J. Biol. Res. 2011 n.1 - Vol. LXXXIV - Rubbettino - Soveria Mannelli

Is the expression of [-G93A(+)] human SOD1 a model to study neurodegenerations?

R. Stifanese*, M. Averna, M. Pedrazzi, R. De Tullio, F. Salamino, S. Pontremoli, E. Melloni

DIMES -Biochemistry Section- and CEBR, University of Genoa, Viale Benedetto XV,1–16132 Genoa, Italy
* stifanese@unige.it

KEY WORDS: Ca^{2+}-dependent proteolysis; calpain; calpastatin; NOS; ALS.

Abstract

To relate the alterations occurring in neurodegenerations with Ca^{2+} homeostasis dysregulation, we analyzed the functional properties of the Ca^{2+}-dependent calpain/calpastatin system in neuronal cells of transgenic mice overexpressing human mutated –G93A(+) SOD1. Motor cortex and spinal cord lower segments from transgenic mice show a very large increase in free Ca^{2+} ions and evident calpain activation, identified on the basis of its consumption and substrates digestion. Changes in calpastatin intracellular localization and calpain conformation state further support these observations. Moreover, calpastatin is significantly expressed, counteracting its calpain-mediated degradation. Thus, the calpain/calpastatin system may be a target for new therapeutic approaches to neurodegenerative diseases.

Introduction

A transition of calpain activity from physiological to pathological neuronal functions can be induced by prolonged $[Ca^{2+}]_i$ elevation, as it seems to occur in many human pathologies including neurodegenerations [1,2]. This event causes Ca^{2+}-dependent proteases and NO synthase activation, both processes of potential risk for cell integrity. Indeed, their activation has been related to a number of cellular damages including digestion of structural and functional proteins and production of highly reactive species damaging lipids and nucleic acids [3]. To verify the possible relationship between Ca^{2+} homeostasis dysregulation and cellular damages, we used transgenic mice overexpressing the mutated human SOD1, an animal model known to develop a pathological state similar to human fALS [4]. In these animals Ca^{2+} level and the properties of both calpain and calpastatin were studied. Intracellular calpain activation can be identified by measuring its consumption and the appearance of the modified forms of its target proteins such as fodrin [5]. Moreover, the changes in calpastatin localization and its interaction with calpain are other effects promoted by $[Ca^{2+}]_i$ elevation accompanying the protease activation [6]. Calpastatin degradation and its expression were further investigated.

Materials and methods

Animals

B6SJL-TgN SOD1-G93A(+)1Gur mice expressing high copies of mutant human SOD1 with Gly93Ala substitution ("transgenic") and B6SJL-TgN (SOD1)2Gur mice expressing WT human SOD1 ("control") were obtained from Jackson Labs and sacrificed 120 days aged [7,8].

Antibodies

Anti-calpastatin 35.23 [6], anti-μ-calpain 56.3 [9], anti-m-calpain (Sigma), anti-fodrin (Chemicon), anti-nNOS (Transduction Labs) mouse mAbs were used.

Calcium imaging

Tissues were cut into thin slices and incubated in darkness (30 min, 37°C) in 10 mM HEPES containing 0.14 M NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, pH 7.4 (buffer A), and 15 μM Calcium Green™ 1-AM (Molecular Probes). The fluorescence was detected by BioRad MRC1024 confocal microscope and quantified by LaserPix Software (BioRad) [10,11].

Immunoblot (IB)

Tissues were homogenized in 50 mM Na-borate buffer, pH 7.5 containing 0.5 mM βMSH (buffer B), 2 mM EDTA, 0.1 mg/ml leupeptin, 10 μg/ml aprotinin and 2 mM AEBSF, and lysed by sonication. Aliquots (25 μg protein) of crude extracts were blotted, probed with the specific mAbs, detected by BioRad ChemiDoc XRS, and quantified by QuantityOne 4.6.1 software (BioRad).

Calpain activity assay

Tissues were homogenized in buffer A containing 2 mM EDTA and lysed as above. The particulate was discarded by centrifugation (60,000 x g, 10 min, 4°C) and 35 mg protein of the supernatants were submitted to ion-exchange chromatography [11]. μ- and m-calpain activity was then assayed [11].

Confocal microscopy

The frozen tissues were included in KILLIK™ (Bio-Optica), cut into 10 μm thick slices (Reichert-Jung Frigocut 2800 cryostat, Cambridge Instruments GmbH), and layered on microscope slides. Cells were fixed and permeabilized by the Triton/PFA method [6]. Calpastatin and calpain were detected by the specific mAbs. The fluorescence was detected and quantified as above [10-12].

Calpastatin level and forms

Aliquots (20 mg protein) of the tissues crude extracts were heated (5 min, 100°C). The particulate was discarded by centrifugation (60,000 x g, 10 min, 4°C) and 150 μg protein of the supernatants were submitted to SDS-PAGE. Active calpastatin forms and their level were evaluated following protein extraction from gel and assay of the inhibitory activity, whereas the molecular weights by their migration in SDS-PAGE [10,11].

RNA isolation and cDNA synthesis. Total RNA was extracted by guanidium thiocyanate and Nucleo Spin RNAII kit (Macherey Nagel). cDNA was synthesized from 3 μg of total RNA by ThermoScript RT-PCR system (Invitrogen). Semiquantitative PCR products [7] were resolved on 1% agarose gel. Calpastatin mRNA content was detected and quantified by ChemiDoc XRS (BioRad).

Results

Ca^{2+} level and calpain activation in CNS areas

Intracellular free [Ca^{2+}] was 4-fold higher in motor cortex and lower spinal cord segments of transgenic mice, whereas in cerebellum was significantly lower. In remaining CNS regions no alterations were present. Thus, calpain could be activated in precise CNS areas. In motor cortex and in total spinal cord of transgenic mice, μ- and m-calpain protein level and catalytic activity were largely reduced. Calpain activation was further indicated by fodrin degradation, a known calpain substrate [5,7]. In cerebellum all these processes were reduced, becoming undetectable in remaining CNS regions. Neuronal NO synthase was also degraded to the active 130 kD form [13].

Functional state of the calpain/calpastatin system in CNS areas

Following $[Ca^{2+}]_i$ elevation, calpastatin diffuses from the perinuclear aggregated form and associates with calpain in cytosol. The formation of such complex can be identified in cells by monitoring the conformation changes at which calpain undergoes following interaction with calpastatin. This process can be visualized by confocal microscopy using the 56.3 mAb and a fluorescent secondary Ab. Calpastatin, normally localized in aggregates close to nucleus, was largely diffused in cytosol of motor cortex neurons of transgenic mice. Calpain-induced fluorescence, evaluated by 56.3 mAb, was from 8- to 10-fold increased and in co-localization with calpastatin. The binding of 56.3 mAb to calpain established also that the protease was in a preactive state. Spinal cord was dissected into 4 segments, starting from cervical to sacral regions. In all segments from control animals calpastatin was in perinuclear aggregates. At difference, in segments 3 and 4 of transgenic mice, it was largely present in cytosol and the cytosolic calpain fluorescence was highly increased, indicating that the alteration in $[Ca^{2+}]_i$ promoted calpastatin redistribution and association to calpain. Segments 1 and 2 were undistinguishable from controls. In cerebellum of transgenic mice, these events were much less pronounced.
Inhibitory capacity and molecular properties of calpastatin in CNS areas.
The formation of the enzyme-inhibitor complex is accompanied by calpastatin degradation producing still active 15 kD fragments [10]. In those CNS areas of transgenic mice characterized by modifications on the calpain/calpastatin system, total calpastatin inhibitory activity was increased. In remaining CNS areas no changes were observed. Calpastatin 15 kD fragments, undetectable in control animals, were 50% of the total inhibitory activity in spinal cord, 20-25% in cerebellum, and poorly detectable in remaining CNS regions of transgenic mice. The presence of high levels of the 15 kD forms in those CNS areas in which $[Ca^{2+}]_i$ was highly modified can be visualized as a mechanism aimed to compensate the concomitant inactivation of calpastatin thus preventing the impairment of the system. Moreover, in the spinal cord upper segment and in cerebellum of transgenic mice, calpastatin mRNA level was 3-fold higher than in controls. In motor cortex, in spinal cord lower segments, and in remaining CNS regions of transgenic mice calpastatin synthesis was not significantly increased. Calpastatin overexpression can provide a second defence mechanism against unregulated protein digestion.

Functional state of the calpain/calpastatin system in skeletal muscle

Also in miocytes of transgenic mice elevation of $[Ca^{2+}]_i$, μ- and m-calpain consumption, fodrin and nNOS degradation, increase in calpain fluorescence intensity, and calpastatin diffusion extensively occurred as well as also total calpastatin inhibitory activity was 2.2-fold increased and calpastatin mRNA was 4-fold higher.

Discussion

In transgenic mice overexpressing the mutated –G93A(+) SOD1, high degree of calpain activation was selectively detectable in motor cortex, lumbar, and sacral spinal cord

segments being limited in cerebellum and undetectable in spinal cord upper segments and in remaining CNS. Only in motor neurons of those CNS areas in which $[Ca^{2+}]_i$ increased, calpastatin diffused in cytosol becoming colocalized and associated to calpain. However, massive calpain activation was prevented by its association to the inhibitor protein in cytosol, a process that prevents translocation of too large calpain amounts to plasma membrane inner surface, the preferred activation site of the protease. Another defence mechanism was represented by the generation of high amounts of 15 kD fragments accumulated in cells following increase in $[Ca^{2+}]_i$ and calpain activation. Moreover, calpastatin overexpression, occurring in those CNS areas affected by $[Ca^{2+}]_i$ dysregulation, maintained the inhibitory capacity still efficient. Thus, the appearance of irreversible damages in neurons was depending on the rates of both calpain activation and defence mechanisms (Fig. 1).

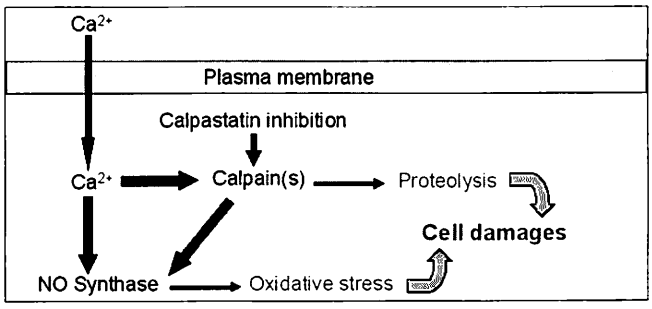

Figure 1. Schematic model of the defence mechanism acting on the regulation of calpain activity in conditions of prolonged increase of $[Ca^{2+}]_i$.

However, in those CNS areas in which calpain activation was more pronounced, these defence mechanisms became defective. Moreover, the conservative nNOS degradation by calpain producing still active 130 kD forms can be of potential cellular toxic effects. The fact that these findings were obtained in transgenic mice is of particular relevance to understand the pathological events occurring in neurodegenerative diseases in which alteration in Ca^{2+} homeostasis is present and indicates the calpain/calpastatin system as a suitable target for new therapeutic approaches to these diseases.

Acknowledgments

This work was supported by MIUR (PRIN Projects E.M. 2008) and University of Genoa.

References

[1] Wu H.Y., Lynch D.R. 2006. Calpain and synaptic function. Mol. Neurobiol., 33: 215-236.

[2] Grosskreutz J., Van Den Bosch L., Keller B.U. 2009. Calcium dysregulation in amyotrophic lateral sclerosis. Cell Calcium, 47: 165-174.

[3] Araujo I.M., Carvalho C.M. 2005. Role of nitric oxide and calpain activation in neuronal death and survival. Curr. Drug. Targets CNS Neurol. Disord., 4: 319–324.

[4] Gurney M.E. 1994. Transgenic-mouse model of amyotrophic lateral sclerosis. *N. Engl. J. Med.*, 331:1721-1722.

[5] Goll D.E., Thompson V.F., Li H., Wei W., Cong J. 2003. The calpain system. Physiol. Rev., 83: 731-801.

[6] Tullio R.D., Passalacqua M., Averna M. Salamino F., Melloni E., Pontremoli S. 1999. Changes in intracellular localization of calpastatin during calpain activation. Biochem. J., 343: 467-472.

[7] Stifanese R., Averna M., De Tullio R., Pedrazzi M., Beccaria F., Salamino F., Milanese M., Bonanno G., Pontremoli S., Melloni E. 2010. Adaptive modifications in the calpain/calpastatin system in brain cells after persistent alteration in Ca^{2+} homeostasis. J. Biol. Chem., 285: 631-643.

[8] Turner B.J., Talbot K. 2008. Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. Prog. Neurobiol., 85: 94-134.

[9] Pontremoli S., Melloni E., Damiani G., Salamino F., Sparatore B., Michetti M., Horecker B.L. 1988. Effects of a monoclonal anti-calpain antibody on responses of stimulated human neutrophils. Evidence for a role for proteolytically modified protein kinase C. J. Biol. Chem., 263: 1915-1919.

[10] Averna M., Stifanese R., De Tullio R. Passalacqua M., Defranchi E., Salamino F., Melloni E., Pontremoli S. 2007. Regulation of calpain activity in rat brain with altered Ca^{2+} homeostasis. J. Biol. Chem., 282: 2656-2665.

[11] Stifanese R., Averna M., Salamino F. Cantoni C., Mingari M.C., Prato C., Pontremoli S., Melloni E. 2006. Characterization of the calpain/calpastatin system in human hemopoietic cell lines. Arch. Biochem. Biophys., 456: 48-57.

[12] Stifanese R., Averna M., De Tullio R. Salamino F., Cantoni C., Mingari M.C., Prato C., Pontremoli S., Melloni E. 2008. Role of the calpain-calpastatin system in the density-dependent growth arrest. Arch. Biochem. Biophys., 479: 145-152.

[13] Averna M., Stifanese R., De Tullio R. Salamino F., Bertuccio M., Pontremoli S., Melloni E. 2007. Proteolytic degradation of nitric oxide synthase isoforms by calpain is modulated by the expression levels of HSP90. FEBS J., 274: 6116-6127.