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Plasma glutamate and glycine levels in patients with amyotrophic lateral sclerosis: The effect of riluzole treatment

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Abstract

Objectives: Defective glutamate (glu) metabolism and excitotoxicity have been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS). Moreover, glycine (gly) has been shown to potentiate excitatory transmission. The "antiglutamatergic" agent riluzole has been shown to prolong survival in ALS. The aim of the study was to investigate a possible effect of riluzole on plasma glu and gly levels, correlating with clinical response to treatment.

Patients and methods: Plasma concentrations of glu and gly were measured in 20 healthy volunteers and 22 ALS patients before treatment and after 6 months on riluzole.

Results: At baseline, increased plasma glu correlated with spinal onset and male gender whereas gly levels did not differ between patients and controls. No significant change was observed for both amino acids post-treatment, despite a lower rate of disease progression.

Conclusion: These results suggest that riluzole may affect disease progression without a significant impact on plasma glu and gly levels, possibly indicating different mechanisms of drug action.

Keywords: Amyotrophic lateral sclerosis (ALS); Glutamate (glu); Glycine (gly); Plasma; Riluzole

1. Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by progressive degeneration of the motor neurons in the spinal cord, brainstem and cerebral cortex. The pathogenetic mechanism of the disease remains unclear; however excitotoxicity may play a role, both as a primary action and as an indirect consequence of oxidative stress [1–4]. Glutamate (glu) is the major excitatory neurotransmitter of the corticospinal pathway. It has been suggested that the chronic loss of glutamate transport protein of the glial subtype GTL-1 (EATT 2) at specific sites of the CNS of patients with ALS results in increased concentrations of extracellular glutamate and consequently in overstimulation of glutamate receptors. This leads to degeneration of the motor neurons, probably through calcium overload [5,6].

Glycine (gly), an inhibitory neurotransmitter of the spinal cord and brainstem, has been shown to potentiate glutamatergic neurotransmission. It acts on a strychnine insensitive allosteric site of the NMDA receptor (glycine site) by increasing the frequency of channel opening, thus accelerating recovery of the receptor from glutamate-induced desensitization [2]. Consequently it enhances the excitotoxic effect of glutamate.

Riluzole (2-amino-6-trifluoromethoxy benzothiazole) has shown mild benefits in ALS, prolonging survival by 2–3 months [7,8]. Despite being considered as an antiglutamatergic agent, the exact mechanism of its action has not been completely elucidated yet, while the "glutamatergic hypothesis" of ALS has not been fully accepted.

The aim of this study was to investigate whether an effect of riluzole on plasma glu and gly levels exists in

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ALS patients, possibly correlating with clinical response to treatment.

2. Material and methods

2.1. Patients

A total of 42 subjects were enrolled in the study. Informed consent was obtained from each subject and the study protocol had the approval of the scientific committee of our hospital. The study was in accordance with the Declaration of Helsinki ethical guidelines.

The patient group comprised 22 adult patients with sporadic ALS according to WFN El Escorial criteria [9]. All patients were free of any medication. Heavy metal intoxication, monoclonal gammopathy, endocrine abnormalities, multifocal conduction block, B12 or folate deficiency, neoplasms, cervical myelopathy and β-hexosaminidase deficiency were excluded by appropriate biochemical tests, imaging, electromyographic and nerve conduction studies. Lumbar puncture was performed in 20 of the patients, revealing normal biochemical and immmunoelectrophoretic cerebrospinal fluid profile, as well as trace elements. The functional status of the patients was assessed with the Appel score [10]. Following initial evaluation patients were started on riluzole (100 mg/day) and were re-evaluated after 6 months.

The control group comprised 20 healthy volunteers, without any family history of ALS. The clinical characteristics of patients and controls are summarized in Table 1.

2.2. Methods

Sample handling and biochemical assay by high performance liquid chromatography were performed as previously described [11,12]. The blood samples were obtained after overnight fasting at 8:00–10:00 h. In order to avoid contamination, tubes, pipettes and eppendorf tubes were soaked in perchloric acid (6N) solution for 24 h and rinsed 5–6 times with Milli-Q water. All samples were immediately (within 5 min) transferred on ice to the laboratory and deproteinized by ultrafiltration (Ultrafree-MC UFC3GC filters, Millipore). All aliquots were stored at −80 °C until assay.

The determination of glu and gly was performed by reverse phase high performance liquid chromatography (Waters system) and pre-column derivatization with phenyl isothiocyanate. The column (pico-tag for free amino acids analysis, Waters) was set at 46 °C. The detection was made at 256 nm with an ultraviolet/visible detector (Waters 486). A computer-based software (Maxima-820, Waters) was used for quantitation of peaks and modulation of the pumps. Lyophilized serum from the ERNDIM Quality Control program for amino acid analysis was regularly used to check the method [13].

2.3. Statistical analysis

Some variables (including glu but not gly concentration) did not follow the normal distribution and are presented in terms of median values and quartiles. Analysis of covariance (ANCOVA) (after logarithmic transformation when appropriate) was used to compare glu and gly levels between the studied groups, pre-treatment. Repeated measures ANOVA was used to compare glu and gly levels pre- and post-treatment. Multiple regression, χ^2-test, Mann–Whitney *U*-test and the Spearman correlation coefficient were also used as appropriate.

3. Results

Clinical and biochemical data of patients are summarized in the table. ANCOVA revealed that patients with ALS, prior to treatment, presented with higher plasma glu levels. Age, disease duration, Appel score and the degree of diagnostic confidence during initial evaluation (possible, probable, definite) did not affect glu, but the type of ALS did, since the

Table 1
Clinical and biochemical data of studied groups

	Controls	ALS	
		Pre-treatment	6 months post-treatment
n	20		22
Sex (males/females)	12/8		15/7
Age (years)[a]	60 ± 10.2	57.4 ± 12	
Age of onset (years)[a]		55 ± 12.9	
ALS duration (months)[b]		20 (12–27)	
Type (spinal/bulbar)			15/7
Initial diagnostic confidence (possible/probable/definite)			6/9/7
Appel score[b]		62 (53–80)	81 (64–101)**
Plasma Glu (μM)[b]	32.8 (22.3–41.6)	45 (29.8–53.2)*	47.2 (33.2–51)*
Plasma Gly (μM)[a]	211.9 ± 63.5	202.4 ± 43.7	215.3 ± 89.2

Only significant differences are marked: *$P = 0.05$ vs. controls. **$P = 0.0033$ vs. pre-treatment.
[a] Mean ± S.D.
[b] Median (25th–75th percentile).

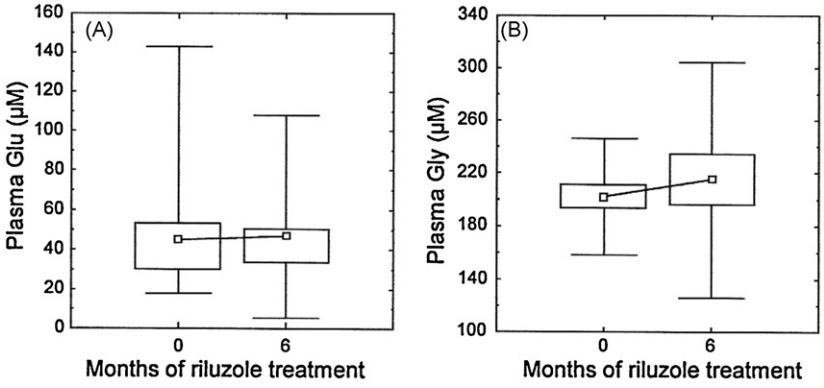

Fig. 1. Box and whiskers plots indicating median value, quartiles, range of plasma Glu levels (A) and mean, S.D. and range of plasma Gly levels (B), before and 6 months post-treatment. No significant change was noted.

spinal type presented with higher glu compared to the bulbar type [median (quartiles) 50 (38.4–63.4) vs. 29.8 (21.5–46.4), respectively, $P = 0.04$], the latter being comparable with the controls. Sex also affected glu in ALS only [males 52.1 (43.6–64.4), females 29.7 (21.5–41.8), $P = 0.03$]. As regards plasma gly, untreated patients did not differ from controls, with no effect of age, sex, ALS duration, subtype of the disease and levels of diagnostic confidence during initial evaluation. Glu and gly levels did not correlate significantly in either ALS or controls. Pre- and post-treatment characteristics of patients are shown in the table. After 6 months of treatment the disease significantly progressed, as judged by deterioration of the Appel score. No significant change from baseline plasma levels was observed for both glu and gly post treatment, with no effect of age, sex, disease duration at initiation of treatment, subtype of the disease and level of diagnostic confidence (Fig. 1). However the degree of post-treatment glu and gly change correlated positively with the Appel score change ($R_S = 0.65$, $P = 0.03$ and $R_S = 0.56$, $P = 0.07$, respectively).

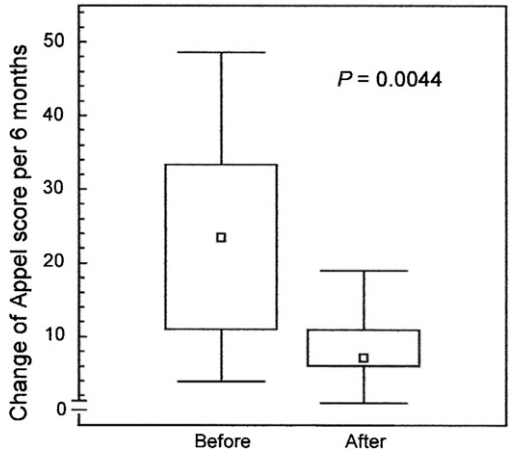

Fig. 2. Box and whiskers plots (median, quartiles, range), of rate of disease progression before and after treatment (P value of Wilcoxon matched pairs test), indicating significant reduction post-treatment. As a measure of the rate of disease progression, the change of Appel score per 6 months was used. Before treatment the mean change of Appel score was calculated according to the formula: (baseline Appel score/disease duration in months) × 6.

After treatment, despite progressive deterioration the rate of disease progression was lower as compared to the pre-treatment rate [7 (6–11) vs. 23 (11–34) points of Appel score per 6 months, respectively, $P = 0.0044$], with no difference between the two subtypes (Fig. 2).

4. Discussion

The present study confirmed a previously reported increase of plasma glu levels in patients with ALS [1,14–16]. In addition, our results indicate that a biochemical difference between ALS subtypes may be present, with plasma glu being increased only in the spinal but not in the bulbar type, in accordance with the observations of Camu et al. [15] and Spreux-Varoquaux et al. [17]. Excitotoxicity can be induced either by an elevation of the extracellular glu concentration or by an increased sensitivity of the postsynaptic neuron to glu stimulation, in the presence of normal synaptic glu levels [4]. Moreover, selective vulnerability of motor neurons may be related to glu receptor type, density, and subunit composition [18] as well as to decreased resistance to oxidative stress due to dysfunction of endogenous antioxidant mechanisms [3]. In vitro data support this hypothesis. Changes in the number or function of glutamate receptors in the CNS as well as differences in the glutamate receptor density between affected and spared motoneurons have been observed in ALS. These changes may be partly related to NMDA receptors [19]. However, the most important receptors involved in excitotoxicity seem to be the Ca^{2+}-permeable AMPA receptors [4], with selectively affected motor neurons having an increased proportion of this type of receptors, while AMPA (but not NMDA) receptor agonists induce spinal motor neuron damage in experimental animals [20]. Furthermore the differential expression and activity of the glutamate tranporters, Ca^{2+}-binding proteins such as parvalbumin and calbindin and metabotropic glu receptors in

specific cell types [4,5] may differentially affect glutamate uptake and consequently neuronal vulnerability. Therefore, the increase of plasma glu concentrations only in the spinal onset patients could suggest the possibility of different susceptibility of motor neuron subpopulation in this subtype of the disease. The observation that spinal onset disease is common in SOD-1 associated ALS, supports this hypothesis [21], as well as the observation that patients with spinal onset have increased CSF levels of vascular endothelial growth factor (VEGF) compared to patients with bulbar onset, possibly indicating a differential activation of neuroprotective mechanisms in response to excitotoxicity and oxidative stress [22]. Male patients had also higher glu concentrations compared to females. This could reflect possible gender-related variations in glu receptor density and subunit composition and might possibly explain the slightly higher incidence of the disease in males. Gly in the plasma of ALS patients has been reported to be elevated [14], decreased [16], or normal [1,15] and our results are compatible with the latter observation.

At 6 months post-treatment we observed no significant difference of plasma levels of both amino acids in relation to the pretreatment values, unlike the findings reported by Niebroj-Dobosz et al. [23]. However, given the positive correlation of glu with disease duration pretreatment, the levels of glu could be higher if the patients had been left untreated. Moreover, it should be mentioned that the degree of post-treatment glu and gly change (Δglu and Δgly) correlated positively with the degree of the Appel score change (Δscore), which should be viewed as an indirect slight indication that the lower the increase of glu and gly, the better the clinical outcome Furthermore, we found a lower rate of disease progression after treatment, in both subgroups of patients, in accordance with the minimal survival benefit of patients with ALS, previously reported [8,9]. However, despite being statistically significant and compatible with the "glutamatergic hypothesis" of ALS, we consider the correlation between Δglu and Δscore alone, inadequate to explain the therapeutic benefit of riluzole.

Although it is generally accepted that riluzole has a minimal and transient impact on ALS, the relation between the mode of action of the drug and the glutamate hypothesis remains to be clarified. Riluzole has been shown to inhibit glutamate release, facilitate glutamate uptake, block the increase in extracellular Ca^{2+} levels evoked by NMDA or glu and enhance gly receptor desensitization [24–26]. Despite its modulating role in glutamatergic transmission, the drug is able to interfere with the other pathogenic mechanisms that have been suggested in ALS, i.e. oxidative stress [27], neurotrophic factors [28] and apoptotic degeneration [29]. An antioxidative effect probably through inhibition of phospolipase A2 and an antiapoptotic effect possibly by protecting from mitochondrial alteration have been reported [29]. Furthermore the neuroprotective effect of riluzole may be related to its activity on the G-protein mediated signaling pathway [24]. Another important mechanism of action of the drug is its interaction with the voltage dependent sodium channel: it has been shown to block inactivated sodium channels and increase the recovery time from inactivation in a dose dependent manner, thus preventing neuronal depolarization [30]. The observed increase of *N*-acetylaspartate (NNA) in the cerebral cortex of ALS patients after a short period of riluzole treatment, using magnetic resonance spectroscopic imaging, probably related to the modulation of the firing rate of cortical neurons induced by the drug, supports the contribution of this mechanism of action [31]. The interference of riluzole with all these pathogenic mechanisms could possibly explain our results.

In conclusion, the present data are compatible with a biochemical difference between ALS subtypes, plasma glu being increased in the spinal type, while it remains normal in bulbar ALS. Despite this difference, both subgroups exhibited a lower rate of disease progression after treatment with riluzole, without any significant impact on plasma glu or gly levels, leading to the speculation that the slight benefit on disease progression could be attributed to additional mechanisms of action of the compound and not merely to its antiglutamatergic properties.

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