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TLQP Peptides in Amyotrophic Lateral Sclerosis: Possible Blood Biomarkers with a Neuroprotective Role

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Abstract—While the VGF-derived TLQP peptides have been shown to prevent neuronal apoptosis, and to act on synaptic strengthening, their involvement in Amyotrophic Lateral Sclerosis (ALS) remains unclarified. We studied human ALS patients' plasma (taken at early to late disease stages) and primary fibroblast cultures (patients *vs* controls), in parallel with SOD1-G93A transgenic mice (taken at pre-, early- and late symptomatic stages) and the mouse motor neuron cell line (NSC-34) treated with Sodium Arsenite (SA) to induce oxidative stress. TLQP peptides were measured by enzyme-linked immunosorbent assay, in parallel with gel chromatography characterization, while their localization was studied by immunohistochemistry. In controls, TLQP peptides, including forms compatible with TLQP-21 and 62, were revealed in plasma and spinal cord motor neurons, as well as in fibroblasts and NSC-34 cells. TLQP peptides were reduced in ALS patients' plasma starting in the early disease stage (14% of controls) and remaining so at the late stage (16% of controls). In mice, a comparable pattern of reduction was shown (*vs* wild type), in both plasma and spinal cord already in the pre-symptomatic phase (about 26% and 70%, respectively). Similarly, the levels of TLQP peptides were reduced in ALS fibroblasts (31% of controls) and in the NSC-34 treated with Sodium Arsenite (53% of decrease), however, the exogeneous TLQP-21 improved cell viability (SA-treated cells with TLQP-21, *vs* SA-treated cells only: about 83% *vs.* 75%). Hence, TLQP peptides, reduced upon oxidative stress, are suggested as blood biomarkers, while TLQP-21 exerts a neuroprotective activity. © 2018 Published by Elsevier Ltd on behalf of IBRO.

Key words: TLQP-peptides, neurodegeneration, ALS, motor neurons, NSC-34 cells, human fibroblasts.

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease characterized by neuronal degeneration in frontal cortex, brainstem and spinal cord. Virtually all muscles are gradually affected, with difficulties in speaking, swallowing and breathing, and death ensues 3–5 years after appearance of the first symptoms. Currently, no treatment is effective in stopping the progression of the disease, nor is any early diagnostic test available. While the etiology of ALS is unknown, mutations of the superoxide dismutase 1 (SOD1) gene, or of the TARDBP (TAR DNA Binding Protein) gene have been hypothesized as common causes (Chiò et al., 2011; Zarei et al., 2015). In fact, oxidative stress, characterized by an altered equilibrium between the production of reactive oxygen species (free radicals) and antioxidant reactions, has been related to motor neuron degeneration in ALS (Bergeron, 1995; Robberecht, 2000). TLQP peptides are a family of peptides derived from the VGF (non acronymic) precursor protein, some of these originally identified in rat brain (Trani et al., 2002). They share a common N-terminal "TLQP" (Thr-Leu-Gln-Pro) amino acid sequence, are cleaved from the primary VGF product at the specific R-P-R (Arg-Pro-Arg) processing site found at rat/mouse $VGF_{553–555}$ ($VGF_{551-553}$ in human), and variably extend

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Abbreviations: ALS, Amyotrophic Lateral Sclerosis; ALSFRS-R, ALS Functional Rating Scale-Revised; C3AR, complement component 3a receptor 1; gC1q-R, receptor for the globular heads of c1q; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; NSC-34, mouse motor neuron-like hybrid cell line; PBS, phosphate-buffered saline; PIC, protease inhibitor cocktail; PFA, paraformaldehyde; SA, Sodium Arsenite; SOD1, superoxide dismutase 1; VAChT, vesicular acetylcholine transporter; TARDBP, TAR DNA-Binding Protein.

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to the VGF precursor C-terminus (Brancia et al., 2010). In the brain, TLQP peptides appear to show a restricted localization compared to other VGF-derived peptides, including a subpopulation of hypothalamic neurons projecting to a discrete area of median eminence (Brancia et al., 2010; Noli et al., 2014). Recently, a differential expression of several TLQP peptides was reported in the Syberian hamster brain. Namely, TLQP-21 (21 amino acid in length, rat $VGF_{556–576}$) was well represented in both hypothalamus and cortex while the longer form of TLQP-62 (rat $VGF_{556–617}$ encompassing the VGF precursor's C-terminus) was abundant in cortex, and less expressed in hypothalamus (Noli et al., 2015). TLQP peptides were also found in hypothalamic–pituitary axis and plasma, differently expressed during the oestrous cycle phases (Noli et al., 2014) as well as in several peripheral locations including adrenal and stomach, changing in condition of stress and upon fasting, respectively (D'Amato et al., 2008; Brancia et al., 2010). Additional molecular forms compatible with predicted TLQP-30 and TLQP-42 peptides were revealed in certain endocrine organs (Cocco et al., 2007; Brancia et al., 2010) but have not been further studied so far. In human plasma, TLQP peptides were upregulated upon hyperglycemia, and downregulated in obese subjects (D'Amato et al., 2015). As to bioactivity and possible role/s, TLQP-21 has been shown to be involved in the regulation of metabolic mechanisms (Bartolomucci et al., 2006; Jethwa et al., 2007; Lewis et al., 2017), reproduction (Aguilar et al., 2013; Noli et al., 2014), chronic stress (Razzoli et al., 2012) and inflammatory pain (Rizzi et al., 2008). The same peptide prevented apoptosis of rat cerebellar granules upon serum and potassium deprivation, with modulation of kinase phosphorylation (Severini et al., 2008). Also, it protected human umbilical vein endothelial cells against high-glucose-induced apoptosis, by enhancing glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate dehydrogenase, hence reducing reactive oxygen species (Zhang et al., 2013). Two receptor molecules have been identified for TLQP-21, namely the complement component 3a receptor (C3a-R: Hannedouche et al., 2013; Cero et al., 2014, 2016) and the receptor for the globular heads of c1q (gC1q-R: Chen et al., 2013) and involved, with TLQP-21, in modulating lipolysis (Cero et al., 2016) and neuropathic pain (Chen et al., 2013), respectively. While the precise mechanisms involved are not entirely known, there is strong evidence that the TLQP-21 may act by increasing intracellular calcium in Chinese hamster ovary cells (Cassina et al., 2013), microglia (Chen et al., 2013) and cerebellum (Severini et al., 2008). The longer form of TLQP-62 has been widely investigated in hippocampus where it enhances synaptogenesis (Behnke et al., 2017), regulates memory formation, and induces both synaptic potentiation (Bozdagi et al., 2008; Lin et al., 2015) and neurogenesis (Thakker-Varia et al., 2014). It can also cause dorsal horn cell hyper-excitability and behavioral hypersensitivity in rats (Moss et al., 2008). No specific receptor has been identified so far for TLQP-62. In ALS, despite the reported evidence that VGF expression is modulated in the animal model and humans (Pasinetti et al., 2006; Zhao et al., 2008), limited information is available regarding TLQP peptides. We have previously reported the involvement of the VGF C-terminal peptides in ALS, modulated in the SOD1 mutant mice and patient's plasma, but exclusively at the final disease phase (Brancia et al., 2016). Afterward, we aimed at specifically investigating the role of the TLQP peptides in ALS, by studying their expression and changes (using ELISA and immunohistochemistry) in transgenic mice (SOD1-G93A) and the mouse motor neuron-like hybrid cell line (NSC-34), as experimental models. In parallel, we also investigated, by ELISA, ALS patients' plasma and primary fibroblast cultures, the latter being considered a good cellular model used in human ALS research (Sabatelli et al., 2015; Yang et al., 2015) and also, contain VGF (Brancia et al., 2016). Moreover, in the NSC-34 cells, the neuroprotective role of the TLQP-21 was addressed in parallel with the presence of its two known receptors (gC1q-R and C3a-R), examined by both western blot and immunocytochemistry.

EXPERIMENTAL PROCEDURES

Human subjects

Subjects of Sardinian descent were studied, including ALS patients (females: $n = 20$, males: $n = 24$, age range: 25–85 yrs), and age-matched controls (unaffected by either neurological conditions, or diabetes; females: $n = 20$, males: $n = 26$). In patients, ALS-related mutations were studied as follows: exon 6 of the TARDBP gene, and all five coding exons of the SOD1 gene were screened by polymerase chain reaction and sequenced using the Big-Dye Terminator v3.1 kit (Applied Biosystems Inc) and an ABI Prism 3130 Genetic Analyzer. A repeat-primed polymerase chain reaction assay was used to screen for the GGGGCC hexanucleotide expansion in the first intron of C9ORF72 (DeJesus-Hernandez et al., 2011; Renton et al., 2011). ALS patients studied showed either: TARDBP-A382T mutation ($n = 16$), SOD1-G93A mutation ($n = 3$); expansion in the C9ORF72 gene ($n = 5$), or no identifiable ALS-related mutation ($n = 20$). The patients' motor and functional (which incorporates additional assessments of dyspnea, orthopnea, and the need for ventilatory support) performance was assessed at the time of blood sampling, by at least two experienced neurologists, according to the ALS Functional Rating Scale Revised (ALSFRS-R: Cedarbaum et al., 1999). Patients' data (summarized in Appendix A: Table 1A), including: age, gender, genetic mutation, ALSFRS-R score and co-morbidity at the time of blood sampling, as well as their clinical condition one year later (whether alive, or not, with or without tracheostomy). On the latter basis, patients were assigned to either group I, "early disease stage" ($n = 25$): patients who were alive and free of tracheostomy one year after blood sampling; or group II, "late disease stage" ($n = 19$): patients who were deceased, or had undergone tracheostomy. The present study was approved by the Ethics Committee at the Cagliari AOU ("Azienda Ospedaliero Universitaria di Cagliari"), protocol n. 450/09/C.E. All patients provided

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