

The regulation of phosphorylation of τ in SY5Y neuroblastoma cells: the role of protein phosphatases

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Abstract In Alzheimer disease brain the microtubule associated protein (MAP) τ is abnormally hyperphosphorylated. The role of protein phosphatases (PP) in the regulation of phosphorylation of τ was studied in undifferentiated SY5Y cells. In cells treated with 10 nM okadaic acid (OA), a PP-2A/PP-1 inhibitor, the PP-1 and -2A activities decreased by 60% and 100% respectively and the activities of MAPKs, cdc2 kinase and cdk5, but not of GSK-3, increased. OA increased the phosphorylation of τ at Thr-231/Ser-235 and Ser-396/404, but not at Ser-262/356 or Ser-199/202. An increase in tyrosinated/detyrosinated tubulin ratio, a decrease in the microtubule binding activities of τ , MAP1b and MAP2, and cell death were observed. Treatment with 1 μ M taxol partially inhibited the cell death. These data suggest (1) that OA induced hyperphosphorylation of τ is probably the result of activated MAPK and cdk5 in addition to decreased PP-2A and PP-1 activities and (2) that in SY5Y cells the OA induced cell death is associated with a decrease in stable microtubules.

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Key words: Microtubule associated protein; Microtubule; Protein phosphatase 2A; Proline dependent protein kinase; Alzheimer disease; SY5Y cell

1. Introduction

Accumulation of paired helical filaments (PHFs) in selected neurons is one of the histopathological hallmarks of the Alzheimer disease (AD) brain, and the microtubule associated protein (MAP) τ is the major protein subunit of PHF [1,2]. In the AD brain, τ protein, the biological activity of which is regulated by its degree of phosphorylation [3], is abnormally hyperphosphorylated [4]. The AD hyperphosphorylated τ (ADP- τ) contains 5–9 mol phosphate per protein molecule as compared with 2–3 mol phosphate per mole normal τ [5]. To date, 21 phosphorylated Ser/Thr sites have been identified in PHF- τ [6]. The ADP- τ does not promote assembly of tubulin into microtubules [7]; it sequesters normal τ and high molecular weight MAPs, MAP1 and MAP2, and causes disassembly of microtubules in vitro [8]. The disruption of microtubules and the consequent compromised axoplasmic transport are believed to be responsible for the retrograde degeneration in neurons with neurofibrillary pathology in AD brain [7,8]. Therefore, understanding of the processes leading to hyperphosphorylation of τ is important.

Several kinases and phosphatases are known to be involved in the regulation of phosphorylation of τ . The proline directed kinases, i.e. mitogen activated protein kinases (MAPKs) [9,10], cyclin dependent kinases (cdks) [10,11], and glycogen

synthase kinase-3 (GSK-3) [12], are thought to be active participants in τ phosphorylation in the brain since these kinases can phosphorylate τ in vitro at many of the sites characteristic for AD.

To date, phosphorylation of only Ser and Thr and not of Tyr has been observed in AD τ . Among the Ser/Thr protein phosphatases, PP-1, -2A and -2B have been shown to catalyze dephosphorylation of the ADP- τ [13,14]. Furthermore, the activities of PP-1 and PP-2A towards phosphorylase kinase (Phk) and the phosphatase activity towards the ADP- τ are compromised in AD brain [15,16].

Previously we reported that SH-SY5Y human neuroblastoma cells, if grown in 5% fetal calf serum, contained τ hyperphosphorylated at several of the same sites as in AD and it had less ability to bind to microtubules [17]. Furthermore, τ hyperphosphorylation by treatment with okadaic acid (OA), a PP-2A and PP-1 inhibitor, and its decrease in biological activity had been reported in different cell lines [18]. However, the protein kinase and protein phosphatase activities involved in the phosphorylation of τ in these cells were not known. In the present study, phosphatase inhibitors were employed to elucidate the role of protein phosphatases in the phosphorylation of τ and the regulation of the microtubule system. We found that in SY5Y cells OA inhibits the activities of PP-2A and PP-1 and increases in an acute fashion the activities of MAPK, cdc2 kinase and cdk5. These changes in phosphatase and kinase activities alter the levels of phosphorylation of τ . Furthermore, OA and other PP-1 and -2A inhibitors produce degeneration of SY5Y cells that is accompanied by a decrease of detyrosinated tubulin (stable microtubules), and this degeneration is inhibited by taxol, a microtubule stabilizer.

2. Materials and methods

2.1. Reagents

OA, calyculin A, and tautomycin were purchased from Calbiochem (San Diego, CA), deltamethrin from Alomone Labs (Jerusalem, Israel) and taxol (paclitaxel) from Sigma (St. Louis, MO.). Cyclosporin A was a gift from Sandoz (Tokyo, Japan).

2.2. Antibodies and recombinant τ

The following site specific phosphorylation dependent antibodies to τ , τ -1 (τ not phosphorylated at Ser-195, Ser-198, Ser-199, or Ser-202 [19]), PHF-1 (τ phosphorylated at Ser-396, Ser-404 [20]), M4 (τ phosphorylated at Thr-231, Ser-235 [21]), 12E8 (τ phosphorylated at Ser-262, Ser-356 [22]) and R145d (τ phosphorylated at Ser-422) were employed. Rabbit antiserum R145d was raised against the synthetic peptide corresponding to residues 417–426 of τ_{410} , with Ser-422 phosphorylated. The peptide was conjugated to keyhole limpet hemocyanin and injected into two rabbits (400 μ g peptide at the initial immunization and 200 μ g peptide for the booster injections in 3 weekly intervals). Serum from one of the rabbits reacted strongly with neurofibrillary tangles in AD hippocampus and on Western blots with ADP- τ but not with recombinant or normal brain τ . Other primary

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antibodies employed were monoclonal antibodies YL 1/2 (anti-Tyr tubulin, Sera Lab, Westbury NY), anti-Glu tubulin [23], 3G5 (anti-MAP1B), HM2 (anti-MAP2A-C, Sigma), anti-MAP kinase antibody and agarose conjugated anti-MAP kinase antibody (clone Z033, Zymed, South San Francisco, CA), anti-cdc2 kinase antibody (sc-53, Santa Cruz Biotechnology, Santa Cruz, CA), anti-cdk5 antibody (sc-173, Santa Cruz). Antibodies sc-53 and sc-173 are highly specific and do not crossreact (information obtained from the supplier). The following rabbit antisera to synthetic peptides of PP-1, -2A and -2B [24] and glycogen synthase kinase-3 [25] were employed: R129d (anti-PP-1), R123d (anti-PP-2A), R126d (anti-PP-2B), R133d (anti-GSK-3). Secondary antibodies employed were alkaline phosphatase conjugated anti-mouse and anti-rabbit IgG antibodies (Sigma), 125 I-conjugated anti-mouse and anti-rabbit IgG antibodies (0.1 μ g/ml, Amersham, Arlington Heights, IL), and fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG antibodies (Cappel, Durham, NC). τ_{410} (3-repeat τ with two N-terminal inserts) was prepared as previously described [26].

2.3. Cells, protein and viability assays

SH-SY5Y human neuroblastoma cells were obtained from Dr. June L. Biedler (Sloan Kettering Institute, NY). Cells were cultured up to about 70–80% confluence in 100 mm or 60 mm diameter dishes at 5% CO₂ and 37°C, employing D-MEM/F-12 medium (Gibco BRL, Gaithersburg, MD) supplemented with 5% fetal calf serum (FCS), 100 IU/ml of penicillin, and 100 μ g/ml of streptomycin. The medium was changed every 3 days. For harvesting, the cells were first washed with warm (37°C) PBS to remove the loosely floating dead or dying cells and then collected by trituration. Cells were lysed on ice for 20 min in lysis buffer (100 mM PIPES, pH 6.8, 0.1% Triton X-100, 2 mM MgCl₂, 1 mM EGTA, 0.1 mM EDTA, 25 mM NaF, 1 mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 2 μ g/ml pepstatin A, 25 μ g/ml phosphoramidon), and centrifuged at 200 000 \times g for 30 min. The supernatants, called 'cell extracts' in the following, were used for Western blots, radioimmuno-dot-blot and microtubule binding assays. The protein concentrations were assayed by the modified Lowry method [27]. Live/Dead Eukolight Viability/Cytotoxicity kit (Molecular Probes Inc., Eugene, OR) was employed to assay cytotoxicity.

2.4. Assay of site specific τ phosphorylation

Levels of phosphorylation of τ at τ -1 and PHF-1 sites were assayed by radioimmuno-dot-blot as described previously [17,28]. Triplicate samples (from three individual dishes) of cell extracts containing 1, 2, and 3 μ g of protein were applied to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and dried at 37°C. As a standard for τ -1 antibody 0.5, 1 and 2 ng of τ_{410} was applied to the membranes. τ -1, with or without prior dephosphorylation of the protein on the membrane with alkaline phosphatase (258 U/ml, 7–10 h, 37°C, Boehringer-Mannheim, Indianapolis, IL), and PHF-1 were employed as primary antibodies and 125 I-conjugated anti-mouse IgG as a secondary antibody. The phosphorylation of τ at M4, R145d and 12E8 sites were assayed by Western blotting using 125 I-conjugated anti-mouse IgG as the secondary antibody.

All radioblots were scanned with a Fuji BAS 1500 Bio Image analyzer (Raytest USA Inc., Wilmington, DE). Images were processed using the Tina software system and expressed as PSL (photo stimutable luminescence units). From the different sample concentrations for the dot blots used, curves were constructed and the slopes (PSL/ μ g protein) determined. Values for phosphorylation of the τ -1 epitope were determined by subtraction of the values of the untreated blot from those of the alkaline phosphatase treated blot (total τ). Changes of phosphorylation at the PHF-1, M4 and 12E8 sites were calculated from the differences between the immunoreactivity levels of the untreated (100%) and the OA treated cells.

2.5. Protein phosphatase assays

Cells were lysed in lysis buffer containing no NaF and centrifuged as above (see Section 2.3). For the PP-1 and -2A assays, 32 P-labeled phosphorylase b was prepared as a substrate by phosphorylating phosphorylase b with Phk [15]. PP-1 and -2A activities were assayed in the reaction mixture containing 20 mM Tris, pH 7.0, 0.1 mM EGTA, 15 mM β -mercaptoethanol, 5 μ M caffeine, 0.2 mg/ml 32 P-phosphorylase and 0.03 μ g/ml of cell extract protein in the absence or presence of 0.2 mg/ml of PP-1 inhibitor-I, respectively.

For PP-2B assay, Phk 32 P-labeled catalytic subunit of cAMP de-

pendent kinase (Sigma) [15] was employed as a substrate. PP-2B activity was assayed in the reaction mixture containing 50 mM Tris, pH 7.0, 10 mM β -mercaptoethanol, 4.0 μ M 32 P-Phk, and 0.025 μ g/ml of cell extract protein in the presence or absence of 1 mM CaCl₂ and 1 μ M calmodulin. PP-2B activity was calculated by subtraction of phosphatase activity in the absence of CaCl₂/calmodulin from the activity in the presence of CaCl₂/calmodulin.

2.6. Protein kinase assays

Activities of cdc2 kinase, cdk5 and MAPK were determined as described previously [26] but in immunoprecipitates of these enzymes isolated from the cell extracts with appropriate specific antibodies. 2 μ g of antibodies to cdc2 kinase or cdk5 together with immobilized protein G (Pierce, Rockford, IL) or 20 μ l of agarose conjugated antibody to MAPK were mixed with 50 μ g of cell extract protein in 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 2 mM EGTA, 1 mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 2 μ g/ml pepstatin and 25 μ g/ml phosphoramidon. After incubation at 4°C overnight the mixtures were centrifuged and precipitates were washed three times. The precipitates were then dissolved in 50 mM Tris, pH 7.4, containing 10 mM MgCl₂. Cdc2 protein kinase peptide substrate (Promega, Madison, WI) was employed for the cdc2 kinase and cdk5 assays, and MAPK substrate peptide (UBI, Lake Placid, NY) was employed for the MAPK assay. Precipitates were suspended in 20 μ l of buffer and incubated at 30°C for 30 min in the reaction mixture containing 30 mM Tris, pH 7.4, 10 mM MgCl₂, 10 mM NaF, 1 mM Na₃VO₄, 2 mM EGTA, 10 mM β -mercaptoethanol, and 200 μ M [γ - 32 P]ATP with each substrate.

For the GSK-3 assay, 20 μ l of cell extracts (diluted to 1 mg/ml) was incubated at 30°C for 30 min in the same reaction buffer as above except that phosphoglycogen synthase peptide 2 (UBI, Lake Placid, NY) was used as a substrate. GSK-3 activity was also determined by immunoprecipitating the enzyme from 50 μ g of cell extracts with 2 μ l of R133 antibody which recognizes both GSK-3 α and β [25]. Non-specific phosphorylation was subtracted from each total kinase activity.

2.7. Microtubule binding assay

MAPs binding to taxol stabilized microtubules was assayed as described previously [17]. Briefly, 900 μ g of cell extracts was mixed with 100 μ g of taxol polymerized microtubules (from MAPs free tubulin), in lysis buffer without Triton X-100 but containing 20 μ M taxol, and incubated at 37°C for 30 min. The microtubule bound proteins (pellet) were separated from proteins not bound to microtubules (supernatant) by centrifugation at 70 000 \times g for 30 min. The pellets were suspended in the same volume as that of the supernatants and 1/10 of the volume analyzed on Western blots. τ , MAP1b and MAP2 were detected with antibodies τ -1 (after dephosphorylation of the blot), 3G5 and HM2. Levels of bound and unbound MAPs were determined with 125 I-labeled secondary antibody. The radioactivities were scanned and quantified and amounts of both microtubule bound and unbound MAPs determined. The percentages of the bound MAPs were calculated from the amounts of total, i.e. bound and unbound, MAPs.

3. Results

3.1. Effect of protein phosphatase inhibitors on cell viability

OA is a specific inhibitor towards PP-2A and PP-1. This compound, though tumorigenic in most organs, causes degeneration of cells of neuronal origin [29]. To establish conditions of phosphatase inhibition least cytotoxic to SY5Y cells, various concentrations of the PP-1 and PP-2A inhibitors, OA (Fig. 1a), calyculin A (Fig. 1b) and tautomycin (data not shown) were tested for their cytotoxicity towards SY5Y cells. The three inhibitors, though chemically different from one another, were all cytotoxic. However, the toxicity was observed at about 10–100 times the concentration of the in vitro IC₅₀ of each inhibitor. In contrast, the PP-2B inhibitors deltamethrin (Fig. 1c) and cyclosporin A (data not shown) did not show any cytotoxic effect even though the concentrations employed were 100 times more than their IC₅₀. Since 10 nM

OA was the least cytotoxic of the three PP-1 and -2A inhibitors (even at 48 h 50% of the cells were viable), for further studies this PP-2A/PP-1 inhibitor was used.

3.2. Effects of OA and cyclosporin A on the activities of PP-1, PP-2A and PP-2B

To confirm the inhibitory effect of OA and cyclosporin A on PP-2A/-1 and PP-2B respectively, phosphatase activities were assayed in cells treated with these inhibitors. In cells treated with 10 nM OA the PP-2A activity decreased gradually starting from 1 h and was not detectable after 24 h, whereas the PP-1 activity was only significantly decreased (to 40%) after 12 h (Fig. 2a,b).

In contrast, no PP-2B activity was detected in untreated SY5Y cells or cells treated with 10 μ M cyclosporin A (data not shown). Furthermore, on Western blots no PP-2B staining was detected whereas in brain homogenate the 61 kDa band of the catalytic subunit of PP-2B was clearly observed. In contrast PP-1 and PP-2A were expressed in SY5Y cells (Fig. 2c).

3.3. Levels of site specific τ phosphorylation

The degree of phosphorylation of τ at different sites was assayed by radio-immuno blots. The τ -1 site is already phosphorylated 70–80% in the untreated cells and the treatment of the cells with 10 nM OA did not induce a significant increase of phosphorylation (Fig. 3), whereas the phosphorylation of

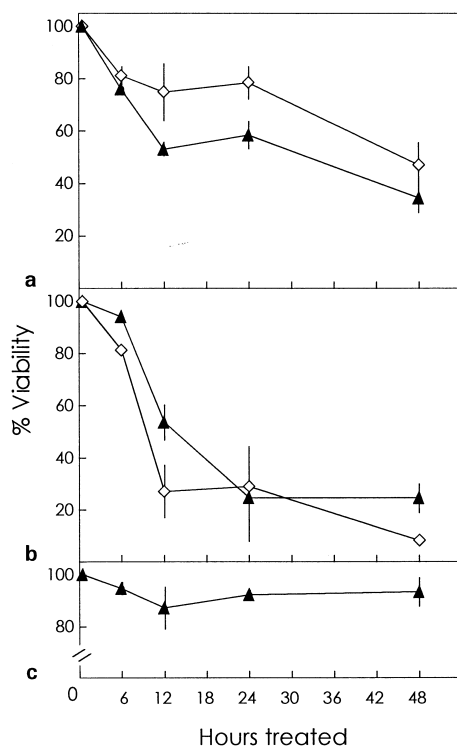


Fig. 1. Cytotoxic effects of PP-1, PP-2A and PP-2B inhibitors and their expression in SY5Y cells. SY5Y cells were treated with (a) 10 nM (\diamond) and 100 nM (\blacktriangle) OA, (b) 1 nM (\blacktriangle) and 10 nM (\diamond) calyculin A, and (c) 1 μ M deltamethrin and cells were counted in a microscope using the live/dead assay. PP-1 and PP-2A inhibitors (OA and calyculin A) were cytotoxic whereas PP-2B inhibitor (deltamethrin) was not. 100 nM and 1 μ M tautomycin (PP-1 and PP-2A inhibitor) also induced cytotoxicity whereas 1 μ M and 10 μ M cyclosporin A (PP-2B inhibitor) did not (not shown).

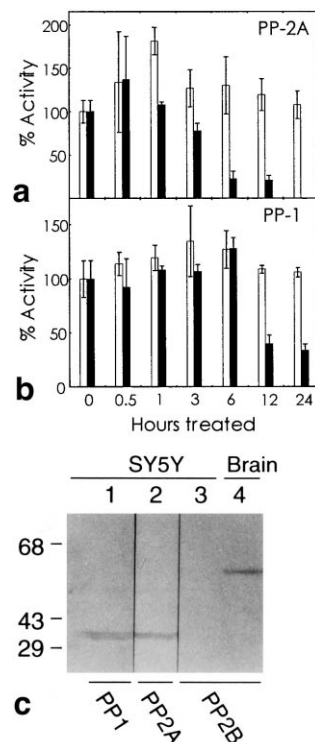


Fig. 2. Activities of PP-1 and PP-2A in SY5Y cells treated with 10 nM OA. a,b: PP-1 and -2A activities were assayed using 32 P-labeled phosphorylase as a substrate. PP-2A activities were determined in the presence of inhibitor-I. PP-1 activities were calculated by subtraction of PP-2A activities from the total activities (without inhibitor-I). Medium containing either 10 nM OA (\blacksquare) or no OA (\square) was added at the 0 time point and protein phosphatase activities were measured. Each data point represents the mean \pm S.D. of three different experiments. c: Expression of PP-2A, PP-1 and PP-2B in SY5Y cells. The expression of PP-2A, PP-1 and PP-2B was studied by Western blots: 50 μ g of 200 000 \times g cell extract/lane was labeled with anti-PP-1 (lane 1), anti-PP-2A (lane 2), and anti-PP-2B (lanes 3 and 4).

the PHF-1 and R145d sites increased respectively up to two- and five-fold of the control level at 24 h. In contrast, the phosphorylation of τ at the M4 site increased more than two-fold between 30 min and 1 h after exposure of the cells to OA and then decreased rapidly thereafter to the control value. The phosphorylation at the 12E8 site was not changed significantly in the OA treated cells. Unlike the τ -1, R145d, PHF-1 and M4 sites, the 12E8 site is canonical for a non-proline dependent protein kinase.

3.4. Stimulation of proline directed protein kinase activities in cells treated with OA

PP-2A has been hypothesized to play a role in several signal transduction cascades. Western blots showed that cdc2, cdk5, MAPK and GSK-3 α/β were expressed in SY5Y cells (data not shown). These kinases have been previously shown *in vitro* to phosphorylate τ at the sites found in PHF- τ [10–12].

Cdc2 kinase and cdk5 activities in control cells increased two- to four-fold at 30 min after medium change, and were decreased to baseline at 1 h (Fig. 4). Addition of OA to the medium resulted in a prolonged increase of cdk5 and cdc2 kinase activities reaching the maximum at 1 h and then a return to about baseline between 3 and 6 h. An additional peak of activity, especially in the case of cdk5, was observed

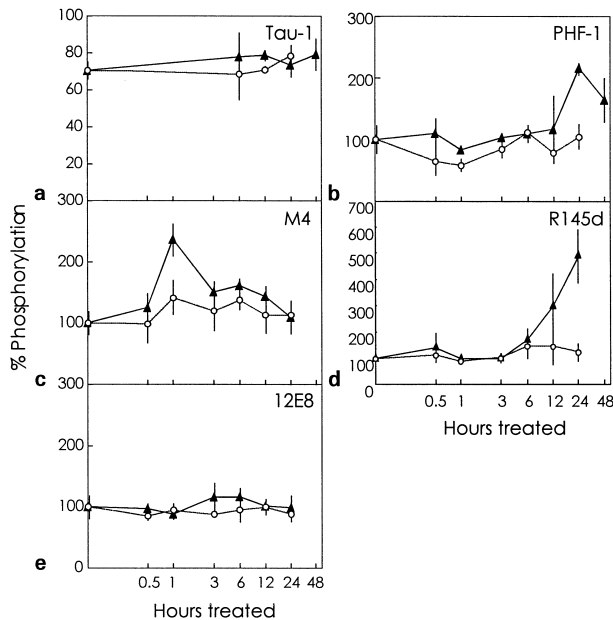


Fig. 3. Levels of site specific phosphorylation of τ in SY5Y cells. The phosphorylation levels of τ were assayed with five different antibodies employing ^{125}I conjugated anti-mouse IgG antibody as a secondary antibody: (a) τ -1; (b) PHF-1; (c) M4; (d) R145d; (e) 12E8. Phosphorylation levels were normalized by taking the starting time ('0' points) as 100% except in the case of the τ -1 site. τ -1 antibody recognizes only non-phosphorylated epitope. Therefore, 100% phosphorylation means no binding of this antibody to cell extracts (theoretically complete phosphorylation). At '0' point medium containing 10 nM OA (\blacktriangle) or no OA (\circ) was added and phosphorylation levels of τ were measured at different time periods.

at 12 h. MAP kinase activity increased up to approximately 10-fold at 30 min after medium change in the control cells whereas it increased to approximately 20-fold at 1 h in the OA treated cells. In contrast, the GSK-3 activity was almost constant in both control and OA treated cells.

All assays were performed employing specific peptides as substrates. To confirm the kinase activities towards τ , the

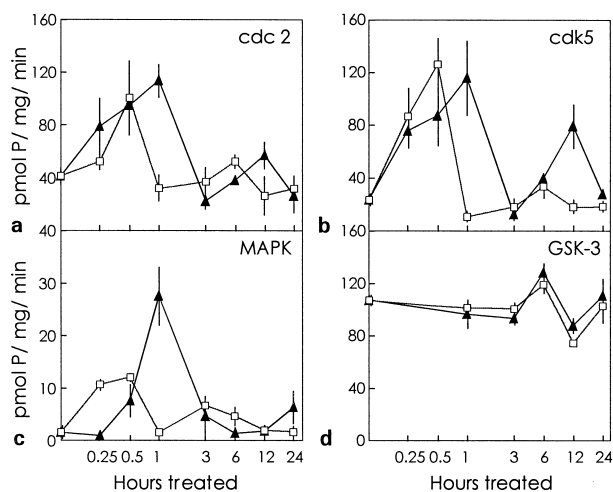


Fig. 4. Activities of cdc2 kinase (a), cdk5 (b), MAPK (c) and GSK-3 (d). 36 h before the start of the experiment the medium was changed. At the starting point ('0') medium without inhibitor (\square) or medium plus 10 nM OA (\blacktriangle) was added. Cdc2, cdk5 and MAPK activities were assayed after immunoprecipitation from cell extract at different times; GSK-3 activity was assayed directly.

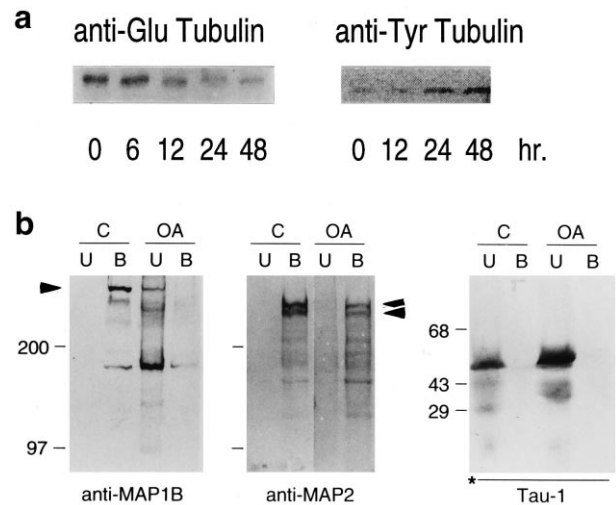


Fig. 5. Changes in cytoskeletal protein in SY5Y cells treated with OA. a: Effect of OA on Glu-tubulin. Western blots of cell extracts (5 and 2 μg) prepared from cells that had been exposed to OA for different time periods were labeled with antibodies to Glu-tubulin and Tyr-tubulin. Glu-tubulin, a marker for stable microtubules, was decreased and Tyr-tubulin, a marker for rapid turnover microtubules, was increased after OA treatment. b: Microtubule binding of MAPs from the OA treated and control SY5Y cells. Cells were treated with culture medium alone (C) or culture medium containing 10 nM OA (OA) for 24 h and cell extracts incubated with taxol polymerized microtubules. The microtubules with bound MAPs (B) were separated from the unbound MAPs (U) by centrifugation and analyzed as described in Section 2 except that the blots were developed with the PAP reaction. *Treatment of the membranes with alkaline phosphatase prior to application of the antibody. Arrow-heads indicate the position of MAP1B (left panel) and MAP2A, b (middle panel).

kinases were first immunoprecipitated and then activities assayed employing recombinant τ as a substrate. Each of the immunoprecipitated kinases had activity towards τ . The increases in the activities of cdc2, cdk5 and MAPK towards τ in the OA treated cells were not as large as those observed using their respective substrate peptides (data not shown).

3.5. OA induces changes in tubulin and microtubule associated proteins

In cells treated with OA, processes were retracted and the cell bodies rounded up (data not shown). In order to investigate whether these obvious changes in cell morphology were associated with the changes of cytoskeletal components, the expression of tubulin and the binding to microtubules of MAPs including MAP1b, MAP2 and τ in the treated and the untreated cells were analyzed. The carboxyl terminal of tubulin is catalyzed by both carboxyl peptidase and tubulin-tyrosine ligase resulting in dephosphorylated tubulin (Glu-tubulin) and tyrosinated tubulin (Tyr-tubulin) respectively [30]. The Glu-tubulin is a marker of stable microtubules, and the Tyr-tubulin is a marker of dynamic (rapid turnover) microtubules. Western blots developed with specific antibodies against both Glu-tubulin and Tyr-tubulin revealed that in cells treated with 10 nM OA over a period of 48 h the levels of Glu-tubulin decreased whereas the Tyr-tubulin levels increased (Fig. 5a).

To study whether the microtubule binding activities of MAP1b, MAP2 and τ might be affected by the OA treatment, the in vitro binding activity of the different MAPs to taxol

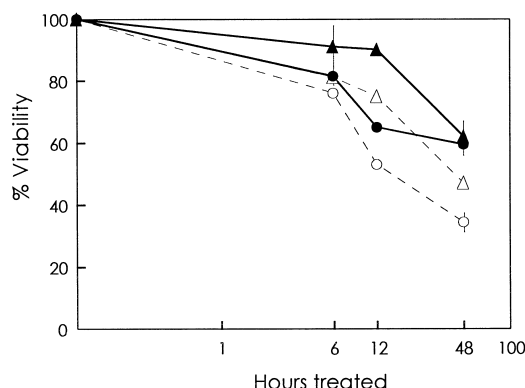


Fig. 6. Inhibition of OA mediated cell death by taxol. SY5Y cells were treated either with 10 nM (Δ) or 100 nM (\circ) OA alone (broken lines) or in the presence of 1 μ M taxol (solid lines). After different time periods the viability of the cells was determined as in Fig. 1. The cell death that had been induced by 10 nM or 100 nM OA was significantly inhibited in the taxol treated cell cultures after 12 h.

stabilized microtubules was determined. Western blots showed that the microtubule binding of MAP1b and MAP2 was decreased (Fig. 5b and Table 1). Furthermore, MAP2 and more so MAP1b were partially degraded in the OA treated cells. In the case of MAP1b some of the fragmented protein was bound to the microtubules. The binding activity of τ which was already strongly compromised because of hyperphosphorylation in the untreated SY5Y cells, was decreased further by the OA treatment (Fig. 5b, Table 1).

3.6. The effect of taxol on the OA induced cell death

Since the treatment of SY5Y cells with OA induced a decrease in the microtubule binding activity of the MAPs and a decrease of the stable microtubules, the effect of the microtubule stabilizer taxol on the OA induced death of the cells was investigated. The addition of taxol was found to cause a significant reduction of the OA induced cell death after 12–48 h (Fig. 6).

4. Discussion

The abnormal hyperphosphorylation of τ protein is thought to be an important initial step to the neurodegeneration in AD brain, because hyperphosphorylated τ is not only incompetent in promoting microtubule assembly and binding to microtubules but also disassembles microtubules and inhibits their assembly by sequestering τ , MAP1 and MAP2 [8,31].

Table 1
Binding of MAPs in SY5Y cell extracts to taxol stabilized microtubules

MAPs	% MAP bound	
	Control	OA treated
τ	9.0 \pm 3.6	4.8 \pm 1.8
MAP1b	81.1 \pm 6.1	10.0 \pm 1.6***
MAP2	76.2 \pm 4.6	55.4 \pm 4.1**

Cell extracts of SY5Y cells that had been either control treated (Control) or treated with 10 nM OA for 24 h (OA treated) were incubated with taxol stabilized microtubules and the levels of MAPs bound to the microtubules determined as described in Section 2.

** $P < 0.005$; *** $P < 0.0005$.

However, the processes which lead to hyperphosphorylation of τ are still unclear. Proline dependent protein kinases cdc2, cdk5, MAPK, and GSK-3 are known to phosphorylate τ in vitro at several of the same sites as the τ in AD brain [9,10,26], but increase in the activity of any of these kinases in AD brain except in the case of cdc2 [32] has not yet been demonstrated. On the other hand, the abnormally hyperphosphorylated τ is readily dephosphorylated in vitro at several sites by protein phosphatases PP-2A and PP-1 which restore the normal biological activity of τ [13,14,33] and the activities of these phosphatases are compromised in AD brain [15,16].

In the present study, the inhibition of the PP-2A and PP-1 activities in SY5Y cells, which have minimal PP-2B activity, produced hyperphosphorylation of τ at specific sites and up-regulation of the activities of cdc2 kinase, cdk5 and MAPK but not of GSK-3. These findings suggest that the inhibition of PP-2A and PP-1 may lead to hyperphosphorylation and decrease in microtubule binding of τ not only by decrease in dephosphorylation but also by increased phosphorylation activity. Furthermore, the decrease in the OA induced cell death by taxol, the decrease in the level of Glu-tubulin and the degradation of MAP1b and MAP2 suggest that the inhibition of PP-2A and PP-1 might cause the disruption of the cytoskeleton and degeneration of SY5Y cells not only by hyperphosphorylation of MAPs but possibly also by directly decreasing the number of the stable microtubules and promoting the proteolysis of the HMW-MAPs. The stimulation of activities of cdc2 kinase, cdk5 and MAPK for extended periods of time in the OA treated cells demonstrates the involvement of PP-2A in signal transduction mechanisms and suggests that in AD brain in which the PP-2A and PP-1 activity is impaired [15], cdk5 and MAPK might be involved in the hyperphosphorylation of τ .

OA, calyculin A and tautomycin are specific inhibitors of PP-2A and, at a lower potency, of PP-1. These inhibitors, despite their chemically unrelated structure, all cause the death of SY5Y cells. However, cytotoxicity is not seen in all cell types. For example, in lymphoid tumor cells like a β -cell lymphoma cell line and a T-cell leukemia line OA and calyculin A have been shown to prevent radiation, heat shock or UV induced apoptosis [34]. The cell death observed in SY5Y cells upon exposure to OA and the other phosphatase inhibitors might therefore be a direct consequence of the inhibition of PP-2A and PP-1.

Upon OA treatment a significant decrease in PP-2A activity was seen as early as after 1 h whereas distinct inhibition of PP-1 was only measurable at 6–12 h. This delay is probably due to the gradual diffusion of the OA through the cell membrane so that for PP-2A with an in vitro IC_{50} of 0.07 nM the critical level of OA was reached much faster than for PP-1 for which the IC_{50} is around 3.4 nM [35].

In the OA treated SY5Y cells, the phosphorylation level of the PHF-1 and the R145d sites increased at 24 h and the phosphorylation level of the M4 site increased at 1 h and decreased until 24 h. In contrast, the phosphorylation levels of the τ -1 site and the 12E8 site remained relatively constant. All these sites, except the 12E8 site, are canonical sites for proline dependent kinases and are dephosphorylated by PP-2A or PP-1 in vitro [13,14]. The exact cause of these differences remains unknown. The τ -1 site is highly phosphorylated (70–80%) in SY5Y cells and probably this site is easily accessed by active kinases. The plausible reason why complete

phosphorylation of the τ -1 site was not induced by the inhibition of phosphatases is as follows. In SY5Y cells grown in 5% fetal calf serum, non-phosphorylated τ at the τ -1 site is predominantly localized in the nucleus [17]. In the nucleus, this site might be dephosphorylated by phosphatases that are not sensitive to OA such as PP-4 and PP-5 that have been localized in the nucleus [36]. However, the expression of these phosphatases in SY5Y cells is not known.

The phosphorylation of the PHF-1 and 145d sites was increased within 24 h whereas the phosphorylation of the M4 site rapidly increased and decreased back to the control level within 3 h. The M4 site might be easily accessible to one or more of the activated proline dependent kinases, followed by its dephosphorylation by the remaining PP-1 activity. In contrast, the PHF-1 and R145d sites might not be favorable substrates available to the activated kinases but the inhibition of phosphatases, especially PP-2A, might have allowed the increased phosphorylation level. Thus the rapid and slow changes of phosphorylation might depend on the accessibilities and/or kinetics of the phosphorylating and dephosphorylating enzymes for each site.

The differential activation of various proline directed kinases by OA observed in the present study is probably due to different activation mechanisms. Inhibition of PP-2A may directly block the inactivation of MAPK [37] by not dephosphorylating it at Thr-183 whereas inhibition of PP-2A indirectly activates cdc2 kinase through the upregulation of cdc25, the activating dual specific phosphatase for cdc2 [38].

The activation mechanism of cdk5 has not yet been demonstrated. The present study is the first report in the cell system which shows that like cdc2 kinase, cdk5 is activated by serum containing medium (see Fig. 4b). Like with cdc2 kinase the total period of activated cdk5 was prolonged by OA suggesting that cdk5 might also have a PP-2A sensitive regulatory mechanism.

The activation of GSK-3 depends on the dephosphorylation of Ser-9 (β isoforms) and Ser-21 (α isoforms), and PP-2A is known to catalyze these sites [39]. Although PP-2A was fully inhibited by 10 nM OA, no significant change of the GSK-3 activity was seen, probably because GSK-3 was dephosphorylated by another protein phosphatase(s).

The breakdown of microtubules which is caused by hyperphosphorylation, is believed to be one of the main causes of neuronal death in AD brain. A previous study has shown that OA treated fibroblasts predominantly contain dynamic microtubules by immunofluorescence. [40]. In the present study we found that Glu-tubulin was decreased and Tyr-tubulin was increased in the OA treated SY5Y cells and the ability of τ , MAP1b and MAP2 from these cells to bind to microtubules was decreased. The decreased binding of MAP1b and MAP2 to microtubules may be due to hyperphosphorylation and/or their increased degradation. Generally in the process of cell death or neurodegeneration components of the cytoskeleton are degraded by activated proteases like cathepsin, calpain or interleukin 1 converting enzyme [41]. Lysosomal abnormalities have been indeed found in OA treated SY5Y cells [42]. OA has been reported to affect the function of several other proteins via the up-regulation of phosphorylation of, for example, calcium channel protein [43], and a transcriptional factor [44]. Thus, the degeneration of SY5Y cells by OA treatment observed in the present study might be due to several causes. However, one major factor is probably the breakdown

of the microtubule network due to the hyperphosphorylation and degradation of the MAPs because the microtubule stabilizer taxol partly inhibited the cell death; co-treatment of the SY5Y cells with OA and taxol does not cause dephosphorylation of τ [Zhong et al., in preparation]. In addition to the cytoskeletal function the microtubule network is critically important in the intracellular transport system [45]. A premature activation of cdc2, i.e. activation of the kinase at an inappropriate time in the cell cycle, is believed to be a cause of apoptosis [46]. Furthermore, the activation of cdc2 has also been reported to induce a catastrophe of the microtubule system [47]. Thus, the increase in the activities of cdc2 and cdk5 in the OA treated SY5Y cells observed in the present study might be involved in the breakdown of the microtubule system and consequently cell death.

In the AD brain due to the decreased activities of τ phosphatases, processes similar to those in the OA treated SY5Y cells might be triggered that lead to hyperphosphorylation of τ , the breakdown of the microtubule system in the affected cells and finally cell death.

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