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Glycogen Synthase Kinase-3β Expression and Phosphorylation in Peripheral Blood Mononuclear Cells of Patients with Amyotrophic Lateral Sclerosis

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Authors' contributions

This work carried out in collaboration between all authors. Authors MGM, IM, AM, JSM designed the study. Authors AIRM and CG performed cellular extract preparation and enzyme quantifications. Author YM collected and managed patients' clinical data. Author JSM assessed patients. All authors contributed to the discussion. Author MGM wrote and edited the manuscript. All authors read and approved the final manuscript.

Short Communication

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ABSTRACT

Aims: To quantify total glycogen synthase kinase (GSK)-3β and GSK-3β phosphorylated at serine 9 in the peripheral blood mononuclear cells from Amyotrophic Lateral Sclerosis (ALS) patients and to assess if GSK-3β could be a biomarker for ALS.
Study Design: Cross-sectional observational study.
Place and Duration of Study: Department of Immunology and Amyotrophic Lateral Sclerosis Unit, Hospital Carlos III, Madrid, Spain, between February 2011 and August 2012.
Methodology: Blood samples were drawn from 44 ALS patients and 41 healthy controls. Peripheral blood mononuclear cells were isolated and cellular extracts were obtained to assess GSK-3β and serine 9 phosphorylated GSK-3β concentrations. Enzymes were measured by a quantitative enzyme-linked immunoassay in the peripheral blood

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mononuclear cell extracts. Patients were divided into two groups according to the Amyotrophic Lateral Sclerosis Functional Rating Scale-revised (ALSFRS-R) median value for the comparative analysis.
Results: Patients (n=22) showing a high functional impairment (ALSFRS-R ≤ 32) had GSK-3β levels (11.2±3.6 pg/µg protein) higher than healthy controls (8.7±4.7 pg/µg protein; P=0.04) and than those patients (n=22) with ALSFRS-R > 32 (6.9±4.4 pg/µg protein; P<0.01). A negative correlation between GSK-3β concentration and ALSFRS-R values (r = −0.39; P=0.006) was also observed.
Conclusion: Our results show that GSK-3β expression is altered in non-neural cells of ALS patients and suggest that its overexpression may play a role in the pathogenesis of ALS. The quantification of GSK-3β in peripheral blood mononuclear cells may be used as a potential biomarker of ALS progression.

Keywords: Amyotrophic lateral sclerosis; glycogen synthase kinase-3β; peripheral blood mononuclear cells; biomarker.

1. INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a progressive, fatal neurodegenerative disease characterized by a preferential loss of motoneurons. The pathogenesis of motoneuron death remains to be clearly elucidated. Several mechanisms have been proposed to be underlying the neurodegeneration including oxidative stress, glutamate toxicity, environmental toxins, genetic factors, immune inflammation and abnormal protein accumulation. An enzyme that is thought to be involved in ALS pathogenic mechanisms is glycogen synthase kinase-3 (GSK-3). GSK-3 is a ubiquitous and multifunctional serine/threonine kinase which plays a key role in the regulation of numerous signalling pathways from gene expression to cellular architecture and apoptosis [1]. Two closely related isoforms of GSK-3, GSK-3α and GSK-3β, exist in mammals which are constitutively active. There is a widespread expression of GSK-3β in adult brain suggesting that it has a fundamental role in neuronal signalling pathways [2]. GSK-3β is constitutively active, being highly active in cells due to phosphorylation of a conserved tyrosine residue on the activation loop of the kinase domain (Tyr216). On the contrary, phosphorylation of a serine at the N-terminus (Ser9) inhibits GSK-3β kinase activity [3]. This phospho-serine prevents interaction with its substrates. GSK-3β has been reported to play an important role in neurodegeneration in both mouse models and neurodegenerative diseases in human [4]. Increased expression and activity of GSK-3β have been associated with neuronal death and neurofibrillary tangle formation in Alzheimer's disease [5,6]. Several studies have reported that an increase in GSK-3β has also been found in vitro and in vivo models of ALS, in the thoracic spinal cord tissue of patients with sporadic ALS, and in the frontal and temporal cortices of ALS patients [7-10]. Furthermore, inhibition of GSK-3β delays disease onset and increases survival in ALS animal models [11-13].

The aim of this work was to quantify GSK-3β and the enzyme phosphorylated at serine 9 ([pSer9]GSK-3β), the inactive form, in the peripheral blood mononuclear cells from ALS patients and to compare its expression with healthy controls as primary outcome. Analysis of the changes in enzyme expression related to the functional impairment was defined as secondary outcome.

2. MATERIALS AND METHODS

2.1 Patients

Forty four patients with sporadic ALS were recruited for the study. Patients were diagnosed of probable or definite ALS according to the El Escorial criteria for ALS diagnosis [14], and the patient's degree of functional impairment was assessed according to the Amyotrophic Lateral Sclerosis Functional Rating Scale-revised (ALSFRS-R) [15], at the ALS Unit in Hospital Carlos III, Madrid. Electromyography was performed in all patients. None of them had cognitive impairment. All patients were taking riluzole, 50 mg BID. Clinical data comprised gender, age, site of onset (spinal or bulbar), age at onset and duration of disease. The age of onset was defined as the time at which first motor alteration (weakness and/or atrophy) was noted by the patient, and the duration of the disease was the interval of time between the age of onset and time at which blood sample was collected. Sex and age matched healthy controls were included in the study (n= 41). The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983. The study was approved by the Ethics Committee at the Hospital Carlos III and written informed consent was obtained from all participating subjects.

2.2 Cellular Extract Preparation

Blood samples were drawn in CPT cell preparation tubes with sodium citrate (Becton Dickinson, Erembodegem, Belgium) and processed within 60 minutes. Peripheral blood mononuclear cells (PBMC) were isolated after tube centrifugation (400g, 30 min, 21ºC). The interphase with the PBMC was removed and washed twice in pre-cooled PBS and the pellet was immediately frozen at -80ºC until use. Cellular extract was obtained by adding 600 µl buffer lysis (0.5% Sodium Deoxycholate, 1% Nonidet P-40, 0.1% Sodium dodecyl sulfate, PBS, pH 7.3) supplemented with 10 mM sodium fluoride, 2 mM sodium pyrophosphate, 2 mM β-glycerophosphate and a protease inhibitor cocktail (Sigma-Aldrich, MO, USA). Then, cellular extract was centrifuged (16,000g, 10 min, 4ºC) and the supernatant was used for the quantification of GSK-3β and [pSer9]GSK-3β. Total protein was determined by bicinchoninic acid assay (Thermo Scientific, IL, USA) using bovine serum albumin as a standard.

2.3 Quantification of GSK-3β

GSK-3β and [pSer9]GSK-3β were quantified by a commercial enzyme-linked immunoassay (ELISA; Thermo Scientific) following manufacturer's instructions. Samples were analysed in duplicates and concentration was determined by interpolation on a standard curve. The GSK-3β ELISA detected 100% GSK-3β with a cross-reactivity <0.01% for GSK-3α. Sensitivity was 74.4 pg/mL (range 78.1- 5,000 pg/mL). The [pSer9]GSK-3β ELISA detected 100% [pSer9]GSK-3β (cross-reactivity 5.4% GSK-3α) with a sensitivity of 9.0 pg /mL (range 62.5- 2,000 pg /mL). Enzyme concentrations are reported as pg of enzyme/µg of total protein.

2.4 Statistics

Statistical analysis was performed with the SPSS v 15.0 software (IBM SPSS Statistics, NY, USA). Qualitative variables were described by frequency and quantitative variables were described by median and interquartile range (IQR) or mean±standard deviation (SD). Comparison of qualitative variables was performed with the Chi-square test. Quantitative

variables were analysed with the Kruskal-Wallis test for comparison of more than two groups and the Mann–Whitney U test for pairs of groups' comparison. Bivariate correlation between was determined by the Pearson coefficient.

3. RESULTS

3.1 Characteristics of the Study Subjects

Characteristics of the sporadic ALS patients and healthy controls are shown in Table 1. Twenty four patients met criteria for probable ALS and the remaining were diagnosed with definite ALS according to the revised El Escorial criteria for ALS diagnosis.

Table 1. Characteristics of the study subjects

	ALS Patients (n=44)	Healthy controls (n=41)	P
Age (years)	57.5, IQR = 47.0-62.7	55.0, IQR = 49.5-63.7	0.6
Sex male	33	25	0.16
age of disease onset (years)	50.5 (IQR = 46.0-60.0)	-	-
Duration of the disease (months)	24 (IQR = 17.0-37.0)	-	-
ALSFRS-R value	32.5 (IQR = 28.0-37.0)	-	-
Onset (spinal/bulbar)	39/5	-	-

3.2 PBMC GSK-3β and [pSer9]GSK-3β Quantification

GSK-3β and [pSer9]GSK-3β concentrations in PBMC of patients were compared to those of healthy controls. There were no significant differences between patients and controls in concentration of GSK-3β and [pSer9]GSK-3β or [pSer9]GSK-3β/GSK-3β ratio (Table 2). Patients were divided into two groups according to the median value of ALSFRS-R. Comparison between patients' groups and controls showed that there were significant differences in GSK-3β (P=0.005) and [pSer9]GSK-3β (P=0.03) concentrations. Comparison between pairs of groups showed that patients with higher functional impairment (ALSFRS-R ≤ 32) had GSK-3β levels higher than controls. Furthermore, both GSK-3β and [pSer9]GSK-3β concentrations were significantly higher in patients with ALSFRS-R ≤ 32 than in those with ALSFRS-R > 32 (Table 2).

Table 2. GSK-3β and [pSer9]GSK-3β levels (mean ± SD) in PBMC of the study subjects

	ALS patients (n=44)	ALSFRS-R > 32 (n=22)	ALSFRS-R ≤ 32 (n=22)	Controls (n=41)
GSK-3β (pg/μg of total protein)	9.0±4.5	6.9±4.4	11.2±3.6*	8.7±4.7
[pSer9]GSK-3β (pg/μg of total protein)	4.5±3.8	3.1±2.7	5.9±4.3**	3.9±2.8
Ratio [pSer9]GSK-3β /GSK-3β	0.4±0.3	0.4±0.3	0.5±0.3	0.4±0.2

** P=0.04 ALSFRS-R ≤ 32 vs. controls; P=0.01 ALSFRS-R ≤ 32 vs. ALSFRS-R > 32.*
***P=0.01 ALSFRS-R ≤ 32 vs. ALSFRS-R > 32.*

A significant difference was found in the frequency of probable ALS (5/24) and definite ALS (17/20; P<0.001) in the group with ALSFRS-R ≤ 32. Therefore, a similar statistical analysis was performed between probable and definite ALS patients and healthy controls. We found only a statistically difference in GSK-3β concentration between probable ALS (7.3±4.1) and definite ALS (11.1±4.4; P=0.006).

3.3 GSK-3β levels negatively correlated with ALSFRS-R

There was no association of the GSK-3β, [pSer9]GSK-3β concentrations and ratio [pSer9]GSK-3β/GSK-3β with patients' age, age at onset and duration of disease. No differences were found in enzyme concentrations between bulbar- and spinal-onset ALS. In regard to the functional impairment, there was a negative correlation between GSK-3β levels and ALSFRS-R values (r = −0.39; P=0.006; Fig. 1).

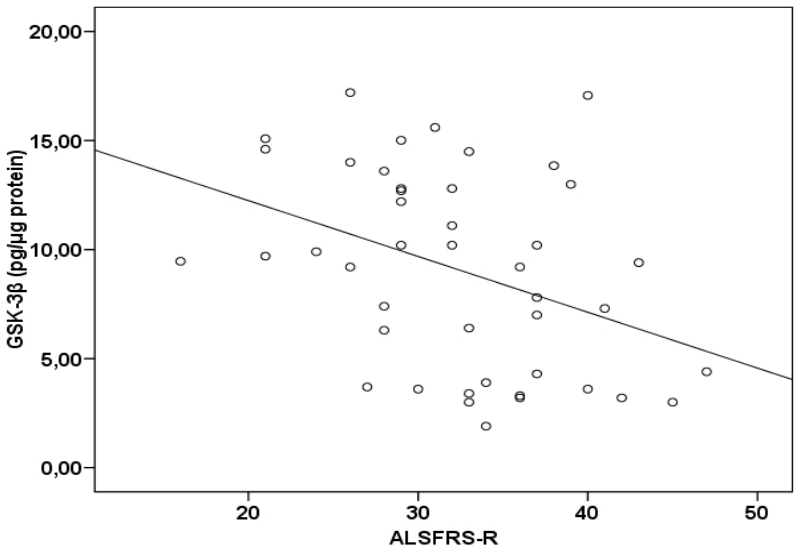

Fig. 1. Relationship between ALSFRS-R scores and GSK-3β levels in PBMC from ALS patients

ALS patients (n=44) are represented by single dots. Pearson's r = −0.39; P=0.01.

4. DISCUSSION

We evaluated the concentrations of total GSK-3β and GSK-3β phosphorylated at serine 9 (inactive form) in PBMC from ALS patients using a quantitative method. Our results showed that there is an over-expression of GSK-3β in ALS patients with lower ALSFRS-R values relative to controls and this increase of enzyme concentration is associated with functional impairment. These data indicate that changes in GSK-3β expression observed in human post-mortem tissues [8-10] can be reflected in non-neural cells, specifically PBMC from alive patients. Blood was chosen because it represents an easily accessible clinical sample. To search if there is a correlation between the enzyme values in neural tissue and blood, GSK3β determinations had to be performed simultaneously in both compartments and the study on patient's neurons is not possible. The limitation of our observations is that the

present work is a descriptive cross-sectional study and these results have to be supported by a longitudinal study.

Previous works on GSK-3β expression in PBMC have been conducted in patients with Alzheimer's disease (AD). Total GSK-3β has been reported to be increased in patients with AD and mild cognitive impairment (MCI) relative to controls while [pSer9]GSK-3β expression was similar to that of controls, suggesting an increase in GSK-3β activity in PBMC from AD patients [16]. In contrast to this study, a later work [17] reported a significant reduction of GSK-3β in PBMC from patients with MCI and less pronounced in AD. Our results indicate that there was an increase in GSK-3β and [pSer9]GSK-3β levels in patients with higher functional impairment but only the GSK-3β concentrations were significantly higher than those found in healthy controls, suggesting an increased enzymatic activity. It is worth mentioning that GSK-3β concentration here measured is the pool of constitutively active native GSK-3β and the super active phosphorylated form [pTyr216]GSK-3β without the possibility of determining the final total activity of the enzyme.

An important role of GSK-3 in PBMC is the regulation of the inflammatory response. Stimulation of monocytes or PBMC with toll-like receptor agonists induces substantial increases in interleukin(IL)-10 production while suppressing the release of pro-inflammatory cytokines after GSK-3 inhibition [18]. Furthermore, it has been shown that active GSK-3β is a critical mediator of the differentiation of pathogenic Th17 lymphocytes [19]. These cells secrete the pro-inflammatory cytokine IL-17 and interestingly, increased IL-17 serum levels were found in ALS patients [20]. The observed overexpression of GSK-3β in ALS patients may indicate a pro-inflammatory profile of PBMC associated with a worsening of symptoms which may suggest a potential therapeutic use of GSK-3 inhibitors for the treatment of ALS.

Lithium is the first GSK-3β inhibitor discovered with a weak inhibition *in vitro* (IC_{50}=2 mM) and dual inhibition *in vivo* by increasing levels of [pSer9]GSK-3β [21] and it has been successfully used in animal models of ALS [22]. It has many other targets for pharmacological action being the standard therapy for bipolar disorders. Although its narrow therapeutic window, it has been used in clinical trials to assess whether lithium improves survival in patients with ALS. First positive results [23] have not recently confirmed and no evidence of benefit can be assured [24]. However, since GSK-3β is overexpressed in ALS, the down regulation of this kinase, without the side effects produced by lithium itself, may be a therapeutic strategy for ALS therapy. Thus, other small molecules specifically designed to target GSK-3 without off target effects would be of therapeutic interest [25].

5. CONCLUSION

Our results show that GSK-3β expression is altered in non-neural cells of ALS patients and suggest that its overexpression may play a role in the pathogenesis of ALS. The quantification of GSK-3β in peripheral blood mononuclear cells may be used as a potential biomarker of ALS progression.

CONSENT

All authors declare that "written informed consent was obtained from all participating subjects".

ETHICAL APPROVAL

All authors hereby declare that the study was approved by the Ethics Committee at the Hospital Carlos III, Madrid, Spain.

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COMPETING INTERESTS

Authors declare that no competing interests exist.

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