Experimental Neurology 271 (2015) 308–318

Contents lists available at ScienceDirect

Experimental Neurology

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

Research Paper

Inhibition of GSK3β by pharmacological modulation of sphingolipid metabolism occurs independently of ganglioside disturbance in a cellular model of Alzheimer's disease

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ARTICLE INFO

Article history:
Received 26 February 2015
Received in revised form 19 June 2015
Accepted 22 June 2015
Available online 24 June 2015

Keywords:
Alzheimer's disease
Ceramides
Gangliosides
Glycogen synthase kinase 3
Sphingolipids

ABSTRACT

Accumulating evidence implicates ganglioside and/or related-sphingolipid disturbance in the pathogenesis of Alzheimer's disease (AD). However, it is not known whether these lipidic alterations are connected with other important features of AD, such as deregulated insulin/Akt/GSK3 signaling. In this study, we have treated neuroglioma cells expressing the double Swedish mutation of human amyloid precursor protein (H4APPsw) with several glycosphingolipid (GSL)-modulating agents, and we have analyzed the impact of the aberrant ganglioside composition on the GSK3 activation state. We found that both ceramide analogs D- and L-PDMP (1-phenyl 2-decanoylamino-3-morpholino-1-propanol), which have opposite effects on ganglioside synthesis, selectively inhibited GSK3β via Ser9 phosphorylation independently of the upstream insulin/Akt pathway. Conversely, the iminosugar N-butyldeoxynojirimycin (NB-DNJ) which displayed similar reduction of gangliosides as D-PDMP, did not affect the phosphorylation state of GSK3β. Concurrently, while NB-DNJ did not modify the cellular ceramide content, both PDMP enantiomers strongly and equally reduced the levels of long-chain ceramide species.

Altogether, our findings led us to hypothesize that the PDMP-induced altered ganglioside composition is not the principal mechanism involved in the inhibition of GSK3β, but seems to implicate, at least in part, their ability to reduce ceramide levels. Nevertheless, this study provides new information regarding the possibilities to target GSK3β through modulation of sphingolipid metabolism.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder characterized neuropathologically by intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein, senile plaques of aggregated amyloid-β peptide (Aβ) and neuronal death. Besides these well-known pathological hallmarks of AD, other metabolic disorders, dysfunctional sphingolipid metabolism and abnormal activation of glycogen synthase 3β (GSK3β) play important roles in the pathogenesis of AD.

GSK3 is a serine/threonine kinase which was initially discovered as a kinase involved in the regulation of glycogen metabolism, but was later shown to affect many cellular processes, including transcription, translation, cell cycle regulation, and apoptosis (Doble and Woodgett, 2003; Rayasam et al., 2009). In mammals, GSK3 exists as two isoforms (GSK3α and GSK3β). Unlike most protein kinases, GSK3 is constitutively active in cells. The activity of GSK3 is dependent on phosphorylation on specific sites (Medina and Wandosell, 2011); phosphorylation of Ser9 of GSK3β, or Ser21 of GSK3α inhibits activity. The best-studied GSK3 regulation pathway is through Akt activation. Insulin stimulation, for example, can activate phosphatidylinositol 3-kinase (PI3K), which phosphorylates Akt (protein kinase B) and in turn inhibits GSK3. Conversely, tyrosine phosphorylation (Tyr216 in GSK3β and Tyr279 in GSK3α) increases the activity of the enzyme. To date, dysregulation of GSK3 is widely recognized to contribute to the generation of multiple lesions associated with AD — including amyloid plaques, neurofibrillary tangles (NFTs), and neuronal loss (for review see Llorens-Martin et al., 2014). Although GSK3 is known to be activated in the brains of AD patients (Leroy et al., 2007), it is not clear what factors activate it.

GSK3 acts downstream of the insulin receptor (IR) signaling through the PI3K/Akt cascade. There is growing evidence that defective insulin signaling is partly responsible for the cognitive decline in AD (De la Monte and Wands, 2005; Gasparini and Xu, 2003, Watson and Craft, 2004). Indeed, one impairment that has been repeatedly described is the observation that in AD, insulin resistance in the CNS develops due

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http://dx.doi.org/10.1016/j.expneurol.2015.06.021
0014-4886/

to alterations of insulin receptor sensitivity. The insulin receptor (IR), like many tyrosine kinase receptors (RTK), is localized in specialized membrane microdomains (lipid rafts) which are highly enriched in cholesterol and sphingolipids, especially gangliosides. Gangliosides are complex glycosphingolipids (GSLs) containing sialic-acids found in all mammalian cells, but they are especially abundant in neuronal and glial membranes (Yu and Saito, 1989). These lipids have been receiving increasing attention because they were shown to be involved in the pathogenesis of AD (Ariga et al., 2008). Indeed, several studies reported significant alterations of ganglioside composition in the brains of AD patients and in transgenic mouse models of AD (Barrier et al., 2007; Kracun et al., 1992; Molander-Melin et al., 2005; Svennerholm, 1994). This is manifested as a reduction in complex gangliosides (GT1b, GD1b, GD1a and GM1), whereas simpler gangliosides (GM2 and GM3) tend to accumulate. However, little is known about the role of these lipidic changes in AD pathology. Concurrently, a growing body of evidence implicates gangliosides in the pathogenesis of insulin resistance (Holland and Summers, 2008; Langeveld and Aerts, 2009). Indeed, Tagami et al. (2002) have demonstrated that insulin resistance in adipocytes is accompanied by an increase in GM3 expression, and that depletion of gangliosides using D-PDMP, an inhibitor of glucosylceramide synthesis, prevents the defect in IR signaling. Similarly, pharmacological inhibition of glycosphingolipid synthesis was also found to markedly improve insulin sensitivity in rodent models of insulin resistance (Aerts et al., 2007; van Eijk et al., 2009; Wang et al., 2014; Zhao et al., 2007).

Based on this information, the aim of the present study was to determine whether ganglioside disturbance could be involved in the upstream molecular mechanism responsible for GSK3 overactivation in AD. To address this issue, we used H4 neuroglioma cells expressing the double Swedish mutation (K595N/M596L) of human APP, which is characterized by a 15 fold increased Aβ production, reproducing a "degenerative state" that mimics the AD pathological condition (Colombo et al., 2009). The endogenous ganglioside composition of H4APPsw cells was manipulated by glucosylceramide synthase (GCS) modulators, i.e. ceramide analogs D-PDMP and its enantiomer L-PDMP, as well as the iminosugar N-butyldeoxynojirimycin (NB-DNJ). The impact of the lipidic modifications on insulin/Akt/GSK3 signaling pathway was examined.

2. Material and methods

2.1. Reagents and antibodies

Cell culture reagents including Opti-MEM, penicillin, streptomycin, Blasticidin S, and fetal bovine serum (FBS), were from Invitrogen (Gibco-Invitrogen, Cergy Pontoise, France). Hygromycin B was purchased from InvivoGen (Cayla-Invivogen, Toulouse, France). All reagent-grade chemicals for buffers were obtained from Sigma (St. Quentin Fallavier, France) and all organic solvents (analytical grade) were from VWR International (Strasbourg, France). Antibodies used for western blot analysis were obtained from the following sources: antibodies against phosphorylated GSK3α/β (pGSK3αSer21 and pGSK3βSer9), total GSK3α/β, phosphorylated Akt (pAktSer473), total Akt, phosphorylated glycogen synthase (pGS Ser641) and total GS were from Cell Signaling (Ozyme distributor, St. Quentin Yvelines, France). Antibody against phosphorylated GSK3α/β (pGSK3αTyr279 and pGSK3βTyr216) was from Biosource (distributed by Fisher Bioblock Scientific, Illkirch, France). Anti-β-actin was from Sigma (St. Quentin Fallavier, France). Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse) were from Cell Signaling and Amersham Biosciences (Orsay, France), respectively. D- and L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP and L-PDMP) and all lipid standards were purchased from Matreya (Biovalley, Marne La Vallée, France). N-butyldeoxynojirimycin (NB-DNJ) was from EnzoLife Sciences (distributed by Covalabs, Villeurbanne, France).

2.2. Cell culture and treatments

The H4 neuroglioma cell line expressing the double Swedish mutation (K595N/M596L) of human APP (APPsw), was a kind gift from Dr A. Colombo and Dr T. Borsello (Institute for Pharmacological Research "Mario Negri", Milan, Italy). The cells were obtained at passage 4 and were used within 6 passages for all experiments. H4APPsw cells were cultured in Opti-MEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml Hygromycin B and 2.5 μg/ml Blasticidin S, 5% CO2, 95% air in a humidified atmosphere, as previously described (Colombo et al., 2009). Cells used for western blot analysis were grown in 6-well plates, whereas those used for lipid extraction were grown in 75 cm^2 culture flasks. Cells were treated either with 20 μM D-PDMP, 20 μM L-PDMP or 50 μM NB-DNJ for times specified in results. D- and L-PDMP were dissolved in ethanol at concentration of 10 mM. NB-DNJ was dissolved at a concentration of 20 mM in sterile water. Control cells were incubated with the carrier alone. For all experiments, the last 24 h of treatment were carried out in serum free medium. For insulin-stimulation experiments, insulin was added to the serum free culture medium at a concentration of 200 nM and the incubation was continued for an additional 10 min.

2.3. Cell lysis

Total protein extracts were obtained by scraping cells in ice-cold lysis buffer (Tris–HCl 50 mM, NaCl 50 mM, pH 6.8, NaF 500 mM, Triton X-100 1%, PMSF 100 mM, protease and phosphatase inhibitors 10 μg/mL). The lysates were sonicated and then centrifuged at 15,000 ×*g* for 15 min at 4 °C. The resulting supernatants were collected for BioRad protein assay and western blot analysis.

2.4. Cell viability assay

Cells were grown in 96-well plates and maintained as described above. Cell viability was determined as previously described (Noel et al., 2011) by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using a commercial kit (MTS CellTiter 96 Aqueous kit, Promega, Madison, WI). The results were expressed as percentages of the untreated controls. All MTS assays were performed in triplicate.

2.5. Western blot analysis

Cell lysate proteins (20 μg) were separated by electrophoresis on a 10% Tris–glycine polyacrylamide gel after denaturation at 100 °C for 5 min and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA) as previously described (Noel et al., 2011). After 2 h blocking at room temperature in Tris-buffered saline containing 5% non fat skim powdered milk, 0.21% NaF and 0.05% Tween 20, membranes were immunoblotted overnight at 4 °C using the following primary antibodies: GSK3α/β (1:1000), pGSK3αSer21/pGSK3βSer9 (1:1000), pGSK3αTyr279/pGSK3βTyr216 (1:1000), pAktSer473 (1:1000), Akt (1:1000), pGSSer641 (1:1000), GS (1:1000) and β-actin (1:10^5). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) and detected with the chemiluminescence ECL plus system (Amersham Biosciences, Orsay, France). The protein bands were analyzed and quantified by G-Box Chemi XL system via GeneSnap and GeneTools softwares (SynGene, Ozyme distributor, St. Quentin Yvelines, France).

2.6. Lipid extraction and analysis

Total lipids from H4APPsw cells were extracted according to previously described procedures (Barrier et al., 2007). Briefly, cells were homogenized with 1.5 ml of distilled water, dispersed in 4 mL of rapidly stirring methanol, and then 2 mL of chloroform were added. At this