Life Sciences 158 (2016) 14–21

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

The Tyr216 phosphorylated form of GSK3β contributes to tau phosphorylation at PHF-1 epitope in response to Aβ in the nucleus of SH-SY5Y cells

Anastasia Noel [a], Laurence Barrier [b], Sabrina Ingrand [b,*]

[a] Université de Poitiers, Groupe de Recherche sur le Vieillissement Cérébral, GRéViC, EA 3808, Poitiers, France
[b] Université de Poitiers, UFR Médecine&Pharmacie, Service de Biochimie et Toxicologie, 6 rue de la Milétrie, TSA 51115, 86073 Poitiers Cedex 9, France

ARTICLE INFO

Article history:
Received 23 October 2015
Received in revised form 9 June 2016
Accepted 20 June 2016
Available online 22 June 2016

Keywords:
Abeta
Glycogen synthase kinase-3 beta
Tyrosine216 phosphorylation
Tau phosphorylation
PHF-1 epitope
SH-SY5Y cell lines

ABSTRACT

Aims: GSK3β activation in Aβ conditions leading to tau phosphorylation at pathological sites is a well-known phenomenon. However, the serine/tyrosine phosphorylation processes implied in Aβ-induced GSK3β activation and responsible for tau phosphorylation, especially at the GSK3β specific Ser396/Ser404 (PHF-1) site, are still debated.
Main methods: Experiments were performed on SH-SY5Y cells exposed to 20 μM $Aβ_{1–42}$ in a time ranging from 5 min to 8 h. The phophorylated forms (Ser9 and Tyr216) of GSK3β and pTau at PHF-1 epitope were measured by immunoblotting in nuclear extracts.
Key findings: We showed a superimposable time-dependent increase of nuclear pGSK3βTyr216 and nuclear pTau at PHF-1 site, both reaching their maximal level after 8 h of $Aβ_{1–42}$ exposure. In addition, nuclear accumulation of pTau is accompanied by its cytoplasmic decrease suggesting that pTau is translocated in response to Aβ treatment. Besides, our experiments showed that specific pGSK3βTyr216 inhibition is required to drop nuclear pTau, ensuring the involvement of Tyr216 phosphorylation in Aβ-mediated tau phosphorylation at PHF-1 epitope.
Significance: These data suggested that in response to Aβ exposure in SH-SY5Y cells, GSK3β activation is performed through Tyr216 phosphorylation and resulted in tau phosphorylation at PHF-1 epitope and in its translocation.

1. Introduction

It is well known that Glycogen Synthase Kinase-3β (GSK3β) whose deregulation has been found in many human diseases especially in Alzheimer's disease (AD) may be the link between Amyloid peptide (Aβ) and tau protein phosphorylation. For a long time, GSK3β, also called Tau Protein Kinase I, was shown to phosphorylate the majority of sites on tau that are abnormally phosphorylated in AD brain including Ser396/Ser404 (PHF-1) epitope. Ser396/Ser404 epitope which is suggested to be directly involved in microtubule destabilization and paired helical filaments (PHF) formation in AD brain is even considered as the preferred GSK3β target site [1–4].

Reciprocal interactions have also been established between Aβ and GSK3β, indicating that Aβ seemed to be strictly linked with GSK3β activation. Studies have shown that Aβ production is promoted by active GSK3β (sometimes GSK3α) and reduced by GSK3 inhibitors [5–6]. In turn, treatment with Aβ activates GSK3β in numerous conditions. However, there is little consensus about how Aβ mediates this activation and the phosphorylation/dephosphorylation processes resulting in GSK3β activation and contributing to tau phosphorylation are still confused.

The insulin/Akt signaling pathway is often suspected to be targeted in Aβ-induced GSK3β activation [7], supposing that reduced Ser9 phosphorylation could be the phosphorylation process implied in Aβ-induced GSK3β activation [8–11]. Besides, studies have shown that reduced pGSK3βSer9 seemed to contribute to tau phosphorylation including Ser202/Thr205 (AT8) phosphorylation [10] but also Ser396/Ser404 (PHF-1) phosphorylation [11–14].

In parallel, the hypothesis that Aβ-mediated GSK3β activation could be achieved through Tyr216 phosphorylation has emerged and an increase of pGSK3βTyr216 (sometimes associated with a decrease of pGSK3βSer9 and sometimes not) was noticed in numerous models of Aβ treatment [10,15–18]. Moreover, Pajak et al. (2009) revealed a co-localization of pGSK3βTyr216 and Aβ deposits in PC12 cells overexpressing human Amyloid Precursor Protein (APP) [16]. These findings indicated the highly relevance in investigating Tyr216 phosphorylation

* Corresponding author.
E-mail address: sabrina.ingrand@univ-poitiers.fr (S. Ingrand).

http://dx.doi.org/10.1016/j.lfs.2016.06.020
0024-3205/

in response to Aβ. Concerning tau phosphorylation, some studies have showed that tyrosine phosphorylated GSK3 isozymes co-localized with tauopathy in neurons in bigenic mouse models [15]. But while an overlap between pGSK3βTyr216 and pTauSer202/thr208 was described [17], very little link has been reported to date between pGSK3βTyr216 and tau phosphorylation at PHF-1 epitope, the characteristic GSK3β motif. Here, using SH-SY5Y cells exposed to $A\beta_{1-42}$, we provided additional data to support that Aβ-induced GSK3β activation is mediated through Tyr216 phosphorylation and contributed to tau phosphorylation at PHF-1 epitope which translocated into the nucleus. In our conditions, a close relationship between pGSK3βTyr216 and pTauSer396/Ser404 could be established since specific pGSK3βTyr216 inhibition is required to prevent tau phosphorylation.

2. Materials and methods

2.1. Chemicals and antibodies

All reagent-grade chemicals for buffers were obtained from VWR International (Starsbourg, France) and those for cell culture were from Invitrogen (Cergy Pontoise, France). SH-SY5Y human neuroblastoma cell lines were obtained from the American Tissue Type Collection (ATCC) (Molsheim, France). Protein assay kit was from BioRad (Marnes-la-Coquette, France). Caspase-Glo® 3/7 assay kit was from Promega (Promega Corp., Madison, WI, USA). $A\beta_{1-42}$ peptide, AR-A014418 (GSK3β inhibitor) and monoclonal antibody Tau-5 against total tau were purchased from Calbiochem VWR International (Starsbourg, France). All-*trans*-Retinoic Acid and LiCl were obtained from Sigma (St Quentin Fallavier, France). Enhanced chemiluminescence (ECL plus) substrate system was from Amersham Biosciences (Orsay, France). Antibodies used for western blot analysis were obtained from the following sources: Monoclonal antibody PHF-1 against phosphorylated tau at the Ser396/404 epitope was a generous gift from Dr. P. Davies (Albert Einstein College of Medicine, Bronx, NY, USA). Polyclonal rabbit antibody anti-mouse FITC was from Dakocytomation. Antibodies against Ser9-phosphorylated GSK3β (pGSK3βSer9), total GSK3β, Histone H3 and secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase were from Cell Signaling (Ozyme distributor, St Quentin Yvelines, France). Polyclonal antibody against Tyr216 phosphorylated GSK3β was obtained from Biosource (Fisher Bioblock Scientific distributor, Illkirch, France). Anti-β-Actin and anti-β-Tubulin were from Sigma (St Quentin Fallavier, France).

2.2. Cell culture

As previously described [19], SH-SY5Y human neuroblastoma cell lines were propagated in minimum essential medium (MEM) mixed with nutrient F12 (1:1, v/v), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (50 U/mL penicillin + 150 μg/mL streptomycin) and were maintained in a humidified 5% CO_2 atmosphere at 37 °C. Cells were seeded at a density of 3.1×10^4 cells/cm^2 in 6-well plates 48 h prior to their differentiation into mature neurons by incubating with 10 μM Retinoic Acid for 7 days. Stock solutions of Retinoic Acid were prepared in dimethylsulfoxide (DMSO) and were diluted into the media with a final concentration adjusted to 0.1%. The media were changed every 48 h.

2.3. Cell treatment and lysis

$A\beta_{1-42}$ was diluted in sterile ultra-high-quality water (H_2O_{UHQ}) and then incubated 48 h at 37 °C to promote peptide aggregation as recommended by the Merck Chemical supplier [20] and as previously described [21–24]. SH-SY5Y cells were exposed to a final concentration of 20 μM $A\beta_{1-42}$ or vehicle for 5 min, 30 min, 2 h, 4 h and 8 h. For GSK3β inhibition, SH-SY5Y were submitted to a 24 h pre-treatment with 10 μM AR-A014418 dissolved in DMSO (0.005% final) or 5 mM LiCl dissolved in H_2O (0.1% final). All treatments were performed in serum-free medium to avoid serum protein-molecules binding. Total protein extracts were obtained as described previously [19].

2.4. Purification of nuclear proteins

Adherent cells were collected in phosphate-buffered saline (PBS) in microcentrifuge tubes and centrifuged at 450 ×*g* for 10 min at 4 °C. The pellet was resuspended in 200 μL of cytoplasmic lysis buffer (Hepes 10 mM pH 7.9, KCl 10 mM, EDTA 0.1 mM, EGTA 0.1 mM, DTT 1 mM, PMSF 0.5 mM, protease and phosphatase inhibitors 10 μL/mL, Igepal 1%), kept on ice for 1 h and centrifuged at 21,900 ×*g* for 30 s at 4 °C. The supernatant containing the cytosolic fraction was removed and stored at −20 °C. The nuclear pellet was resuspended in 50 μL of nuclear lysis buffer (Hepes 20 mM pH 7.9, NaCl 400 mM, EDTA 1 mM, EGTA 1 mM, DTT 1 mM, PMSF 0.5 mM, protease and phosphatase inhibitors 10 μL/mL) and gently rotated for 90 min at 4 °C to release the proteins from ADN. Then, vials were centrifuged at 14,000 ×*g* for 10 min at 4 °C and the supernatant was isolated. Proteins quantification was measured by using a BioRad protein assay kit and samples were stored at −20 °C before used for western blot.

2.5. Caspase-3 activity assay

Caspase-3 activity assay was recorded according to the manufacturer's procedures. Briefly, Caspase-Glo® 3/7 Reagent was added to each well containing treated cells in culture medium and gently scraped resulting in cell lysis. Plates were then incubated for the optimal period of 2 h when caspase cleavage of the substrate occurred generating a luminescent signal, produced by luciferase. Luminescence was directly proportional to the caspase-3 activity present and was measured according to the manufacturers' instructions. All caspase-3 activity assays were performed in triplicate.

2.6. Western blot analysis

Protein (20 μg) were separated on electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes as previously described [19,25]. Immunoblotting were performed using pGSK3βSer9 (1:1000), pGSK3βTyr216 (1:1000), GSK3β (1:1000), PHF-1 (1:1000), Tau-5 (1:500), β-Actin (1:10^5), β-Tubulin (1:10^4) and Histone H3 (1:1000) antibodies. After incubation with horseradish peroxidase-conjugated secondary antibody (1:1000), the blots were detected with the chemiluminescence ECL plus system and quantitative analyses were achieved by G-Box Chemi XL system [19]. The immunoreactivity for β-Actin was controlled to exclude that various amounts of material were loaded. Activation of the proteins was determined as the *ratio* between the phosphorylated and the total form of the corresponding kinases.

2.7. Immunocytochemistry

SH-SY5Y cells were grown on coverslips placed in 6 wells plates. Treatments were performed as outlined above. Coverslips were then washed with PBS and fixed with PFA 4% for 10 min. After incubation 1 h at room temperature in PBS/0.3% Triton ×100/BSA 5%, cells were incubated overnight at 4 °C in primary antibody mouse PHF-1 (1:50) diluted in PBS/0.3% Triton ×100/1% BSA solution. Coverslips were rinsed and incubated in secondary antibody (polyclonal rabbit anti-mouse FITC, 1:20) 1 h at room temperature before DAPI staining (1 μg/mL, 15 min). An Olympus DP70 digital camera and an Olympus DP-soft (Olympus S.A., Rungis, France) were used to perform microscopic visualizations of staining.