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Riluzole increases glutamate uptake by cultured C6 astroglial cells

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ABSTRACT

Riluzole is a drug approved for the treatment of amyotrophic lateral sclerosis (ALS) and may be effective for the treatment of other neurodegenerative and neuropsychiatric disorders. Riluzole exerts diverse actions on the central nervous system, including altering glutamate release and uptake, and therefore act diminishing glutamate extracellular levels, but the underlying mechanism of these actions is still unknown. Here, we demonstrate that riluzole stimulated glutamate uptake and augmented the expression of the glutamate EAAC1 transporter in C6 astroglial cell cultures. The effect of riluzole on glutamate uptake was reduced to below controls when it was co-administered with inhibitors of protein kinase C (PKC; bisindolylmaleimide II), phosphatidylinositol 3-kinase (PI3K; wortmannin) and fibroblast growth factor receptor 1 (FGFR1; PD173074). Riluzole also decreased reactive oxygen species load with no effect on glutathione levels. This study investigates three independent intracellular pathways and the mechanism of action of riluzole on glutamate metabolism.

1. Introduction

Riluzole (2-amino-6-trifluoromethoxy benzothiazole) is the only drug currently approved for the treatment of amyotrophic lateral sclerosis (ALS), and it is also studied as a putative treatment for other neurodegenerative and neuropsychiatric disorders (Bensimon et al., 2009; Pittenger et al., 2008). Multiple mechanisms of action have been described for riluzole, including modulation of cation channels, inhibition of neuronal firing, modulation of excitatory neurotransmitters activity (including glutamate) (Bellingham, 2011) and inhibition of protein kinase C (PKC) (Noh et al., 2000), resulting in a net neuroprotective effect. Some evidence also indicates that riluzole acts through an antioxidant mechanism both in humans (Bonnefont-Rousselot et al., 2000) and rodents (Rinwa et al., 2012), an action that could be particularly relevant considering the oxidative stress damage observed in ALS and other excitotoxic-mediated conditions (Mehta et al., 2013; Trotti et al., 1998; Van den Bosch et al., 2006).

Glutamate is the major excitatory neurotransmitter of the mammalian brain, and appropriate glutamatergic signaling is dependent on precise regulation in the synaptic cleft. A major mechanism for the synaptic clearance of glutamate involves a family of sodium-dependent glutamate transporters, named excitatory amino acid transporter 1–5 (EAAT1-5), also known in rodents as GLAST, GLT-1, EAAC1 and rodent EAAT 4–5, respectively (Beart and O'shea, 2007; Foran and Trotti, 2009). These transporters have various distributions among different cell types, with EAAC1 acting as the main transporter expressed by C6 astroglial lineage cells (Davis et al., 1998). Interference with the proper function of these transporter proteins has been shown to elevate extracellular glutamate levels and induce neuronal damage (Beart and O'shea, 2007; Mehta et al., 2013; Swanson et al., 1997), which sometimes resembles degenerative patterns of human diseases. ALS patients were shown to have diminished levels of both GLT-1 and EAAC1 (Rothstein et al., 1995, 2005), while the loss of GLT-1 was not attributed to cellular death but rather to underexpression. Strategies that modulate glutamate homeostasis, including its transport, have potential therapeutic effects in a range of neurodegenerative diseases, including ALS (Sheldon and Robinson, 2007).

Riluzole was shown to stimulate glutamate uptake in rat cortical astrocyte cultures (Frizzo et al., 2004) and rat spinal cord synaptosomes, as well as to decrease glutamate release (Azbill et al., 2000), and exerted such effects in concentrations that resemble those found in humans during treatment for ALS (Bellingham, 2011). This action could potentially be beneficial for neurodegenerative diseases, although the modulation of glutamate in other cell populations and the mechanism of action are yet to be established. In this study, we tested the effect of riluzole on glutamate uptake in

Abbreviations: ALS, amyotrophic lateral sclerosis; EAAT, excitatory amino acid transporter; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; FGFR1, fibroblast growth factor receptor 1; FRS2α, FGFR substrate 2α; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; EDTA, ethylene-diaminetetracetic acid; ANOVA, analysis of variance; bis II, bisindolylmaleimide II; BDNF, brain-derived neurotrophic factor.

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cultured C6 astroglial cells and the influence of three different cellular pathways: PKC, phosphatidylinositol 3-kinase (PI3K) and fibroblast growth factor receptor 1 (FGFR1). Expression of EAAC1 was also tested after riluzole treatment, as well as the total reactive oxygen species and glutathione (GSH) levels.

2. Materials and methods

2.1. Materials

Riluzole, standard GSH, DCF-DA, Ponceau S staining, and cell culture materials were purchased from Sigma (St. Louis, MO, USA), except for Dulbecco's modified Eagle's medium (DMEM) which was purchased from Gibco BRL (Carlbad, CA, USA). Fetal bovine serum (FBS) was obtained from Cultilab (Campinas, SP, Brazil). L-[3H]Glutamate and ECL was purchased from Amersham International (UK). Polyclonal anti-EAAC1 antibody was purchased from Alpha Diagnostic. All other chemicals were purchased from local commercial suppliers.

2.2. C6 cell culture and pharmacological treatments

The C6 cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and was cultured as previously described (dos Santos et al., 2006). The C6 lineage cells are an astrocyte-like cell line that is widely used to study cellular functions such as glutamate uptake, glutamine synthetase activity, S100B secretion, and oxidative stress (dos Santos et al., 2006; Quincozes-Santos et al., 2009, 2010). The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% FBS, 2.5 mg/mL Fungizone® and 100 U/L gentamicin. Cells were kept at a temperature of 37 °C in an atmosphere of 5% CO_2/95% air. Exponentially growing cells were detached from the culture flasks by using 0.05% trypsin/ethylenediaminetetracetic acid (EDTA) and then seeded (5×10^3 cells/cm^2) in 24- or 6-well plates. When cells reached confluence, the culture medium was removed by suction, and the cells were incubated for 6 h at 37 °C in an atmosphere of 5% CO_2/95% air in DMEM (pH 7.4) without serum in the absence (control) or presence of riluzole (0.1, 1, 10 or 100 μM) for 1–24 h, and in absence or presence of bisindolylmaleimide II (1 μM), wortimannin (1 μM) or PD173074 (10 μM) for 1 h. Membrane integrity loss was determined by fluorescent image analysis of propidium iodide (PI) uptake (dos Santos et al., 2006). Cells were incubated with 7.5 μM PI, concomitantly with treatments ($N = 3$, performed in triplicate). Images were acquired by digital camera (Sound Vision Inc., Wayland, MA, USA) at the end of incubations, using a TE-FM Epi-Fluorescence accessory. Optical density was determined with Optiquant version 02.00 software (Packard Instrument Company). Density values obtained were expressed as density light units (DLU).

2.3. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) assay was conducted in 50 μL of extracellular medium using a commercial colorimetric assay from Doles (Brazil).

2.4. MTT reduction assay

Cells were treated with 50 μg/mL MTT for 30 min in 5% CO_2/95% air at 37 °C. Subsequently, the medium was removed, and the MTT crystals were dissolved in DMSO. Absorbance values were measured at 560 and 650 nm.

2.5. Glutamate uptake assay

Glutamate uptake was performed as previously described (Gottfried et al., 2002) with some modifications. Briefly, C6 glioma cells were incubated at 37 °C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO4{\cdot}7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$, and 5.6 glucose, adjusted to pH 7.4. The assay was started by addition of 0.1 mM L-glutamate and 0.33 μCi/mL L-[2,3-^{3}H]glutamate. Incubation was stopped after 10 min by removing the medium and rinsing the cells twice with ice-cold HBSS. The cells were then lysed in a solution containing 0.5 M of NaOH. Incorporated radioactivity was measured in a scintillation counter. Sodium independent uptake was determined by using N-methyl-D-glucamine instead of NaCl. Sodium-dependent glutamate uptake was obtained by subtracting the sodium independent uptake from the total uptake in order to obtain the specific uptake. Values are expressed as nmol/mg protein/min.

2.6. Western blot analysis

Equal amounts of proteins from each sample were boiled in sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue] and submitted to electrophoresis in 10% (w/v) SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma). After overnight incubation with the polyclonal anti-EAAC1 antibody (1:1000) antibody at room temperature, filters were washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG) at a dilution of 1:1000 for 1 h. The chemiluminescence signal was detected using an ECL, after the films were scanned and bands were quantified using the Scion Image software.

2.7. Evaluation of intracellular ROS levels

Intracellular ROS levels were measured using the nonfluorescent cell permeating compound 2′,7′-dichlorofluorescein diacetate (DCF-DA) method. DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound, 2′,7′-dichlorofluorescein (DCFH). C6 cells were treated with DCF-DA (10 μM) for 30 min at 37 °C. Following DCF-DA exposure, the cells were rinsed and then scraped into PBS with 0.2% Triton X-100. The fluorescence was measured in a plate reader (Spectra Max Gemini XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm (Quincozes-Santos et al., 2009). Values were obtained as fluorescence units/mg protein and expressed as % of control.

2.8. Glutathione (GSH) content assay

Glutathione levels (nmol/mg protein) were measured as previously described (Browne and Armstrong, 1998). Cell homogenates were diluted in 10 volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and proteins were precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdeyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured by using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 μM).

2.9. Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard (Lowry et al., 1951).

2.10. Statistical analysis

Data represent means ± SE and were analyzed statistically by one-way analysis of variance (ANOVA), followed by the Turkey test. Values of $p < 0.05$ were considered to be significant.

3. Results

3.1. Riluzole has dose- and time-dependent toxic effects on C6 astroglial cell cultures

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released into the extracellular space upon loss of membrane integrity. Riluzole significantly increased LDH levels in the incubation medium of C6 astroglial cell cultures in a dose- and time-dependent manner. This effect occurred after a short treatment with high doses (1 h and 500 μM – 120% of control; $p < 0.05$) and with doses as low as 25 μM after prolonged exposure (24 h – 136% of control, $p < 0.05$), whereas no significant effect was observed with doses lower than 25 μM in the same prolonged treatment period (data not shown).

Cell viability assays were performed using the colorimetric [3(4,5-dimethylthiazol-2-yl)]-2,5-diphenyl tetrazolium bromide (MTT) assay (Hansen et al., 1989). Yellow MTT is reduced to purple formazan by the metabolic activity of living cells. The alterations of LDH levels described above were accompanied by a corresponding decrease in cellular viability as measured through the MTT assay, which was reduced to 87% of the control after treatment with 500 μM riluzole for 1 h ($p < 0.001$) and to 88% of the control after treatment with 25 μM riluzole for 24 h ($p < 0.05$). Such data indicate a dose and time threshold for cellular damage mediated by riluzole and was corroborated by significant propidium iodide incorporation after 1 h of treatment with doses of 300, 500 and 1000 μM riluzole (data not shown).

3.2. Riluzole increases glutamate uptake and EAAC1 expression

We studied the influence of riluzole on glutamate uptake at doses that did not induce damage on the studied cells within a 1 h time frame (0.1–100 μM). One hour pre-incubation of riluzole significantly enhanced glutamate uptake in C6 astroglial cell cultures