blotting with anti-p35 and anti-cdk5 antibodies. We observed conversion of p35 into p25, but no alteration in cdk5 expression, during okadaic acid-induced neurodegeneration (Fig. 2).

Conversion of p35 into p25 has been reported to cause prolonged activation and mislocalization of cdk5 (Patrick et al., 1999). To investigate whether treatment with okadaic acid induces a change in localization of cdk5, we immunocytochemically stained cultured rat neurons with antibody to cdk5 (Fig. 3). In control neurons, cdk5 was mainly localized in the cytoplasm, whereas, following okadaic acid treatment, cdk5 immunoreactivity in degenerating neurons was concentrated in the nuclei. Most of the okadaic acid treated neurons were devoid of neurites and cytoskeletal collapse, including tau phosphorylation, was observed in their cytoplasm (Kim et al., 1999). Thus, in addition to disrupting the cytoskeleton, cdk5 may contribute to neurodegeneration by its aberrant nuclear localization.

Two regulators of the eukaryotic cell cycle, cyclin-dependent kinase 4 and cell division cycle 2, have been reported to be related to AD pathology (Busser et al., 1998; Tsujioka et al., 1999). Mitotic phosphoepitopes (MPM-2), which are common to mitosis and degenerating neurons, also reappear during neurodegeneration in AD (Vincent et al., 1998). These findings are compatible with the hypothesis that the

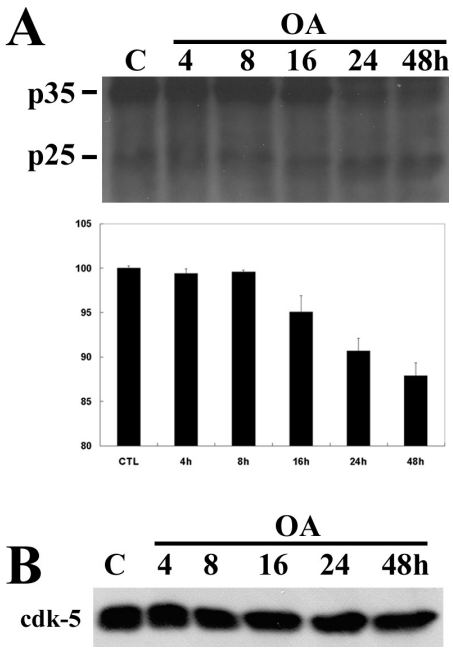


Fig. 2. Western blot analysis of p35 (A) and cdk5 (B). Equal amounts of protein (20μg) from lysates of okadaic acid treated neuronal cultures were loaded. The C-19 polyclonal antibody recognizes p35 and p25, whereas the anti-cdk5 antibody recognizes a single 33 kDa band, consistent with the reported size of cdk5. Densitometry showed that p35 degradation increased over time. Values shown are means±SEM from three independent experiments.

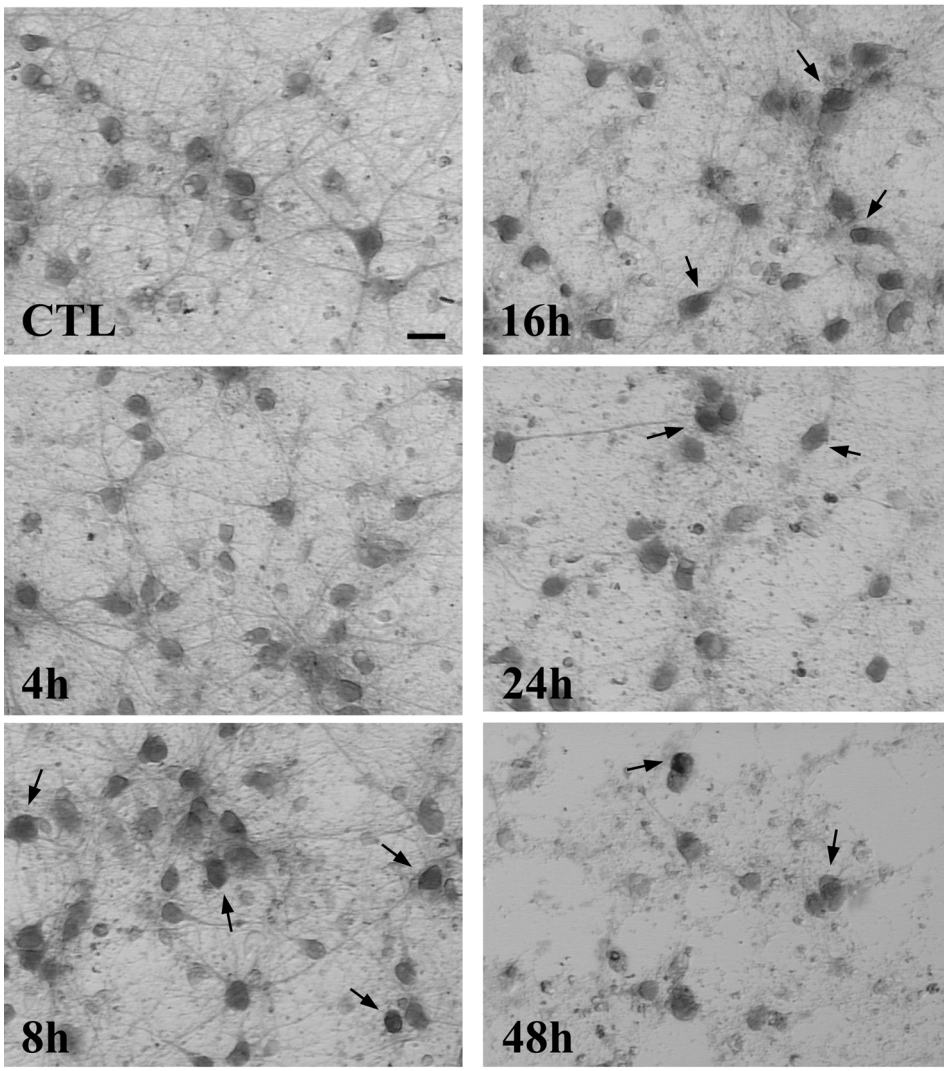
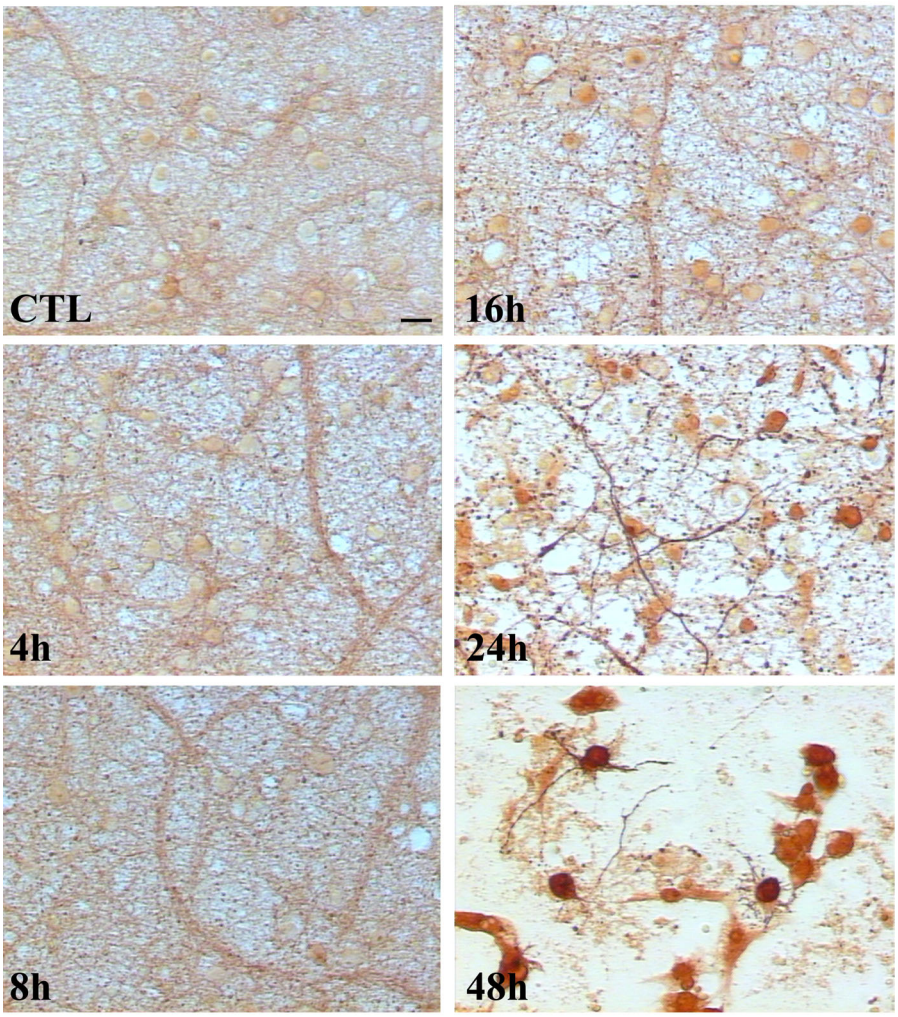


Fig. 3. Temporal changes in cdk5 immunoreactivity in neurons after okadaic acid treatment. Control neurons show moderate immunoreactivity for cdk5 in the cytoplasm and nuclei of neurons (CTL). Following okadaic acid treatment, however, immunostaining for cdk5 was more prominent in nuclei (arrows). Scale bar, 20μm.

activation of cell cycle-promoting factors in postmitotic neurons induces cell death. To determine whether okadaic acid treatment results in characteristic neurodegeneration, cultures were stained with antibody to MPM-2. In vehicle-treated control cultures, MPM-2 immunoreactivity was weak and evenly distributed in the axons and cell bodies. After 24 hr of incubation with okadaic acid, however, dense MPM-2 immunoreactivity accumulated in neuronal cell bodies and degenerating neurites (Fig. 4).

DISCUSSION

Numerous protein kinases and protein phosphatases have been implicated in the dysregulation of tau phosphorylation in the AD brain. Among the kinases involved, GSK-3β and p35/cdk5 have been found to be very important. Recently, we and others showed that GSK-3β was slightly reduced or inactivated during the process of tau phosphorylation (Lu et al., 1999; Swatton et al., 2004; Yoon et al., 2005). Using Western blotting analysis and immunocytochemistry, we assayed the status of p35/cdk5 in the okadaic acid-induced model of neurodegeneration. Western blotting showed conversion of p35 to p25 during okadaic acid-induced neurodegeneration. We also found that calpain-induced cleavage of fodrin and synapsin I occurred (data not shown). The calcium-dependent cysteine protease calpain has been reported to be involved in the generation of p25 from p35 (Lee et al., 2000; Nath et al., 2000). Thus, taken together, these results suggest that accumulation of p25 correlates with increased calpain activity. Aβ peptides have been shown to induce the



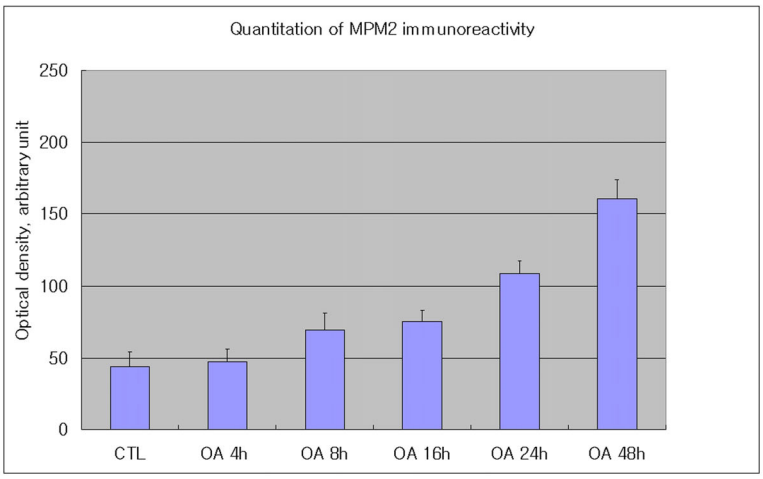


Fig. 4. Temporal changes in MPM-2 immunoreactivity in neurons after okadaic acid treatment. Control neurons show moderate immunoreactivity for MPM2 (CTL). Following okadaic acid treatment, MPM-2 immunoreactivity was increased in neuronal cell bodies with dystrophic neurites, which are likely undergoing degeneration. Scale bar, 20μm.

conversion of p35 to p25 in primary cortical neurons (Lee et al., 2000), as well as to induce cdk5 dependent tau phosphorylation in TG2576 mice (Otth et al., 2002), suggesting that the binding of p25 to cdk5 constitutively activates the latter. Furthermore inhibition of cdk5 and calpain activity reduced cell death in Aβ-treated cortical neurons (Lee et al., 2000), indicating that it may serve as a therapeutic target of AD.

Expression of cdk5 is highest in the brain and

less abundant in other tissues; however, the brain is the only tissue with cdk5 histone H1 kinase activity (Tsai et al., 1994). The regulatory protein for cdk5, p35, is expressed only in the brain (Tsai et al., 1994; Lew et al., 1994). The finding that mice lacking cdk5 or p35 display defects in cortical lamination, as well as seizures and adult lethality, suggests that both cdk5 and p35 are required for cortical lamination (Ohshima et al., 1996; Chae et al., 1997). Expression of dominant-negative mutants of cdk5 inhibited neurite outgrowth, which was rescued by coexpression of the wild-type proteins (Nikolic et al., 1996), demonstrating the critical role of cdk5 in neurite outgrowth during neuronal differentiation. Cdk5 is highly enriched in neurons that colocalize to the cytoskeleton and serves as an important regulatory linker between environmental signals (e.g. laminin) and constituents of the intracellular machinery (e.g. MAP1b) involved in axonal formation (Pigino et al., 1997), supporting its role in neurite outgrowth. Recent evidence also suggests that cdk5 is involved in learning and memory in the adult brain (Fischer et al., 2002; Tan et al., 2003). The p25/cdk5 system, however, can also be detrimental to neurons and may be involved in neurodegenerative diseases (Kusakawa et al., 2000; Lee et al., 2000; Nath et al., 2000). Although conversion of p35 to p25 causes prolonged activation and mislocalization of cdk5 (Patrick et al., 1999), the exact localization of cdk5 has not yet been characterized. Interestingly, we found that treatment with okadaic acid led to increased localization of cdk5 in the nuclei of neurons, suggesting that the p25/cdk5 system acts via an intranuclear mechanism. Intense cdk5 immunoreactivity was previously observed exclusively in apoptotic cells in neurons and other cells (Henchcliffe and Burke, 1997). Another cell cycle dependent kinase, cdk4, is also increased in the nuclei of degenerating neurons in the AD brain (Busser et al., 1998; Tsujioka et al., 1999).

We also found that MPM-2 was markedly increased in the cytoplasm and nuclei of degenerating neurons. Because MPM-2 monoclonal antibody recognizes a large set of mitosis-specific phosphoepitopes, mitotic mechanisms have been hypothesized to occur in AD (Yaffe et al., 1997; Vincent et al., 1998). Our results therefore suggest that the p35/cdk5 pathway may be involved in the mitotic mechanism of neuronal death.

CONCLUSION

We have shown here that okadaic acid, a protein phosphatase inhibitor, induces the conversion of p35 to p25, the mislocalization of cdk5, and the overexpression of MPM2 immunoreactivity. Although the mechanism by which tau phosphorylation and aberrant expression of phosphoepitopes lead to cell death in the AD brain is still not clear, our findings suggest that the okadaic acid-induced model of neurodegeneration may be a useful tool in the comprehensive understanding of AD pathogenesis.

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