Inactivation of glycogen synthase kinase-3 by protein kinase C δ: implications for regulation of τ phosphorylation

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Abstract The role of the phosphatidylinositol 3-kinase (PI3K) pathway in the hyperphosphorylation of τ was investigated in SY5Y human neuroblastoma cells. Wortmannin, an inhibitor of PI3K, induced transient (after 1 h) activation of glycogen synthase kinase-3 (GSK-3), hyperphosphorylation of τ and dose-dependent cytotoxicity. However, continuous inactivation of protein kinase (PK) B was observed from 1 to 24 h, suggesting the involvement of protein kinase(s) other than PKB in the phosphorylation and inactivation of GSK-3 after 3 h. In cells treated with wortmannin, PKC δ fragments were observed, and the PKC activity increased after 3 h, whereas treatment of cells with z-DEVD-fmk, an inhibitor of caspase 3, also inhibited fragmentation of PKC δ and induced continuous activation of GSK-3. It is suggested that fragmentation of PKC δ during the process of apoptosis results in the phosphorylation and inactivation of GSK-3 and consequently inhibition of the phosphorylation of τ.

Key words: Tau phosphorylation; Protein kinase C δ; Regulation of glycogen synthase kinase-3; Apoptosis; Alzheimer disease; Wortmannin

1. Introduction

Neurofibrillary tangles of paired helical filaments (PHF) are neuropathological hallmarks of Alzheimer disease and abnormally hyperphosphorylated τ is the major protein subunit of PHF [1,2]. However, the mechanisms of phosphorylation of τ and the neurodegeneration in Alzheimer disease are still unclear. It has been reported that abnormally hyperphosphorylated τ strongly inhibits microtubule assembly and is also present in the affected neurons as amorphous aggregates [3–5]. Generally, the level of phosphorylation of a protein is regulated by the equilibrium between the activities of protein kinases and phosphatases. Previously, regulation of phosphorylation of τ has been studied employing SY5Y human neuroblastoma cells [6]. Protein phosphatases (PPs), especially PP-2A and PP-1, were shown to have important roles in the regulation, and the activities of several protein kinases which phosphorylate τ were also regulated by these protein phosphatases [7].

Studies on the protein kinases which phosphorylate τ have also been carried out. At least half of the phosphorylated (Ser/Thr) sites on τ in Alzheimer brains are proline-directed [8]. Proline-directed kinases, including mitogen-activated protein kinase (MAPK) [9], cyclin-dependent kinase [10], and glycogen synthetase kinase-3 (GSK-3) [11], are known to phosphorylate τ at several sites. Among these kinases, GSK-3 has been most implicated because higher levels of phosphorylation of τ were observed in cells transfected with GSK-3 as compared to cells transfected with MAPK [12]. Furthermore, GSK-3 has been co-localized with hyperphosphorylated τ in degenerating neurons in Alzheimer brains and the amount of GSK-3 was increased in the cytosol fraction [13]. One of the intracellular signal transduction pathways which might control the GSK-3 activity is the phosphatidylinositol 3-kinase (PI3K) pathway, which is highly important for the survival of cells, including neuronal cells [14,15]. Activated PI3K is known to convert 4,5-phosphatidylinositol diphosphates to 3,4,5-phosphatidylinositol triphosphates (PI-TP), and phosphatidylinositol-dependent kinase (PDK) is activated by its binding with 3,4,5-PI-TP. Activated PDK is able to phosphorylate at Ser-473 and activate protein kinase (PK) B, which is identical to Akt kinase, and activated PKB is able to phosphorylate GSK-3 β at Ser-9. Phosphorylation of GSK-3 β at Ser-9 is known to inhibit its kinase activity [16] (Fig. 1).

The present study shows that in SY5Y human neuroblastoma cultured cells wortmannin, an inhibitor of PI3K, results in apoptosis. During the early phase (1–3 h) the GSK-3 activity and the phosphorylation of τ were increased, and were followed by a decrease in the enzymatic activity of GSK-3 to the basal level and dephosphorylation of τ during the late phase (3–24 h). The inhibition of GSK-3 activity during 3–24 h resulted from the phosphorylation of GSK-3 by PKC δ which, during the process of apoptosis, was stimulated due to its cleavage by caspase 3.

2. Materials and methods

2.1. Chemicals and antibodies

Wortmannin and z-DEVD-fmk were purchased from Sigma-Aldrich (Japan) and from Enzyme System Products (Rivermore, CA, USA), respectively. GF-109203X was bought from RBI (Natick, MA, USA). Several phosphorylation-dependent antibodies against τ were employed in this study (Table 1). The Tau-1, PHF-1, M4, and 12E8 antibodies were kindly provided by Drs. L. Binder (North Western University, Chicago, IL, USA), P. Davies (Albert Einstein College of Medicine, New York, NY, USA), Y. Ihara (Tokyo University, Tokyo, Japan), and D. Schenk (Athena Neurosciences, San Francisco, CA, USA), respectively. R127 antibody was previously raised against a synthetic peptide corresponding to amino acid residues 353–364 of

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GSK-3 β [13]. Anti-caspase 3 antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-PKC δ and θ antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-protein kinase Akt antibodies (α, β, γ) and phospho-specific anti-Akt antibody, which is dependent on phosphorylation of PKB at Ser-473, were purchased from Upstate Biotechnology (Lake Placid, NY, USA) and New England Biolabs (Beverly, MA, USA), respectively. OPR-1, an anti-GSK-3 β antibody dependent on phosphorylation at Ser-9, was generated as follows.

Antisera against synthetic peptide corresponding to the residues 3–13 (GRPRTS**Sp**FAEG) (**Sp**: phosphorylated Ser) of the human GSK-3 β was raised in rabbits. Synthetic phospho- and unphospho-peptides with an N-terminal cysteine added were prepared by Sawaday Technology (Tokyo, Japan). They were conjugated to sulfo-SMCC-activated keyhole limpet hemocyanin (Pierce, IL, USA) and emulsified with Freund's complete adjuvant for the first immunization and with Freund's incomplete adjuvant for the others. Rabbits were immunized with three serial injections of the phospho-peptide (300 μg/kg). Antibodies were purified from the antisera by immunoaffinity chromatography using the Prot On Kit (Chiron Mimotopes Peptide Systems, CA, USA). The fractions that bound to the column coupled with the phospho-peptide were collected and were passed through the column coupled with the unphospho-peptide. The unbound fractions were used as Ser-9 phosphorylation-dependent anti-GSK-3 β polyclonal antibody, OPR-1. The reactivity of these antibodies was examined by ELISA and Western blotting.

2.2. Cell line and culture conditions

SH-SY5Y human neuroblastoma cells were obtained from Dr. J.L. Biedler (Sloan Kettering Institute, New York, NY, USA) and grown as previously described [6,7].

SY5Y cells were cultured in the absence or the presence of 100 nM, 1 μM or 10 μM of wortmannin. Surviving and dead cells were visualized and counted using the Live/Dead Eukolight Viability/Cytotoxicity kit (Molecular Probes, Eugene, OR, USA). Apoptotic cells containing fragmented nuclei were also visualized by ethidium homodimer in the above kit, and counted.

2.3. Western blots

SY5Y cells were lysed in 100 mM PIPES pH 6.8, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 25 mM NaF, 1 mM Na_3VO_4, 0.1% Triton X-100 on ice, and centrifuged at $200\,000 \times g$ for 30 min. The supernatants were employed for the Western blot analysis and kinase assays. SY5Y cells were cultured in the presence of 1 μM wortmannin, and harvested after 0, 1, 3, 6, 12 or 24 h. Aliquots of 50 μg of the supernatants were separated by SDS–PAGE and transferred to PVDF membranes. As secondary antibodies alkaline phosphatase-labeled goat anti-mouse or anti-rabbit IgG were used and membranes were developed by 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitroblue tetrazolium chloride except for the Tau-1 antibody. For the Tau-1 antibody part of the membranes was treated with alkaline phosphatase prior to the application of primary antibody to visualize total τ, and goat anti-mouse antibody and peroxidase-labeled mouse antibody were used as secondary and third antibodies, respectively. The membranes were developed with 3-diaminobenzidine tetrahydrochloride dihydrate. The total levels of GSK-3 and PKB were visualized using R127 and anti-protein kinase Akt antibodies (α, β, γ), respectively. The phosphorylation of τ was investigated using phosphorylation-dependent anti-τ antibodies as previously described (Table 1), and the phosphorylation of GSK-3 was studied using OPR-1, the phosphorylation-dependent anti-GSK-3 β (Ser-9) antibody. The phosphorylation of PKB was investigated using phospho-specific anti-Akt antibody. The process of apoptosis was analyzed employing anti-caspase 3 antibody, because the cleavage is necessary for activation of caspase 3. The degree and cleavage of PKC δ and θ were determined by Western blots developed with anti-PKC δ and anti-PKC θ antibodies, respectively.

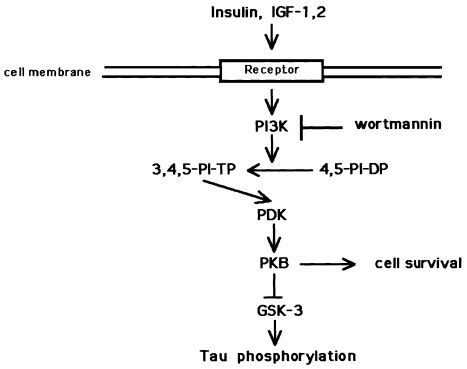

Fig. 1. The phosphatidylinositol 3-kinase (PI3K) pathway. Binding of insulin to insulin receptor leads to autophosphorylation of the receptor, and PI3K is activated by binding to the receptor. Activated PI3K converts 4,5-phosphatidylinositol diphosphates (4,5-PI-DP) to 3,4,5-phosphatidylinositol triphosphates (3,4,5-PI-TP). Phosphatidylinositol-dependent kinase (PDK) is activated by its binding to 3,4,5-PI-TP, and phosphorylates protein kinase B (PKB). Activated PKB (by phosphorylation) phosphorylates GSK-3, and inhibits the kinase activity of GSK-3. Taken together, insulin is able to reduce phosphorylation of τ through inhibition of GSK-3.

2.4. Protein kinase assays

SY5Y cells were cultured in the presence of 1 μM wortmannin alone or together with 50 μM z-DEVD-fmk or with 10 μM GF-109203X, and harvested after 0, 1, 3, 6 or 24 h.

2.4.1. GSK-3. Cell supernatants were prepared, and the GSK-3 activity was assayed using GS-2 phosphopeptide as a kinase substrate

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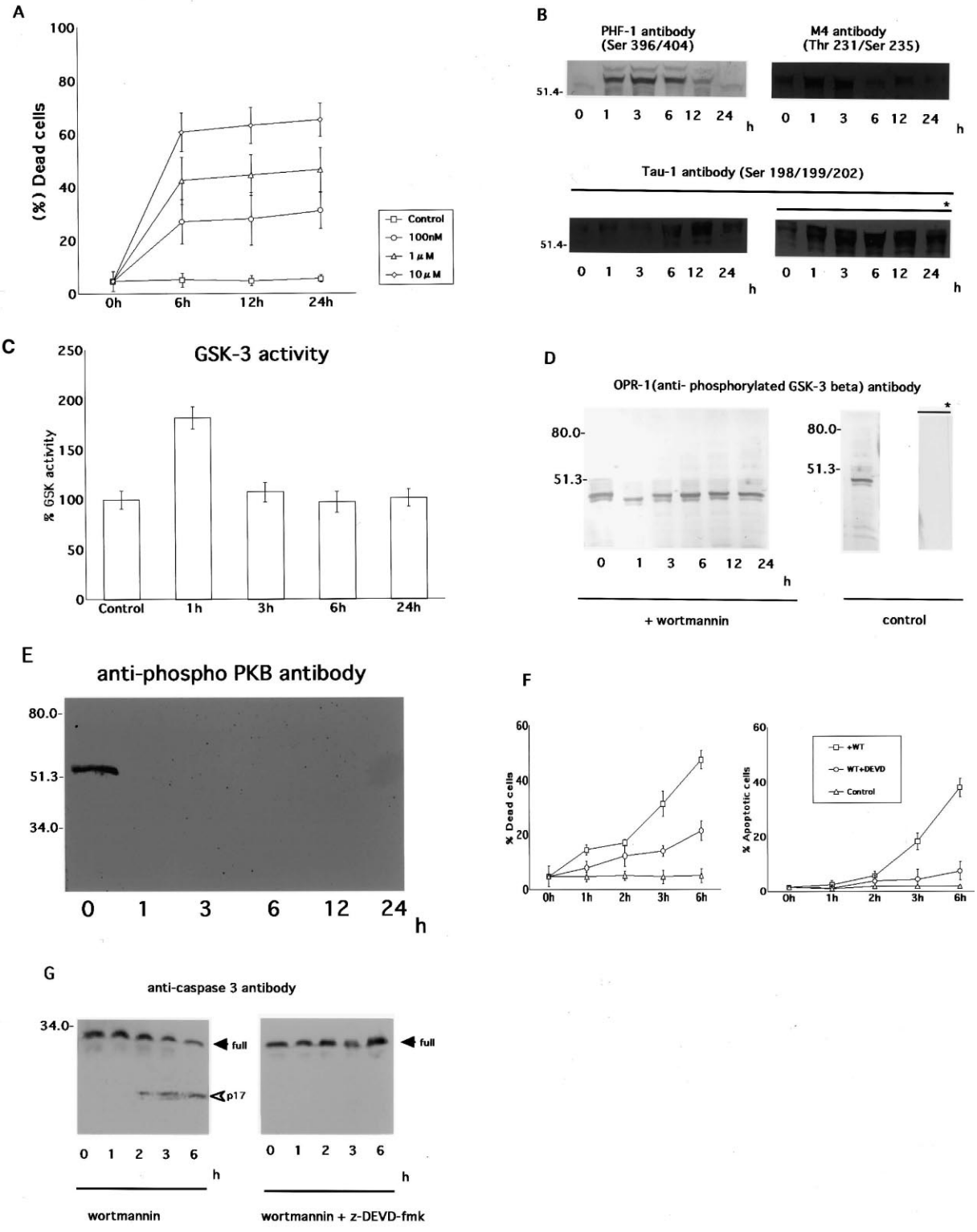
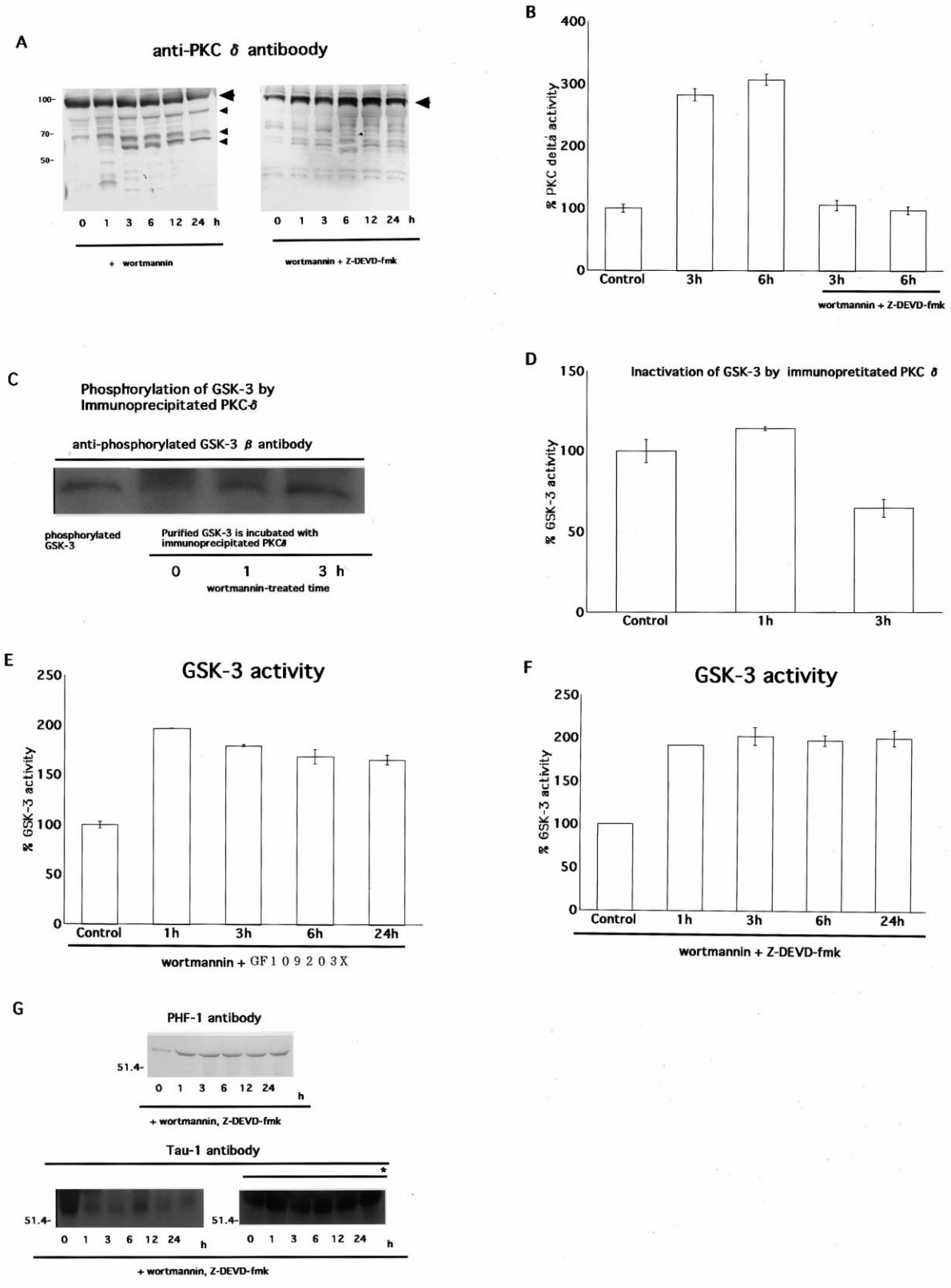
Fig. 2. Effect of wortmannin on SY5Y cells. A: Effect of wortmannin on the percent cell death of SY5Y cells. SY5Y cells were cultured in the absence or presence of different concentrations of wortmannin. The percent dead cells was calculated using the total number of cells as 100%. After 24 h in the presence of different concentrations of wortmannin, the percent dead cells was dose-dependently increased. B: Western blots of τ in SY5Y cells treated with wortmannin. SY5Y cells were cultured in the presence of 1 μM wortmannin, harvested at different time points, and the $200\,000 \times g$ supernatants analyzed by Western blotting for τ phosphorylation. Phosphorylation levels of τ at the PHF-1, M4, and Tau-1 sites were only increased in the early phase (1–3 h), and then decreased until 24 h. The Tau-1 staining after dephosphorylation (*) of the membrane shows that total τ levels were almost the same at each experimental point. C: Kinase activity of GSK-3 in SY5Y cells treated with wortmannin. SY5Y cells were cultured in the presence of 1 μM wortmannin and harvested at different time points. The GSK-3 kinase activity was assayed in the cell lysates using 10 μM GS-2 phosphopeptide as a substrate. The radioactivity of control cells was normalized as 100%. GSK-3 activity increased to 172% at 1 h, and decreased thereafter ($n = 5$). D: Western blots of cell lysates with OPR-1, the phosphorylation-specific anti-GSK-3 β antibody. Decreased phosphorylation of GSK-3 β was observed at 1 h and the level of phosphorylation increased thereafter. The right panel shows the phosphorylation specificity of OPR-1. The antibody stained a 46 kDa band corresponding to GSK-3 β, which is abolished by the dephosphorylation (*) of the membrane. E: Phosphorylation level of PKB in SY5Y cells treated with wortmannin. Western blots of cell lysates were developed with phosphorylation-specific anti-PKB antibody. Decreased phosphorylation (i.e. inactivation) of PKB was observed continuously from 1 h until 24 h. F: Cell death and apoptotic bodies in SY5Y cells treated with wortmannin. Dead cells were increased gradually in cells treated with 1 μM wortmannin (WT) (squares) and with 1 μM wortmannin plus z-DEVD-fmk (WT+DEVD) (circles), but not in control cells (triangles). Apoptotic bodies were observed in cells treated with 1 μM wortmannin, and z-DEVD-fmk almost completely blocked the increase of apoptotic cells. When apoptic cells were visualized by 10 μM of Höchest 33342, the outcome was similar with this result (data not shown). G: Activation of caspase 3 visualized by its cleavage. Wortmannin induced the cleavage of caspase 3. The full length of caspase 3 (MW 32 kDa) (full) was gradually decreased, and its cleaved product (p17) was appeared between 2 and 6 h.



and (Ala-21)–GS-2 peptide as a background substrate (Upstate Biotechnology, Lake Placid, NY, USA). The samples were incubated with the substrates in phosphorylation buffer containing 30 mM Tris pH 7.4, 10 mM $MgCl_2$, 1 mM NaF, 1 mM Na_3VO_4, 2 mM EGTA, 10 mM β-mercaptoethanol, 200 μM [^{32}P]ATP at 30°C for 30 min and the radioactivity incorporated into the peptides was determined as previously described ($n=5$) [13,17].

2.4.2. PKC δ. Cell supernatants were immunoprecipitated using anti-PKC δ antibody, and immunoprecipitates were incubated with 250 μg/ml histone H1 (Sigma-Aldrich, Tokyo, Japan) as a substrate and 10 μg/ml phosphatidylserine (Sigma-Aldrich, Tokyo, Japan) ($n=4$) [18].

2.4.3. Phosphorylation of GSK-3 by PKC δ. SY5Y cells were cultured in the presence of 1 μM wortmannin and harvested after 0, 1 or 3 h. Cell lysates were immunoprecipitated with anti-PKC δ antibody and were incubated with 0.1 units GSK-3 purified from rabbit muscle



(Upstate Biotechnology, Lake Placid, NY, USA) as a substrate, in the enzyme-reactive mixture for PKC described above, at 30°C for 4 h. These samples were immunoblotted with OPR-1 to confirm the phosphorylation of GSK-3 β at Ser-9 by PKC δ. The GSK-3 kinase activity of the samples was assayed employing GS-2 phosphopeptide as a substrate as above ($n = 4$).

3. Results

The effect of wortmannin on the viability of SY5Y cells was investigated. After 24 h, in the presence of 100 nM, 1 μM and 10 μM wortmannin, the cell death of SY5Y cells was approx-

Fig. 3. Upstream regulation of GSK-3 in SY5Y cells treated with wortmannin. A,B: Western blots of cell lysates developed with anti-PKC δ antibody and PKC δ activities in SY5Y cells treated with wortmannin and z-DEVD-fmk. SY5Y cells were cultured in the presence of 1 μM wortmannin with or without 50 μM DEVD-fmk. A: Cell lysates were immunoblotted with anti-PKC δ antibody. Left panel: A main band of PKC δ (90 kDa) (large arrowheads) and an increase of degradative fragments (80 kDa, 60 kDa and 50 kDa) (small arrowheads) after addition of wortmannin were observed. Right panel: Inhibition of caspase 3 (z-DEVD-fmk) blocked the fragmentation of PKC δ. B: SY5Y cells were cultured in the presence of 1 μM wortmannin with or without 50 μM z-DEVD-fmk, and PKC δ was immunoprecipitated. In the absence of z-DEVD-fmk PKC δ kinase activity increased up to 300% (3 h, 6 h), however, in the presence of the caspase 3 inhibitor the kinase activity did not change (3 h, 6 h) ($n=4$). C–E: Phosphorylation and inactivation of purified GSK-3 by immunoprecipitated PKC δ and the effect of PKC on GSK-3. C: GSK-3 purified from rabbit muscle (0.1 U) was incubated with PKC δ that had been immunoprecipitated from wortmannin (1 μM)-treated cells (0, 1, 3 h). The left lane shows the basal phosphorylation of GSK-3 in the GSK-3 preparation. The phosphorylation of GSK-3 β at Ser-9 increased upon incubation with immunoprecipitated PKC δ from the wortmannin-treated cells (0, 1, 3 h). D: Corresponding to C, the immunoprecipitated PKC δ from cells treated with wortmannin for 3 h also decreased the GSK-3 kinase activity ($n=4$). E: In SY5Y cells that were treated with 1 μM wortmannin and 10 μM GF-109203X, an inhibitor of PKC, the GSK-3 kinase activity increased after 1 h up to approximately 200%. But in contrast to the cells treated with wortmannin alone (in C) the kinase activity did not decrease thereafter. F,G: The effect of caspase 3 inhibitor on the cellular GSK-3 activity and phosphorylation of τ. F: In SY5Y cells that had been cultured in the presence of 1 μM wortmannin and 50 μM z-DEVD-fmk, the GSK-3 kinase activity increased in the presence of z-DEVD-fmk, up to approximately 200% at 1 h, and was constant until 24 h. G: Similarly in these cell lysates the phosphorylation of τ at the PHF-1 and Tau-1 sites increased at 1 h, and did not change thereafter up to 24 h studied. (*) Immunolabeling of the membrane after prior dephosphorylation shows that the τ content in each lane was constant.
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imately 30%, 45% and 65% respectively, confirming the dose-dependent cytotoxic effect of wortmannin (Fig. 2A). Western blot analysis revealed that increased phosphorylation levels of τ at the PHF-1 (Ser-396/404), the Tau-1 (Ser-198/199/202) and the M4 (Thr-231/Ser-235) sites appeared in the early phase (1–3 h), and thereafter decreased until 24 h (Fig. 2B). However, no change of the level of phosphorylation of τ at the 12E8 site (Ser-262/356) was observed (data not shown). To determine the effect of wortmannin on GSK-3 activity and the level of phosphorylation of GSK-3 β at Ser-9, SY5Y cells were cultured in the presence of 1 μM wortmannin, and harvested at 0, 1, 3, 6 or 24 h. The GSK-3 activity increased to 170% at 1 h, and decreased thereafter to the basal level (Fig. 2C). Similarly a decreased level of phosphorylation of GSK-3 β at Ser-9 was observed at 1 h, but the level increased thereafter with a time course comparable to that of the changes in the GSK-3 kinase activity (Fig. 2D). These data raised the question of why wortmannin induced only transient activation of GSK-3. The decreased activity of GSK-3 beyond 1 h was not due to the poor stability of wortmannin since fresh supply of the inhibitor after every 6 h did not change the outcome of this experiment (data not shown). Furthermore, even though the cells received only a single treatment with 1 μM wortmannin, decreased phosphorylation of PKB was observed from 1 until 24 h (Fig. 2E). This implied that although PKB was inhibited continuously GSK-3 was still inactivated through its phosphorylation at Ser-9. Therefore, we examined whether activation of other kinase(s) led to the change of the phosphorylation level of GSK-3 β, and also whether apoptosis was involved in this cell death process, because apoptosis is known to induce dramatic biochemical changes including activation of some kinases. Between 1 and 6 h wortmannin induced cell death and apoptotic bodies were also increased enormously between 3 and 6 h (Fig. 2F). The inhibitor of caspase 3, z-DEVD-fmk, attenuated cell death, and moreover almost completely inhibited the production of apoptotic bodies, suggesting that apoptosis was involved in this process. In fact activation of caspase 3 by wortmannin was revealed by the appearance of p17 (molecular weight 17 kDa), a cleaved fragment of the enzyme, between 2 and 6 h, and this cleavage was blocked by z-DEVD-fmk (Fig. 2G). Therefore the activated kinases during apoptosis were studied because the process of apoptosis is known to induce cleavage of kinases, which is tightly linked to their enzymatic activation through deletion of their regulatory domains. Possible cleavage of PKB in SY5Y cells treated with wortmannin was therefore investigated, but no fragmentation of active PKB was observed in SY5Y cells (Fig. 2E). Then another candidate pathway, involving PKC, was investigated. According to the homology study of protein kinases, PKB is a member of the PKC family and it phosphorylates a RXRXXS sequence motif, while PKC phosphorylates RXS, RXXS, or RXXSXR motifs and these are close to the motif of PKB. Indeed, GSK-3 was previously shown to be phosphorylated by PKC [19,20], and it has been reported that PKC δ and θ were cleaved by caspase 3 in cells in the process of apoptosis [21,22]. Therefore we studied the possibility of involvement of PKC in regulating GSK-3 activity in cells treated with wortmannin. After 3 h of treatment with wortmannin, the fragmentation of PKC δ in SY5Y cells gradually increased (Fig. 3A), but no fragmentation of PKC θ was observed (data not shown). In the presence of z-DEVD-fmk (an inhibitor of caspase 3) with wortmannin, the fragmentation of PKC δ disappeared (Fig. 3A). Next it was examined whether wortmannin affected PKC δ activity. SY5Y cells were cultured in the presence of 1 μM wortmannin and in the absence or presence of 50 μM z-DEVD-fmk, harvested after 0, 3 or 6 h, and the PKC δ activities were assayed after immunoprecipitation. The radioactivities of substrate peptides in the case of control cells were normalized as 100%. Wortmannin induced the increase of PKC δ activity, but wortman-

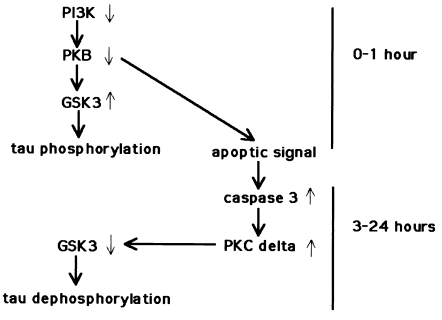

Fig. 4. The signal transduction pathway that mediates the regulation of phosphorylation of τ and survival signaling pathways by wortmannin in SY5Y cells. In the early phase (0–1 h) GSK-3 is transiently activated by the inactivation of PKB, however in the late phase (3–24 h) GSK-3 is inactivated by activated PKC δ in the process of apoptosis.

Table 1
Phosphorylation-dependent anti-τ antibodies employed in this study

Antibody	Type	Epitope	Phosphorylated (P)/ non-phosphorylated (N)
Tau-1	mono	Ser-198/199/202	N
PHF-1	mono	Ser-396/404	P
M4	mono	Thr-231/Ser-235	P
12E8	mono	Ser-262/356	P

nin together with z-DEVD-fmk did not (Fig. 3B). To confirm the phosphorylation of GSK-3 by PKC δ, GSK-3 purified from rabbit muscle was phosphorylated by immunoprecipitated PKC δ from wortmannin-treated cells, and the immunoreactivity with OPR-1 (phosphorylation at Ser-9) and the kinase activity were investigated. Indeed, PKC δ from the cells treated with wortmannin for 3 h increased the immunoreactivity of GSK-3 with OPR-1 (Fig. 3C). Corresponding to the increase in the immunoreactivity with OPR-1, the activity of the GSK-3 preparation was decreased by PKC δ from the cells treated with wortmannin for 3 h (Fig. 3D). To confirm the effect of PKC on the GSK-3 kinase activity, cells were treated with 1 μM wortmannin together with 10 μM GF-109203X, an inhibitor of PKC. In contrast to the cells treated with wortmannin alone, the GSK-3 activity increased after 1 h and did not decrease thereafter (Fig. 3E). Furthermore, it was examined whether PKC δ fragmentation might affect GSK-3 activity and phosphorylation of τ. When SY5Y cells were cultured in the presence of wortmannin and z-DEVD-fmk, and harvested after different time intervals, the GSK-3 activity was assayed in cell supernatants. The GSK-3 activity was increased at 1 h and did not decrease until up to 24 h (Fig. 3F). Being compatible with the time course of the activity of GSK-3, the phosphorylation of τ at the PHF-1, Tau-1 (Fig. 3G) and M4 sites (data not shown) increased at 1 h and thereafter no dephosphorylation of τ was observed during the 24 h observation period.

4. Discussion

A major biochemical abnormality in Alzheimer disease brains is the abnormally hyperphosphorylated τ, which has been shown to be phosphorylated by several kinases (for review see [23]). One of these kinases, GSK-3, might be involved in the mechanism of neurodegeneration in Alzheimer disease, because in cultured cells transfected GSK-3 effectively phosphorylates τ [12] and in Alzheimer brains GSK-3 co-localizes with hyperphosphorylated τ in degenerating neurons, and the levels of GSK-3 are increased by approximately 50% in the postsynaptosomal supernatants from Alzheimer brains as compared to controls [13]. One of the intracellular signal transduction pathways which might control GSK-3 activity and the phosphorylation of τ is the PI3K pathway (Fig. 1), which has been reported to have a critical role in the survival of neuronal cells [14,15]. Therefore, employing wortmannin (an inhibitor of PI3K), which indirectly inhibits PKB and activates GSK-3, we examined the relationship between cell death and the phosphorylation of τ. In the present study, wortmannin had cytotoxic effects on SY5Y cells (Fig. 2A), and actually inhibition of PI3K temporally induced the increase of phosphorylation of τ (Fig. 2B). It is known that GSK-3 alone phosphorylates τ at the Tau-1 (Ser-198/199/202), PHF-1 (Ser-396/404) and M4 (Thr-231/Ser-235) sites, but not at the 12E8 (Ser-262/356) site in vitro and in vivo [24,25]. In the present study, the phosphorylation of τ was increased at the same sites in SY5Y cells by wortmannin (Fig. 2B) and no change in the level of phosphorylation of τ at the 12E8 site was observed throughout the observation period of 24 h.

The present results suggest that the degree of phosphorylation of τ is regulated by the PI3K pathway, including GSK-3. However, we found that the increased levels of phosphorylation of τ and GSK-3 activity were observed only in the early phase but decreased later. And decreased levels of phosphorylation of GSK-3 β were observed after 1 h, and the levels increased thereafter. This increased phosphorylation of GSK-3 was not due to the instability of wortmannin since repeated addition of wortmannin to cells caused results similar to those with a single dose, nor was it due to a reactivation of PKB since dephosphorylation of PKB was observed after the treatment of cells with wortmannin. Therefore activation of other kinase(s) might have led to these changes in the level of phosphorylation of GSK-3. Apoptosis is controversial in the neurodegenerative mechanism of Alzheimer disease and the involvement of apoptosis was also examined in our experiments. In fact involvement of apoptosis in cells treated with wortmannin was strongly suggested (Fig. 2F,G). The production of enormous apoptotic bodies, which implies the activation of endonuclease resulting in fragmented nuclei, was observed and it was blocked by z-DEVD-fmk, an inhibitor of caspase 3. The reason for the incomplete inhibition of cell death by z-DEVD-fmk, in contrast to the complete inhibition of production of apoptotic bodies, might be explained probably by a caspase-independent programmed cell death mechanism which was recently reported [26,27]. The process of apoptosis is known to induce cleavage of kinases, which is tightly linked to their enzymatic activation by deletion of their regulatory domain. Thus, in apoptosis, the cleavage of the regulatory domain by caspase activates kinases such as PAK2 and MEKK-1 [28,29]. PAK2 and MEKK-1 phosphorylate $p47^{phox}$ and MKK3/6/7, respectively, and the sequence motifs of the phosphorylation site by both kinases differ from that of GSK-3 by PKB. Therefore we first examined whether through the wortmannin-induced apoptosis PKB might be proteolysed, and found that in SY5Y cells, no cleavage of PKB was observed. Other studies have shown that the PKC δ and θ isoforms are activated by the caspase 3 cysteine protease in cells induced to undergo apoptosis [21,22]. Moreover, it has been reported that GSK-3 β (not α) is phosphorylated in vitro by particular forms of PKC [19,20]. We inferred from this that PKC δ or θ fragmentation might affect GSK-3 activity. We found that the treatment of SY5Y cells with wortmannin for 3 h led to a gradual increase of the fragmentation of PKC δ (Fig. 3A), but not to an increase of the fragmentation of PKC θ (data not shown). Addition of z-DEVD-fmk (an inhibitor of caspase 3) along with wortmannin inhibited fragmentation and activation of PKC δ (Fig. 3A,B). The effect of PKC on GSK-3 was confirmed in several ways in this study. Activated PKC δ phosphorylated GSK-3 at Ser-9 and inactivated GSK-3 (Fig. 3C,D). In the presence of GF-109203X, an inhibitor of PKC, wortmannin induced activation of GSK-3 without consequential inactivation and also phosphorylation of τ without dephosphorylation. These findings suggested that treatment with wortmannin led to

apoptosis in SY5Y cells, but simultaneously activated the survival signal pathway, and consequently the generation of active PKC δ fragment which phosphorylated GSK-3 β and consequently inhibited the phosphorylation of τ (Fig. 4). Our data show that wortmannin increases the level of τ phosphorylation by inhibiting the PI3K signal pathway, but later led to the dephosphorylation of τ. In a study of oxidative stress linked to apoptosis, we have found that hydrogen peroxide, arsenite and 4-hydroxy-2-nonenal induced dephosphorylation of τ (in preparation) with a basically similar mechanism. The staining of neuronal cells in Alzheimer brains with the terminal deoxynucleotidyl transferase method [30], and apoptosis-specific fragmentation of τ, actin and amyloid β precursor protein have been studied [31–33]. However, our results suggest that a simple mechanism of apoptosis cannot explain the several-fold increase of hyperphosphorylated τ in Alzheimer brains. If we can assume the co-existence of pro-apoptotic and anti-apoptotic mechanisms in Alzheimer brains, the slowly progressive neuronal cell death with huge amounts of hyperphosphorylated τ might be explained. These situations were experimentally demonstrated in Figs. 2F and 3G in our present study. The neuronal degeneration in Alzheimer disease must involve more complicated mechanisms, and further studies will be required to clarify this complex process.

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