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Nordihydroguaiaretic acid increases glutamate uptake *in vitro* and *in vivo*: Therapeutic implications for amyotrophic lateral sclerosis

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ABSTRACT

Synaptic accumulation of glutamate causes neuronal death in many neurodegenerative pathologies including amyotrophic lateral sclerosis. Drugs capable of increasing glutamate uptake could therefore be therapeutically effective. We screened in a cell-based assay a library of 1040 FDA-approved drugs and nutrients for compounds that could enhance glutamate uptake. Nordihydroguaiaretic acid (NDGA), an anti-inflammatory drug that inhibits lipoxygensases, potently enhanced glutamate uptake in MN-1 cells. Given subcutaneously at 1 mg/day for 30 days in mice, NDGA increased glutamate uptake in spinal cord synaptosomes persistently throughout the treatment. However, when administered following the same regimen to the SOD1-G93A transgenic mouse model of ALS at disease onset, NDGA did not extend survival of these mice. We found that NDGA failed to sustain increased glutamate uptake in the SOD1-G93A mice despite an initial upregulation measured during the first 10 days of treatment. SOD1-G93A mice displayed a progressive increase in spinal cord expression levels of the efflux transporter P-glycoprotein beginning at disease onset. This increase was not mediated by the NDGA treatment because it was measured in untreated SOD1-G93A mice. Since P-glycoproteins control the extrusion of a broad range of toxins and xenobiotics and are responsible for drug resistance in many diseases including cancer and brain diseases such as epilepsy, we propose that the failure of NDGA in maintaining glutamate uptake upregulated in SOD1-G93A mice and its therapeutic inefficacy are due to acquired pharmacoresistance mediated by the increased expression of P-glycoprotein.

Introduction

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system (CNS). Abnormal accumulation of glutamate in the synaptic cleft and excessive activation of glutamate receptors contribute to neuronal death in acute insults to the CNS. The process, known as 'excitotoxicity', also contributes to neuronal loss in neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) (Pasinelli and Brown, 2006). Excitotoxicity is based on altered extracellular concentrations of glutamate, since it is this pool that can be toxic to neurons. The intracellular concentration of glutamate (1–10 mM) is 1000 to 10,000-fold greater than the extracellular concentration (<1–10 μM) (Danbolt, 2001; Clements et al., 1992; Herman and Jahr, 2007). In the excitatory synapses of the nervous system termination of the excitatory neurotransmission occurs exclusively *via* a re-uptake mechanism mediated by high-affinity glutamate carriers. There are 5 high-affinity, Na^+-dependent glutamate transporters: EAAT1 (GLAST), EAAT2 (GLT-1), EAAT3 (EAAC1), EAAT4, and EAAT5. These transporters exhibit distinct properties in substrates affinity or inhibitors sensitivity. For instance, dihydrokainate (DHK) is a non-transportable EAAT2 (GLT-1) inhibitor ineffective, if not at millimolar concentration, on the other glutamate transporter subtypes.

Several studies indicate that genetic and pharmacological upregulation of expression levels and activity of the glutamate transporter EAAT2 (GLT-1) can offer neuroprotection *in vitro* and *in vivo* in animal models of neurodegeneration (Guo et al., 2003; Rothstein et al., 2005). On the contrary, loss of EAAT2 expression levels can adversely worsen the course of neurodegenerative diseases such as ALS, at least in animal models (Pardo et al., 2006). Therefore, a correct functioning of the glutamate transporters in general and of the EAAT2 subtype in particular is of fundamental importance. Rescuing or increasing glutamate transport in neurological disorders characterized by an excitotoxic component by modulating the activity of a specific glutamate transporter subtype or the overall cellular glutamate uptake could constitute an efficacious treatment for such pathological conditions.

Based on these premises, we established a cell-based assay to screen for compounds with glutamate transport enhancing activity. We screened a structurally diverse library of 1040 FDA-approved drugs and nutrients known to cross the blood–brain barrier (Micro-Source Discovery Systems, NINDS custom collection ™) (Heemskerk et al., 2002) using an *in vitro* activity assay based on a clonal neural

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hybrid cell line, referred to here as MN-1 (Salazar-Grueso et al., 1991). The natural dicatechol nordihydroguaiaretic acid (NDGA), a generic lipoxygenase inhibitor with anti-inflammatory and anti-oxidant properties (Salari et al., 1984), was particularly potent in increasing the high-affinity glutamate transport activity in MN-1 cells. NDGA was also active *in vivo* since we measured increased glutamate transport activity in synaptosomes prepared from mice treated up to 30 days with NDGA. However, when NDGA was chronically administered to the SOD1-G93A mouse model of ALS to determine if it could be therapeutically effective in this chronic neurodegenerative disease characterized by glutamate transport downregulation (Bendotti et al., 2001; Rothstein et al., 1993; Rothstein et al., 1992), we observed an initial increase in glutamate transport in spinal cord synaptosomes after 10 days of treatment that vanished at 20 and 30 days of treatment. Kaplan–Meier survival analysis showed that the SOD1-G93A mice did not benefit from the NDGA treatment, presumably in part because the NDGA treatment failed in keeping glutamate uptake upregulated. Interestingly, SOD1-G93A mice showed increased expression levels of P-glycoprotein (P-gp) in the spinal cord during the progression of the disease. This increase was likely caused by the disease mechanisms and not related to the NDGA administration as we have observed it in untreated SOD1-G93A mice. Moreover, *in vitro* assay showed that NDGA increased P-gp basal activity measured in vesicles strongly suggesting that it could be used as substrate by this transporter *in vivo* (Garrigues et al., 2002; Gebhardt et al., 2002). As these proteins are involved in multi-drug resistance *via* a transport-mediated mechanism of extrusion of xenobiotics out of the cells in which they are expressed, we suggest that their constitutive increased expression levels in the spinal cord of SOD1-G93A mice could be responsible for the poor efficacy of NDGA in maintaining elevated glutamate transport activity *in vivo*, compromising its potential therapeutic efficacy.

Materials and methods

MN-1 cell culture, screening assay and glutamate transport activity measurement

We used a clonal neural hybrid cell line (MN-1) that expresses motor neuron characteristics and showed consistent glutamate transport activity and expression of Na^+-dependent, high-affinity glutamate transporters EAAT1 (GLAST), EAAT2 (GLT-1) and EAAT3 (EAAC1). Cells were grown in 24-well plates in DMEM supplemented with 10% FBS and penicillin/streptomycin. Compounds from the NINDS library collection or pharmacological agents were added for 12 h to the cells at 37 °C in Opti-MEM (Gibco) at 1 μM in 0.1% DMSO or at concentrations otherwise indicated. Control groups received DMSO 0.1%. The next day compounds are removed by three washes with PBS and glutamate transport activity was measured for 15 min at room temperature by adding the uptake buffer in the presence of 1 μM glutamate (1:30 isotopic dilution with ^{3}H-glutamate, NEN). Uptake buffer contained (in mM): 5 HEPES/Na, 145 NaCl, 2.5 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 1.2 K_2HPO_4, 10 glucose, pH 7.4. Glutamate uptake is terminated by rapid aspiration followed by washing the cells with ice-cold uptake stopping buffer containing (in mM): 5 Tris–HCl, 145 CholineCl, 2.5 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 1.2 K_2HPO_4, 10 Glucose, pH 7.4. Cells are harvested with lysis buffer containing 0.5 N NaOH and 0.1% Triton X-100, and accumulated radioactivity measured by liquid scintillation counting. For saturation experiments the uptake solution contained L-glutamate in the concentration range 1–250 μM and 0.5 μCi of L-^{3}H-glutamate as radiotracer.

Synaptosome preparation

Synaptosomes were prepared from spinal cords of mice according to our published protocol with small modifications (Volterra et al., 1992). Synaptosomes (50 μg/ml) were incubated at 37 °C for 15 min under shaking conditions in oxygenated Krebs/bicarbonate buffer containing (in mM): NaCl 124, KCl 4.6, $CaCl_2$ 1.2, $MgCl_2 \times 6H_2O$ 1.3, KH_2PO_4 0.42, $NaHCO_3$ 26.75, glucose 10, adjusted to pH 7.4 and 10 μM [^{3}H]-glutamate (N.E.N. 41.9 Ci/mmol; isotopic dilution 1:4000). The uptake was terminated by addition of ice-cold uptake stopping buffer with 100-fold excess cold glutamate and rapid filtration on 0.45 μm nitrocellulose filters. Filter blotted synaptosomes were counted by liquid scintillography for incorporated radioactivity. Accumulation of glutamate in synaptosomes in the presence of Na^+ is usually between 100,000 and 140,000 d.p.m. at the isotopic dilution indicated above. Radioactivity retained after incubation in Choline$^+$ uptake buffer was used to correct data to represent Na^+-dependent uptake. Experimental samples were run at least in triplicate.

Data analysis

Uptake kinetics was determined by non-linear regression analysis of saturation curves using the Michaelis–Menten equation built in GraFit v5.0 (Eritacus Software). Log concentration–response curve were constructed for the determination of IC_{50} and data were fitted using a four-parameter logistic equation built. IC_{50} values were converted to K_i (inhibition constant) values according to the equation $K_i = IC_{50}/1+[s]/K_m$ where [*s*] equals the concentration of L-glutamate substrate (1 μM) and K_m equals the estimated K_m for glutamate calculated with the Michaelis–Menten transformation. Data reported in this study represent the average ± s.e.m. of at least three independent determinations.

Western Blot analysis

Spinal cords were collected from SOD1-G93A mice at different stages of disease and from SOD1-wild type mice age-matched with end-stage SOD1-G93A mice and immediately homogenized on ice (glass-teflon homogenizer) in 30 volumes of PBS with 1% SDS and protease inhibitor mix (Complete ™). MN-1 cells were also collected and homogenized in the same buffer. The SDS-extracts were then incubated for 10 min at room temperature, sonicated, centrifuged at 1000 ×*g* to remove unsolubilized material and immediately analyzed or stored at −80 °C. Immunoblots were probed with anti-P-glycoprotein (Mdr; polyclonal rabbit antibody H241; Santa Cruz Biotechnology; 1 μg/ml; this antibody recognizes both P-gp isoforms, $Mdr1_a$ and $Mdr1_b$ in rodents). Affinity-purified polyclonal antibodies against glutamate transporter subtypes were A522–541 (0.2 μg/ml; rabbit 8D0161, rat EAAT1 C-terminus), B12–26 (0.1 μg/ml; rabbit 26970, rat EAAT2 N-terminus), B493–508 (0.1 μg/ml, rabbit 84946, rat EAAT2 C-terminus), ABR556–573 (Affinity BioReagent; cat.#PA3–040, 1:1000, mouse EAAT2 -terminus), and C161–177 (0.5 μg/ml, rat EAAT3 extracellular loop, Zymed lab). Band intensity was quantified using the Chemidoc system (BioRad). The anti-EAAT antibodies were raised against identical epitopes in mouse and rat isoforms.

Mice models of ALS and pharmacological treatment

Human SOD1-wild type (B6SJL-TgN(SOD1)2Gur; stock #002297; Jackson Lab) and SOD1-G93A mutant (B6SJL-TgN(G93A-SOD1)1Gur; stock #002726; Jackson Lab) transgenic mice were used for this study. Male SOD1-G93A mice were bred in house with B6SJL females. Offsprings were assessed for the presence of the human SOD1 transgene and copy number by PCR. NDGA and placebo were delivered in a time-controlled release manner using subcutaneous implantation of matrix-driven delivery pellets (Innovative Research of America). The experimental groups receiving NDGA or placebo consisted of 17 mice, 6 males and 11 females. The pellet technology integrates the principles of drug diffusion, erosion and concentration