Glucose deprivation increases tau phosphorylation via P38 mitogen-activated protein kinase

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Summary

Alterations of glucose metabolism have been observed in Alzheimer’s disease (AD) brain. Previous studies showed that glucose deprivation increases amyloidogenesis via a BACE-1-dependent mechanism. However, no data are available on the effect that this condition may have on tau phosphorylation. In this study, we exposed neuronal cells to a glucose-free medium and investigated the effect on tau phosphorylation. Compared with controls, cells incubated in the absence of glucose had a significant increase in tau phosphorylation at epitopes Ser202/Thr205 and Ser404, which was associated with a selective activation of the P38 mitogen-activated protein kinase. Pharmacological inhibition of this kinase prevented the increase in tau phosphorylation, while fluorescence studies revealed its co-localization with phosphorylated tau. The activation of P38 was secondary to the action of the apoptosis signal-regulating kinase 1, as its down-regulation prevented it. Finally, glucose deprivation induced cell apoptosis, which was associated with a significant increase in both caspase 3 and caspase 12 active forms. Taken together, our studies reveal a new mechanism whereby glucose deprivation can modulate AD pathogenesis by influencing tau phosphorylation and suggest that this pathway may be a new therapeutic target for AD.

Key words: Alzheimer’s disease; amyloid beta; glucose deprivation; mitogen-activated protein kinase; neuronal cells; tau phosphorylation.

Abbreviations

AD Alzheimer’s disease
NFT neurofibrillary tangles
CNS central nervous system
Aβ amyloid beta
APP amyloid beta precursor protein
BACE-1 beta secretase-1
GSK3 glycogen synthase kinase 3
CDK5 cyclin kinase 5

MAPK mitogen-activated protein kinase
ASK1 apoptosis signal-regulating kinase 1
DMEM Dulbecco’s modified Eagle’s medium

Introduction

Alzheimer’s disease (AD) is an age-related neurodegenerative disorder with dementia characterized by progressive accumulation of amyloid beta (Aβ) peptides and abnormal aggregates of the microtubule-associated tau protein, also known as neurofibrillary tangles (NFTs) (Giannopoulos & Praticò, 2015). Recent functional evidence suggests that Aβ initiates the AD changes, but pathological tau protein causes neurodegeneration and better correlates with the clinical manifestation of the disease (Arriagada et al., 1992; Riley et al., 2002). While we know that NFTs are mainly composed of abnormally hyperphosphorylated tau that aggregates into paired helical filaments and straight filaments, the mechanisms leading to the abnormal hyperphosphorylation of tau in the brain of AD patients remain unclear.

Glucose is the main source of energy in the central nervous system (CNS), as under physiological conditions neuronal cells are entirely dependent on a continuous supply of glucose for their correct functioning. The CNS is particularly vulnerable to hypoglycemic damage, and changes in glucose metabolism have been observed in a variety of neurodegenerative conditions associated with dementia (Peppard et al., 1990, 1992; De Leon et al., 2007; Mosconi et al., 2007). In particular, positron emission tomography imaging studies have shown that glucose utilization is dramatically lower in AD, compared to aged-matched, nondemented brain (Mosconi et al., 2009; Landau et al., 2012). Moreover, postmortem analysis of AD brain shows down-regulated expression of mitochondrial enzymes indicating a deficiency in energy metabolism (Soane et al., 2007).

In line with this concept, previous studies have demonstrated that using a pharmacological model of energy metabolism inhibition in APP overexpressing transgenic mice (i.e., Tg2576), BACE-1 and Aβ levels become elevated, suggesting that energy deprivation may be amyloidogenic in vivo (O’Connor et al., 2008).

However, to the best of our knowledge no data are available on the effect that a dysregulation of glucose levels may have on tau and its phosphorylation state. To address this important biological question in the current study, we exposed neuronal cells to a glucose-free medium and investigated the effect on tau phosphorylation and the potential mechanisms involved in this biological effect. Under this experimental condition, we found that, compared with controls, glucose-deprived cells had significant increase in tau phosphorylation at specific epitopes, which was dependent on the activation of the P38 mitogen-activated protein kinase (MAPK) pathway.

Results

Glucose deprivation induces tau phosphorylation

N2A cells were incubated for 24 h in either DMEM with glucose, or DMEM without glucose. Compared with controls, no changes in levels of
total tau were observed in cells that were exposed to medium without glucose (Fig. 1A). However, glucose-deprived cells had a significant increase in tau phosphorylation at Ser202/Thr205 as recognized by the antibody AT8, and at Ser404 as recognized by the antibody PHF1. By contrast, compared with controls, the absence of glucose did not alter the phosphorylation status at Thr231/Ser235 as recognized by the antibody AT180, at Thr-181 as recognized by the antibody AT270, and at Ser396 as recognized by the antibody PHF13 (Fig. 1A).

To study the mechanism underlying the increase in tau phosphorylation after glucose deprivation, we next examined some of the kinases and phosphatases which are considered major regulators of this post-translational modification. As shown in Figure 1C, we did not find any changes in the levels of cyclin kinase-5 (CDK5) and its two coactivators P35 and P25, phosphorylated stress-activated protein kinase (SAPK/JNK and the phosphatase 2A (PP2A). By contrast, compared with controls, while no change was observed for the total level of P38 MAPK, glucose-deprived cells shown a significant increase in its active form (phosphorylated at Thr180 and Tyr182 (pP38). Moreover, the absence of glucose elevated the total level of glycogen synthase kinase 3-α (GSK3-α) and phosphorylated (p) GSK3-β and pGSK3-α (Fig. 1C, D).

**P38 mitogen-activated protein kinase mediates glucose deprivation-induced tau phosphorylation**

To provide evidence for a functional role of P38 activation in the glucose deprivation-induced tau phosphorylation, cells were incubated with SB20358, a specific inhibitor of this kinase activation (20 μM) (Katome...
As shown in Figure 2A, we found that the pharmacological blockade of P38 activation prevented the energy deprivation-dependent hyperphosphorylation of tau. These results were confirmed by performing double staining immunofluorescence assays. The pP38 was detected by green fluorescent signal and phosphorylated tau as the red fluorescent signal under a fluorescent microscope. As shown in Figure 2C, while glucose-deprived cells had an increase in the signal for the antibody AT8, which recognizes tau phosphorylated at Ser202/Thr205, this effect was blunted when cells were incubated with the specific P38 inhibitor. The merged images (Fig. 2D) showed that the green fluorescent for pP38 co-localized with the red fluorescent for AT8 antibody.

As it is known that stress-dependent P38 activation requires the participation of the apoptosis signal-regulating kinase 1 (ASK1), next we investigated the involvement of this kinase under our experimental conditions. To this end, we down-regulated ASK1 protein levels using a siRNA approach and found that the reduced availability of this kinase in glucose-deprived cells was sufficient to prevent the phosphorylation/activation of P38 MAPK (Fig. 3). Under the same experimental conditions, we also observed that while total tau levels were unchanged, glucose deprivation-dependent tau phosphorylation as recognized by the AT8 antibody was prevented (Fig. 3).

Discussion

Numerous clinical studies have shown that there is a biological link between impaired energy metabolism, glucose utilization, and AD.
pathogenesis (Craft & Watson, 2004; Steen et al., 2005), which has been confirmed in both cellular and animal models of energy deprivation. Thus, using a pharmacological model of energy metabolism inhibition in Tg2576 mice, Velliquette et al. reported that Aβ levels were significantly increased suggesting for the first time that energy deprivation acts as an amyloidogenic stimulus in vivo (Velliquette et al., 2005). In a later paper that used glucose deprivation in cell culture as a model of energy deficiency, it was demonstrated a post-transcriptional increase in BACE1 level and enhanced Aβ production (O’Connor et al., 2008). This effect was then confirmed in two APP transgenic mouse models (i.e., Tg2576 and 5xFAD mice) in which pharmacological energy deprivation promoted amyloidogenesis via a BACE-1-dependent mechanism (O’Connor et al., 2008).

However, despite this evidence no data are available on the effect that impaired energy metabolism may have on tau phosphorylation. In the current paper, we provide the first in vitro experimental proof that in response to the inhibition of energy metabolism, recreated by a condition of glucose deprivation, neuronal cells manifest a significant increase in tau phosphorylation, which is mediated by the activation of the P38 MAP kinase.

Taken together, our data lend further support to a growing amount of literature showing the central role that different types of cellular stress responses could play in the onset and development of AD neuropathology. The discovery of an involvement of the P38 MAP kinase in this biological effect does not come as surprise considering that this very kinase activation has been observed in response to a variety of extracellular stimuli and stressors such as UV light, heat, osmotic shock, inflammatory cytokines, and growth factors (Munoz & Ammit, 2010). Of interest, it is the other observation that the active phosphorylated form of this kinase has been found to be physically associated with hyperphosphorylated tau within the NFT of AD brain (Munoz & Ammit, 2010).

Although the normal roles of Aβ and tau in the CNS are not completely understood, one of the several proposed possibilities is that both may be involved in response to stress or external stimuli (Tesco et al., 2007; Wen et al., 2008). Impaired energy metabolism is definitely one type of stress that is responsible of modifying APP processing and elevating Aβ formation via a translational control of BACE1. However, whether energy deprivation is capable to alter tau metabolism and its post-translational modification is still not demonstrated.

With this goal in mind, in our studies we use a well-established model of impaired energy metabolism by incubating neuronal cells in a medium not containing glucose. At the end of this treatment, we observed a significant increase in tau phosphorylation at the epitopes recognized by the AT8 and PHF1 antibodies, but not at any of the other epitopes investigated and recognized by AT180, AT270, and PHF13 antibodies, suggesting the specific nature of this biological effect. In search for the molecular mechanism that could be responsible for this selective increase in tau phosphorylation, we assayed several of the kinases that are considered important modulators of tau post-translational modification. Among them, we observed that pGSK3α and pGSK3β were elevated in cells incubated in glucose-free medium. We interpret these changes in the activation state of this kinase isoforms as not relevant to the increase in tau phosphorylation we observed under our experimental conditions as it is known that an increase in pGSK3 is an index of a reduced activity of this kinase (Fang et al., 2000). For this reason, we believe that, as previously reported, the change in pGSK3 in our cells is a generic response to an energy-deprived condition (Planel et al., 2001).
In recent years, the P38 MAP kinase has attracted much attention as an important regulator of tau phosphorylation. P38 exists in four isoforms (α, β, γ, δ), all implicated in tau phosphorylation when activated by dual phosphorylation at Thr180 and Tyr182. Several studies have shown that P38 active form (i.e., pP38) is associated with neuritic Aβ-amyloid plaques and tau NFT in postmortem brains of AD patients and that P38 activation occurs at very early stage of the disease (Reynolds et al., 2000; Peel et al., 2004).

Interesting in our studies we documented not only an activation of this kinase but importantly its co-localization with a phosphorylated tau isoform which is an early component of full-blown tau pathology, that is, NFTs. In addition, using a selective and specific pharmacological inhibitor, we were able to prove that the activation of this kinase played a functional role in the glucose deprivation-dependent changes in tau phosphorylation.

As stress can activate ASK1, a protein kinase of the MAPK kinase family that in turn activates the P38 MAPK signaling cascade, we were interested to investigate whether this was the case in our cells (Tobiume et al., 2001). Thus, by down-regulating ASK1 mRNA and significantly reducing its protein levels, we were able to prove that even in the presence of a glucose-free medium, neuronal cells did not show changes in pP38 and most importantly, the intracellular levels of tau phosphorylated were also unmodified compared with controls.

Accumulating evidence suggests that ASK1 may contribute to the pathogenesis of AD and other neurodegenerative disorders by regulating various cellular responses such as apoptosis, cell survival, and differentiation (Song et al., 2014).

While ASK1 is known to be activated by several internal or external environmental stressors, the extent and duration of the exposure to them is crucial to determine subsequent cell fate. In fact, ASK1 activation as result of short or low stress exposure leads to cell survival/differentiation; by contrast, prolonged exposure leads to apoptosis (Matsuzawa et al., 2002).

In our studies, because of the prolonged duration (24-h glucose deprivation) of the in vitro stress exposure, the activation of ASK1 was associated with cell apoptosis, which was accompanied by the activation of caspase-3 and caspase-12. Importantly, this activation was prevented by the pharmacological inhibition of P38 kinase activity, further supporting the role of this pathway in the cell apoptotic response to the impaired energy metabolism secondary to glucose unavailability.

**Conclusions**

In summary, our work using a model of energy deficiency provides the first in vitro experimental evidence that in response to a condition of glucose deprivation stress, neuronal cells manifest an increase in tau phosphorylation and apoptotic response which were mediated by the activation of the P38 MAPK pathway via the participation of the ASK1 kinase.

Considering that impaired glucose utilization is a well-known AD risk factor, the targeting of this kinase could afford a new therapeutic opportunity for developing preventative and disease-modifying therapies for AD.
Experimental procedures

Cell culture

The N2A (neuro-2 A neuroblastoma) neuronal cells stably expressing human APP carrying the K670 N, M671 L Swedish mutation (APPswe), were grown in Dulbecco’s modified Eagle medium (DMEM) (cat. # 11965-092; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin, and 400 μg mL⁻¹ G418 (Gibco), at 37 °C in the presence of 5% CO₂, as previously described (Lauretti et al., 2015). For each experiment, equal numbers of cells were plated in six-well plates; the day of the experiment media was removed, cells washed with PBS and fresh glucose-free media (cat. #11966-025, Gibco) or regular media was removed, cells washed with PBS and fresh glucose-free media (cat. #11965-092, Gibco) and media was added. After 24-h incubation, cell pellets were harvested in lytic buffer for immunoblot analyses as described below.

Western blot analysis

Proteins were extracted in EIA buffer containing 250 mm Tris base, 750 mm NaCl, 5% NP-40, 25 mm EDTA, 2.5% sodium deoxycholate, 0.5% SDS, and an EDTA-free protease and phosphatase inhibitors cocktail tablet (Roche Applied Science, Indianapolis, IN, USA). Samples were centrifuged at 15.7 g for 20 min at 4 °C, and supernatants were used for immunoblot analysis, as previously described (Di Meco et al., 2014). Total protein concentration was determined using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Samples were electrophoretically separated using 10% Bis-Tris gels (Bio-Rad, Richmond, CA, USA), according to the molecular weight of the target molecule, and then transferred onto nitrocellulose membranes (Bio-Rad). They were blocked according to the molecular weight of the target molecule, and then developed with Odyssey Infrared Imaging Systems (LI-COR Bioscience). Actin was always used as an internal loading control.

Immunochemistry

Cell immunostaining was carried out as previously described (Chu et al., 2015). Briefly, after treatment, N2A-APPswe cells were plated on glass coverslips, and the following day, fixed in 4% paraformaldehyde in PBS for 15 min at 22 °C. After rinsing several times with PBS, cells were incubated in a blocking solution (5% normal serum/0.4% TX-100) for 1 h at 22 °C and then with the primary antibody against AT8 (1:50; Thermo Scientific, Rockford, IL, USA), and the active form of P38 kinase (1:100 Cell Signaling, Danvers, MA, USA) overnight at 4 °C. After several washings with PBS, cells were incubated for 1 h with a secondary Alexa 488-conjugated antibody (1:200; Invitrogen, Camarillo, CA, USA). Coverslips were mounted using VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA) and analyzed with a confocal microscope (Zeiss 710, Carl Zeiss Microscopy GmbH, Carl Zeiss Promenade, Jena, Germany) with a 639 oil objective and sequential with IRDye 800CW or IRDye 680CW-labeled secondary antibodies (LI-COR Bioscience, Lincoln, NE, USA) at 22 °C for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR Bioscience). Actin was always used as an internal loading control.
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P38 phosphorylates tau in glucose-starved cells, E. Lauretti and D. Praticò

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