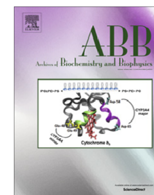
Archives of Biochemistry and Biophysics 562 (2014) 1–8

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi



Role of calpain-1 in the early phase of experimental ALS

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ARTICLE INFO

Article history:
Received 21 May 2014
and in revised form 29 July 2014
Available online 21 August 2014

Keywords:
Ca^{2+}-dependent proteolysis
Calpain
SOD1/G93A transgenic mice
ALS
Neurodegeneration
NR2B
NMDA-R
nNOS

ABSTRACT

Elevation in $[Ca^{2+}]_i$ and activation of calpain-1 occur in central nervous system of *SOD1^{G93A}* transgenic mice model of amyotrophic lateral sclerosis (ALS), but few data are available about the early stage of ALS. We here investigated the level of activation of the Ca^{2+}-dependent protease calpain-1 in spinal cord of *SOD1^{G93A}* mice to ascertain a possible role of the protease in the aetiology of ALS. Comparing the events occurring in the 120 day old mice, we found that $[Ca^{2+}]_i$ and activation of calpain-1 were also increased in the spinal cord of 30 day old mice, as indicated by the digestion of some substrates of the protease such as nNOS, αII-spectrin, and the NR2B subunit of NMDA-R. However, the digestion pattern of these proteins suggests that calpain-1 may play different roles depending on the phase of ALS. In fact, in spinal cord of 30 day old mice, activation of calpain-1 produces high amounts of nNOS active species, while in 120 day old mice enhanced-prolonged activation of calpain-1 inactivates nNOS and down-regulates NR2B.

Our data reveal a critical role of calpain-1 in the early phase and during progression of ALS, suggesting new therapeutic approaches to counteract its onset and fatal course.

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Introduction

Calpains are members of a family of neutral calcium-dependent intracellular proteases whose proteolytic activity plays a crucial role in several physiological functions of the central nervous system (CNS)[1] [41,12,52]. Due to dysregulation of Ca^{2+} homeostasis, the activity of calpain undergoes a transition from a physiological to a pathological role thereby becoming involved in the development of most neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) [50,51,19,6,36,16,9].

The possibility that over-activation of calpain could be implicated in the neurodegenerative scenario that characterizes ALS was supported by the observation that intracellular free Ca^{2+} ion level ($[Ca^{2+}]_i$) is increased in motor nerve terminals of biopsied human ALS muscles [38] and in animal models of familial ALS (fALS) with super oxide dismutase (SOD) 1 mutations, even in the early stage of the disease [39]. Dysregulation of Ca^{2+} homeostasis has been attributed to different factors, such as over-stimulation of post-synaptic NMDA receptors (NMDARs), alteration of mitochondrial electron transport found in patients as well as in the animal model here utilized [45,25], and modification in the expression/function of important Ca^{2+}-binding proteins [1]. Although the role of each of these factors has been well established, their temporal and spatial interplay remains elusive. Moreover, in transgenic mice over-expressing the G93A point mutated human soluble Cu/Zn SOD1 (*SOD1^{G93A}*) form, a widely utilized experimental model of ALS [14,37,47], we have recently demonstrated the presence of excessive glutamate release in spinal cord of 120 day old *SOD1^{G93A}* transgenic mice and found that this phenomenon was already present during the early phase of the disease [26]. This abnormal release was supported by elevated cytosolic $[Ca^{2+}]$ present at synaptic level in the spinal cord of ALS mice. A direct evidence for the occurrence of an increased activity of calpain in nervous tissue of *SOD1^{G93A}* transgenic mice was provided by the elevation in $[Ca^{2+}]_i$ found in spinal cord of these animals in parallel with an increased activation of calpain [43]. It was

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[1] *Abbreviations used:* ALS, amyotrophic lateral sclerosis; fALS, familial ALS; CNS, central nervous system; SOD, super oxide dismutase; nNOS, neuronal nitric-oxide synthase; NO, nitric-oxide; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; NR2B, NMDAR subunit 2B.

http://dx.doi.org/10.1016/j.abb.2014.08.006
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observed that during a clear symptomatic stage of the disease (110–120 day old mice) when the *SOD1*^{G93A} transgenic mice show remarkable pathological symptoms, the dysregulation of $[Ca^{2+}]_i$ is accompanied by a large increase in the activity of both calpain-1 and -2 in distinct regions of CNS. These studies confirmed a concomitant alteration in $[Ca^{2+}]_i$ in those regions of CNS most affected in ALS, leading to the activation of both the classical calpain-1 and -2 isoforms, to adaptive changes of the Ca^{2+}-dependent proteolytic system, and to digestion of calpain sensitive protein substrates [43]. No information is currently available, however, on the possible involvement of the protease during the early, phase of ALS when the animals do not show remarkable clinical symptoms of the disease. In this frame we wanted to establish whether the biochemical alterations observed in symptomatic 120 day old *SOD1*^{G93A} mice also occur at an early phase (30 day old mice) of the disease. The involvement of calpain-1 in the early phase of ALS is herein investigated by determining its consumption and substrate degradation [12,21,42].

The data reported demonstrate that an increase in $[Ca^{2+}]_i$ and the activity of calpain-1 also take place at an early stage of ALS. As for 120 day old *SOD1*^{G93A} mice, also for 30 day old animals the region of CNS showing the more pronounced calpain activation is the spinal cord, mainly in lumbar and sacral segments. However, the increase of the activity of calpain and the extent of digestion of specific substrates of the protease such as αII-spectrin, neuronal nitric oxide synthase (nNOS), and NR2B are much less pronounced in 30 day old than in 120 day old animals.

Our observations could be important in determining the progressive development of ALS disease and therefore utilized to design specific calpain inhibitors that could be useful for preventing the calpain-dependent neurodegenerative process.

Materials and methods

Materials

4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) was obtained from Calbiochem (Billerica, MA, USA). SuperSignal® West Pico Detection System was purchased from Pierce (Rockford, IL USA). Leupeptin, aprotinin, Percoll® and fura-2AM were obtained from Sigma Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-calpain-1 antibody and mouse monoclonal anti-β-actin antibody were purchased from Sigma Aldrich (St. Louis, MO, USA). Mouse monoclonal anti-αII-spectrin antibody was obtained from Chemicon (Billerica, MA, USA). Mouse monoclonal anti-nNOS antibody and mouse monoclonal anti-NMDAR2B (NR2B) subunit were purchased from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Horseradish peroxidase (HRP)-linked anti-mouse and HRP-linked anti-rabbit secondary antibodies were purchased from Cell Signaling (Danvers, MA).

Animals

B6SJL-TgN SOD1-G93A(+)1Gur mice expressing a high copy number of mutant human super oxide dismutase (SOD) 1 with Gly93Ala substitution (*SOD1*^{G93A}, here referred to as transgenic mice), and B6SJL-TgN (SOD1)2Gur mice expressing wild-type human SOD1 (here referred to as control mice), were obtained from The Jackson Laboratories and bred at the animal facility of the Pharmacology and Toxicology Unit, Department of Pharmacy, University of Genoa, Italy. Selective breeding maintained each transgene in the hemizigous state on an F1 hybrid C57Bl6 × SJL genetic background [14]. Animals were housed at a constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular dark-light schedule (light on 7 a.m. to 7 p.m.). Food and water were freely available. Transgenic mutated and age- and gender-(male) matched control mice were sacrificed approximately at 30 days of life (age in which a significant loss of muscle efficiency is still absent), or at 120–130 days of life (due to the presence of significant loss of muscle efficiency according to a five-point score scale as previously described by [48]). The tissues were removed and collected as described elsewhere in this Section. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Unit, Department of Pharmacy, in accordance with the European legislation (European Communities Council Directive of November, 24th, 1986, 86/609/EEC) and were approved by Italian legislation on animal experimentation (approved project protocol number 298223-8). All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable results.

Preparation of synaptosomes

30 day old and 120 day old *SOD1*^{G93A} transgenic mice and control animals were sacrificed, and the spinal cord as well as the brain were rapidly removed. Synaptosomes were prepared essentially as previously described [26]. Briefly, the tissue was homogenized in 14 volumes (w/v) of 0.01 MTris–HCl, pH 7.4, containing 0.32 M sucrose using a glass–teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min, 1000*g* at 4 °C) to remove nuclei and debris and the supernatant was gently stratified on a discontinuous Percoll® gradient (2%, 6%, 10% and 20% v/v in Tris-buffered sucrose). After centrifugation (5 min, 33,500*g* at 4 °C), the layer between 10% and 20% Percoll® (synaptosomal fraction) was collected, washed (15 min, 22,000*g* at 4 °C) and resuspended in 10 mM HEPES, pH 7.4, containing 140 mM NaCl, 3 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, and 10 mM glucose (physiological medium). Protein content was determined according to Bradford method [5].

Determination of intrasynaptosomal [Ca^{2+}*]*

Intracellular Ca^{2+} concentration was determined in the cytosol of spinal cord and brain synaptosomes using the fluorescent dye fura-2/AM as previously described [26]. Briefly, synaptosomes were incubated for 40 min at 37 °C, while gently shaking, in 10 mM HEPES medium, pH 7.4, in the presence of 5 μM fura-2/AM (and 0.5% DMSO). Synaptosomes, incubated in the presence of 0.5% DMSO only, were used to measure autofluorescence. After extrasynaptosomal fura-2/AM removal, the pellets were resuspended in HEPES-buffered medium, divided into 200 μl aliquots (200 μg protein/sample), and stored at 4 °C until use. Measures were obtained within 2 h. Synaptosomes were diluted in HEPES-buffered medium (final volume 2 ml) and equilibrated at 37 °C for 15 min. The measurements were made at 37 °C in a thermostated cuvette under continuous stirring using an RF-5301PC dual wavelength spectrofluorophotometer (Shimadzu, Japan) by alternating the excitation wavelengths of 340 and 380 nm. Fluorescent emission was monitored at 510 nm. Basal fluorescence was recorded for 15 min. Calibration of the fluorescent signals was performed at the end of each measure by adding 10 μM ionomycin in the presence of $CaCl_2$ to obtain F_{max}, followed by 10 mM EGTA (adjusted to pH 8.0 with 3 mM; Tris) to obtain F_{min}. After correcting for extracellular dye, $[Ca^{2+}]$ was calculated with the equation of Grynkiewicz [13] using a K_D of 224 nM for the Ca^{2+}-fura-2 complex.

Immunoblot analysis

30 day old and 120 day old *SOD1*^{G93A} transgenic mice and control animals were sacrificed, and the spinal cord as well as the

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