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Increased platelet GSK3B activity in patients with mild cognitive impairment and Alzheimer's disease

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ABSTRACT

The disruption of glycogen synthase kinase 3-beta (GSK3B) homeostasis has implications in the pathophysiology of neuropsychiatric disorders, namely Alzheimer's disease (AD). GSK3B activity is increased within the AD brain, favoring the hyperphosphorylation of microtubule-associated protein Tau and the formation of neurofibrillary tangles. Such abnormality has also been detected in leukocytes of patients with cognitive disorders. The aim of the present study was to determine the expression of total and phosphorylated GSK3B at protein level in platelets of older adults with varying degrees of cognitive impairment, and to compare GSK3B activity in patients with AD, mild cognitive impairment (MCI) and healthy controls. Sixty-nine older adults were included (24 patients with mild to moderate AD, 22 patients with amnestic MCI and 23 elderly controls). The expression of platelet GSK3B (total- and Ser-9 phosphorylated GSK3B) was determined by Western blot. GSK3B activity was indirectly assessed by means of the proportion between phospho-GSK3B to total GSK3B (GSK3B ratio), the former representing the inactive form of the enzyme. Ser-9 phosphorylated GSK3B was significantly reduced in patients with MCI and AD as compared to controls ($p = 0.04$). Platelet GSK3B ratio was significantly decreased in patients with MCI and AD ($p = 0.04$), and positively correlated with scores on memory tests ($r = 0.298$, $p = 0.01$). In conclusion, we corroborate previous evidence of increased GSK activity in peripheral tissues of patients with MCI and AD, and further propose that platelet GSK may be an alternative peripheral biomarker of this abnormality, provided samples are adequately handled in order to preclude platelet activation.

1. Introduction

Glycogen synthase kinase 3-beta (GSK3B) is a serine–threonine kinase identified in the early 80s as a key enzyme in the regulation of the glycogen synthesis, given its ability to phosphorylate and inactivate glycogen synthase (Embi et al., 1980; Chin et al., 2005; Balaraman et al., 2006). In addition to glucose metabolism, GSK3B is also involved in the regulation of critical intracellular signaling pathways, including cell cycle, gene expression and apoptosis (Jope et al., 2007; Peineau et al., 2008; Muyllaert et al., 2008). Two widely distributed isoforms, alpha and beta (GSK3A and GSK3B) have been described (Gould and Manji, 2005; Forde and Dale, 2007), the latter being the most abundant in the brain (Grimes and Jope, 2001; Planel et al., 2002). In neurons, GSK3B plays a major role in cytoskeletal organization and remodeling, being thus involved in mechanisms of synaptic plasticity, neurogenesis and resilience to neuronal injury (Grimes and Jope, 2001; Gould and Manji, 2005; Muyllaert et al., 2008).

GSK3B is the most important Tau kinase in neurons (Lovestone et al., 1994). The phosphorylation state of Tau is determinant of its ability to stabilize microtubules. In the pathophysiology of AD, hyperactive GSK3B has been associated with the formation of paired helicoidal filaments (PHF-Tau) and, therefore, of neurofibrillary tangles (Frame and Cohen, 2001; Lovestone and McLoughlin, 2002; Kar et al., 2004; Balaraman et al., 2006). In experimental models of AD, GSK3B has been shown to hyperphosphorylate Tau, leading to microtubule disassembly and loss of function (Lovestone et al., 1994). In addition, the activation of GSK3B inhibits the secretory cleavage of the amyloid precursor protein (APP), increasing the production of the amyloid-beta ($A\beta_{42}$) peptide (Rockenstein et al., 2007), and leads to memory impairment in animal models (Grimes and Jope, 2001). Therefore, the deregulation of GSK3B activity has major effects in key pathological features of AD and its abnormal activation may be

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implicated in the early and primary event in the physiopathology of AD (Hooper et al., 2008). Conversely, the phosphorylation of GSK3B at Serine 9 leads to its inactivation (Klein and Melton, 1996; Doble and Woodgett, 2003), which may prevent Tau hyperphosphorylation and also have potential neuroprotective effects against $A\beta_{42}$ toxicity (Koh et al., 2008; Noble et al., 2005). Naturalistic and observational studies carried out in patients with bipolar disorder showed that chronic intake of lithium salts, a potent GSK3B inhibitor, may reduce the prevalence of AD (Nunes et al., 2007; Kessing et al., 2008).

In the search for peripheral correlates of intracerebral GSK3B, Hye et al. (2005) found a marked increase in GSK3B activity in leukocytes of patients with AD and mild cognitive impairment (MCI), as compared to age-matched controls. GSK3B is also expressed in human platelets, in amounts even higher than in leukocytes (personal observation; data available upon request). The precise physiological role of platelet GSK3B has not yet been fully determined, but there is evidence of involvement in the regulation of platelet activation, since both GSK3A and GSK3B are inhibited by phosphorylation at Ser-21 and Ser-9 (respectively) by platelet agonists (Barry et al., 2003). In addition to these mechanisms, platelet GSK3B is subject to similar regulatory mechanisms as those observed in the brain, such as phosphoinositide 3-kinase (PI3K) and PKB signaling. Finally, platelet GSK3B is inhibited by lithium (manuscript in preparation) and other specific inhibitors that have been validated in neuronal models (Martinez et al., 2002; Mendes et al., 2009). In our group, we have accumulated over the past years a reasonable expertise handling human platelets on experiments dedicated to ascertain biochemical abnormalities in patients with MCI and AD (Gattaz et al., 1996; Forlenza et al., 2005). In the present study, we address the feasibility and potential advantages of using human platelets to determine GSK3B activity for clinical–laboratorial explorations. Therefore, the objective of the present study is to determine GSK3B expression and activity in platelets of patients with AD and MCI as compared to cognitively unimpaired controls.

2. Methods

Sixty-nine older adults were recruited to this study (24 patients with mild to moderate AD, 22 patients with amnestic MCI and 23 healthy elderly controls as comparison group). All patients and subjects were recruited at the Memory Clinic of the Laboratory of Neuroscience (LIM 27), Department and Institute of Psychiatry, Faculty of Medicine, University of São Paulo, Brazil. Written informed consent was obtained after the procedures had been fully explained to all patients. The study was approved by the local ethics committee and conducted according to the principles of the Helsinki Declaration.

Patients and controls were examined by geriatric psychiatrists with the aid of the Brazilian version of the CAMDEX (Cambridge Mental Disorders of the Elderly Examination) (Roth et al., 1986), which yields scores for the Cambridge Cognitive Test (CAMCOG) (Nunes et al., 2008) and the Mini-Mental State Examination (MMSE) (Folstein et al., 1975). All participants were further assessed at baseline with a comprehensive neuropsychological battery in order to ascertain the cognitive state, which was discussed at expert consensus meetings in the light of all available clinical data (for details on the assessment protocol, please see Diniz et al., 2008a). Laboratorial exams were carried out for every patient, encompassing thyroid function, complete blood count, blood chemistry serum levels, folic acid and vitamin B12, blood lipid profile, and syphilis tests to rule out potentially reversible causes of cognitive impairment. Neuroimaging studies (computed tomography scans or magnetic resonance imaging) were completed in patients with suspected dementing or cerebrovascular disorders. Exclusion criteria were illiteracy, visual and hearing disabilities, and other relevant health conditions that could either affect cognition or limit the administration of neuropsychological tests. Patients with severe dementia, concomitant psychiatric diseases, and clinical evidence of cerebrovascular disease (as the main cause of cognitive decline) were also excluded.

Diagnoses of AD and MCI were established in accordance to standard diagnostic criteria (McKhann et al., 1984; Petersen et al., 2001). In the presence of common medical comorbidities such as systemic hypertension, diabetes mellitus or hypothyroidism, subjects were included only if clinically stable on the respective treatments. One patient in the AD group and one healthy control were using oral hypoglycemiants, and a statistically similar number across the three groups (AD, 11; MCI, 10; controls, 18) were receiving more than one drug for the treatment of hypertension, gastrointestinal symptoms, thyroid replacement or sleeping aids. All patients with AD (but none in the other groups) were on stable doses of cholinesterase inhibitors when included in this study. The concomitant prescription of antidepressants and antipsychotic drugs was significantly more frequent for patients in the AD group (11 and 5) as compared to MCI (2 and 0) and control subjects (2 and 0).

2.1. Determination of GSK3B protein levels

Blood samples were collected in 0.1 M sodium citrate-coated tubes from a peripheral vein of the forearm of all participants between 8AM and 9AM and observing a 10–12-h fasting. Samples were homogenized in 1 ml of acid citrate dextrose solution (ACD-NIH-formel-A solution) and centrifuged at 1600 rpm for 15 min at 20 °C (room temperature – RT). Platelet-rich plasma (PRP) was pH adjusted to 6.5 and re-centrifuged for 10 min at 2400 rpm (RT). Pellets were resuspended in 5 ml of wash-solution (sodium citrate 30 mM pH 6.5, potassium chloride 5 mM, calcium chloride 2 mM, magnesium chloride 1 mM, glucose 5 mM, albumin 500 mg/ml, apyrase 50 mg/ml) and centrifuged for 8 min at 2400 rpm (RT). Platelet-rich pellets were then resuspended in 500 μl of Tris–sucrose solution (Tris 50 mM pH 7.4, sucrose 233 mM) and stored at −70 °C. Protein levels were determined for each aliquot by a modified Lowry method (Bio-Rad DC Protein Assay).

A pool of platelets from healthy adults was prepared following the same protocol and was used as internal standard (IS) to correct analytical variation. The IS was analyzed in each assay. The IS densitometry was used to calculate the intra- and inter-day coefficient of variation (CV).

2.2. Western-blot analysis

For each sample, 24 μg of protein (prepared in Laemmli sample buffer) was separated by electrophoresis in 10% polyacrylamide gels (150 V, 90 min) and transferred to nitrocellulose membranes (250 mA, 90 min); unspecific binding was blocked with 5% nonfat milk. For the assessment of total GSK3B, membranes were incubated for 1 h with a monoclonal, primary antibody raised against GSK3B (anti-GSK3B, Clone GSK-4B, Sigma), diluted 1:3000 in TBS-T; membranes were then washed and incubated for 1 h with the secondary antibody (Anti-mouse Ig Biotinylated, GE), diluted 1:400 in TBS-T; and subsequently incubated for 1 h with horseradish peroxidase-conjugated (GE), diluted 1:3000 in TBS-T. For the assessment of phospho-GSK3B, membranes were incubated overnight at 4 °C, with a polyclonal, primary antibody raised against the phosphorylated Ser-9 epitope of GSK3B (phospho-ser-9, Cell Signaling), diluted 1:5000 in a 5% (w/v) solution of milk in TBS-T; membranes were then incubated for 1 h with a polyclonal, secondary antibody (Anti-goat Ig Biotinylated, GE) diluted 1:400 in