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THE EFFECTS OF WILD-TYPE AND MUTANT SOD1 ON SMOOTH MUSCLE CONTRACTION

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Abstract – In this work we compared the mutated liver copper zinc-containing superoxide dismutase (SOD1) protein G93A of the transgenic rat model of familial amyotrophic lateral sclerosis (FALS), to wild-type (WT) rat SOD1. We examined their enzymatic activities and effects on isometric contractions of uteri of healthy virgin rats. G93A SOD1 showed a slightly higher activity than WT SOD1 and, in contrast to WT SOD1, G93A SOD1 did not induce smooth muscle relaxation. This result indicates that effects on smooth muscles are not related to SOD1 enzyme activity and suggest that heterodimers of G93A SOD1 form an ion-conducting pore that diminishes the relaxatory effects of SOD1. We propose that this type of pathogenic feedback affects neurons in FALS.

Key words: FALS; mutation; G93A SOD1; WT SOD1; muscle relaxation

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INTRODUCTION

About 2% of amyotrophic lateral sclerosis (ALS) cases are familial (FALS), and have been associated with more than 150 different mutations on the SOD1 gene (Kiernan et al., 2011). SOD1 represents a soluble cytoplasmic and mitochondrial intermembrane space protein, whose function is to convert the superoxide radical anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) (Fridovich, 1989). The redox role of SOD1 represents the foundation of the hypothesis that increased $O_2^{\bullet-}$ production and related oxidative stress are at least partially responsible for neurodegeneration in FALS patients. However, some SOD1 mutations do not result in decreased SOD1 activity, although they are observed in FALS (Goto et al., 2000; Siddique and Siddique, 2008). This implies that the protein structure could play an important role independently of enzyme function. Mutated SOD proteins may self-aggregate, which represents an event that could also be an initial cause of motor neuron malfunction leading to disease onset (Durham et al., 2007). The aggregates may provoke abnormalities in the endoplasmatic reticulum, Golgi complex, and other intracellular structures, leading to neuronal dysfunction and cytotoxicity (Nassif et al., 2010). However, the effects can also be extracellular, since SOD1 is now known to be excreted from the neurons (Urushitani

et al., 2006; Gomes et al., 2007; Vella et al., 2008). In line with recent evidence of SOD1 secretion in diverse cell lines, Turner et al. (2005) have proposed that mutant SOD1 engages in ALS pathogenesis by modulating different secretory pathways. In other words, mutated SOD1 may be involved in extracellular events affecting other cells directly, as previously shown on microglia by Yim et al. (1996). Damage to the vasculature is one of the earliest pathological events in the toxic cascade initiated by both dismutase-active and dismutase-inactive SOD1 mutants (Zhong et al., 2008). The damage includes blood-spinal cord barrier disruption, and it is mediated by the reduction in the levels of essential endothelial tight junction proteins ZO-1, occludin and claudin-5, resulting in both hypoperfusion and microhemorrhages. In the next step, microhemorrhages lead to the release of neurotoxic products into the microcirculation (Zhong et al., 2008). It has been shown that WT SOD isoforms provoke endothelium-dependent relaxation, implying a role of extracellular SOD1 in smooth muscle function (Morikawa et al., 2003). Pertinent to this, Zhong et al. (2008) proposed that the failure of mutated SOD1 to initiate smooth muscle relaxation may be involved in ALS pathogenesis.

In this work, we compared the effects of purified SOD1 isolated from the liver of WT rat with the effects of G93A SOD1 isolated from the transgenic rat model of FALS, on isometric contractions of uteri taken from healthy virgin rats.

MATERIALS AND METHODS

Animals

SODs were taken from the liver of Sprague-Dawley rats: genetically modified, expressing multiple copies of mutated (G93A) human SOD-1 gene (hSOD-1G93A; Taconic Farms Inc. NY) and WT (standard Sprague-Dawley rats). In these animals, ALS was expressed 5 to 7 months after birth and the period from onset to the disease end-stage was about 20 days. The progression of the disease was followed by visually checking the movement of the animal on a flat surface and by testing the regaining of stature after turning the animal on its side. If the latter was not possible, the end-stage of the disease was indicated and the animal was deeply anaesthetized with an i.p. injection of Nembutal and sacrificed. Livers were taken and placed in 0.1 mol/l phosphate buffer pH 7.4 at 4°C.

Isolation

The liver was homogenized on IKA T10 basic ULTRA-TURRAX® in 0.25M saccharose buffer pH=7.4. The other proteins were denatured by heat (60°C) with constant stirring (CuZn SOD is stable up to 70°C). The residue was separated by centrifugation at 5 000 rpm at 5°C. SOD was further purified according to the technique of McCord (1977). The purity of the preparation was tested by polyacrylamide gel electrophoresis.

Biochemical procedures

The activity of SOD1 was assayed using superoxide anion radical-mediated oxidation of epinephrine to adrenochrome at pH 10.2 (Misra and Fridovich, 1972) and it is expressed in U/mg of proteins. The amounts of proteins were determined by the method of Lowry (1951).

Native polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli et al. (1970), under non-denaturation conditions, using 12% acrylamide. SOD1 was diluted to 2 mg/ml of a solution containing 12% glycerol, 0.5 mM Tris-HCl (pH 6.8) and 0.2 M EDTA before loading. To detect proteins, gels were stained with 0.03% Coomassie Brilliant Blue R250. SOD bands were visualized by the activity-staining procedure described by Beauchamp and Fridovich (1971), using the reduction of nitro blue tetrazolium (2.45 mM) with $O_2^{\bullet-}$ produced by photochemical reduction of riboflavin (28 mM) with TEMED (28 mM).

Uterine contraction experimental system

Isolated uteri from virgin Wistar rats (200-250 g) in estrous were used. The estrous phase was determined

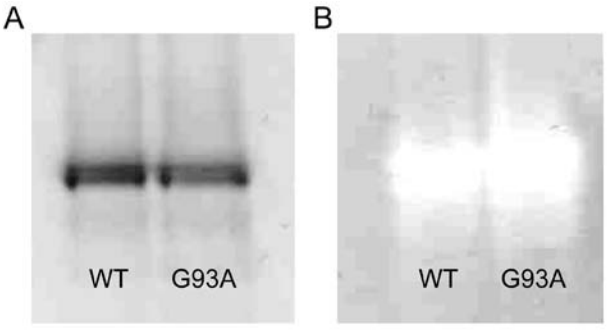

Fig.1. Non-reducing polyacrylamide gel electrophoresis of SOD. Panel **A** – proteins stained with Coomassie blue; **B** – SOD1 activity.

by examination of a daily vaginal lavage (Marcondes et al., 2002). The uterine horns were rapidly excised and carefully cleaned of surrounding connective tissue and mounted vertically in an organ bath containing De Jalon solution, aerated with 95% oxygen and 5% carbon dioxide at 37°C. The preload of the preparation was about 1 g. Experiments were performed on Ca^{2+}-induced uteri exposed to SOD1 activity ~200U, five times. Isometric contractions were recorded by an isometric force transducer (Experimetria, Budapest, Hungary).

Statistical analysis

All experiments were performed in quadruplicate. Statistical difference was determined by means of the non-parametric two-tailed Mann-Whitney test using Statistica 6.0 (StatSoft Inc, Tulsa, OK, USA). Results are presented as means ± S.D. and were taken to be statistically significant if $p < 0.05$.

RESULTS

SOD 1 activity after the heating step was 163.4±6.7 U/mg of proteins, while G93A SOD1 from ALS rat liver showed an activity of 216 ± 7.8 U/mg of proteins. This is a 30% higher activity than in wild type rat. The electrophoresis profile (Fig. 1) confirmed this slight, but statistically insignificant difference. Although the same amounts of proteins were added, the band staining for the activity was obviously slightly lighter for the G93A. After further purification, SOD 1 activity was 3480±190 U/mg of protein for WT and 3856±248 U/mg of protein for G93A SOD1.

The representative results of SOD effects on Ca^{2+}-induced activation of uteri are presented in Fig. 2. The 200U of SOD (WT) had a statistically significant relaxatory effect on isolated smooth muscles ($p<0.005$), while the same activity of the applied G93A SOD1 did not. The applied G93A SOD1 only slightly decreased the frequency of Ca^{2+}-induced uterine contractions.

DISCUSSION

The results presented here show that, in contrast to WT SOD1, G93A SOD1 does not induce smooth muscle relaxation. Since G93A SOD1 has an even higher SOD activity than the wild type of SOD1, it was tempting to speculate that the relaxatory effect of their catalytic activity product (H_2O_2) should be similar. According to the model used in this work, the increased level of H_2O_2 related to the supplementation of SOD1 is not solely responsible for muscle relaxation. Our results, together with those of Allen et al. (2012) suggest that the mutant SOD1 forms tetrameric pore-like structures allowing for non-selective ion conductance, thus diminishing relaxatory effects as compared to WT SOD1 (Museth et al., 2009). It should be stressed that ALS model animals overexpressing the mutant gene exhibit a progressive, ALS-like neurodegenerative condition, characterized by motor neuron degeneration and paralysis. Mutations may cause SOD1 to have an increased propensity to miss-fold or aggregate (Furukawa and O'Halloran, 2006). The G93A mutation in SOD1 results in a local destabilization of the enzyme at the site of the mutation but also in the stabilization on several positions that are apparently scattered over the entire protein surface (Shipp et al., 2003). NMR studies have found that the G93A mutation causes a disruption in the hydrogen-bonding network of the protein (Allen et al., 2012). The mutation selectively destabilizes the remote metal-binding region, which may affect the intermolecular protein-protein interactions (Tradewell et al., 2010). Electrophysio-

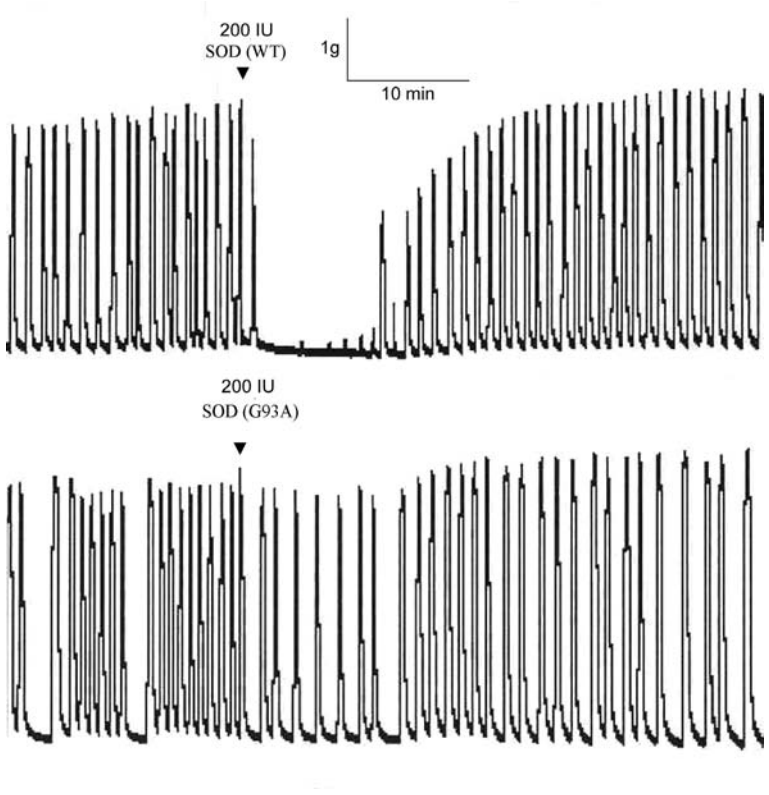

Fig. 2. Original trace of Ca^{2+}-induced uterine contraction monitoring. ▼ – applied SOD points.

logical recordings conducted on research performed on another SOD1 mutation, A4VSOD1, showed distinct ionic conductance across the lipid bilayer for A4VSOD1 and none for the wild-type SOD1. Mouse neuroblastoma cells exposed to A4VSOD1 undergo membrane depolarization and an increase in intracellular calcium. These results have provided compelling new evidence that a mutant SOD1 is capable of disrupting cellular homeostasis via an unregulated ion channel mechanism identified as a "toxic channel" (Museth et al., 2009). Tradewell and Durham (2010) have shown that the reason for increased calpain activity in mutant SOD1 (SOD1 (G93A)) transgenic mice is the increased cytosolic calcium. They have also reported that inhibition of calpain activity using calpastatin prevented the toxicity of SOD1 (G93A) in the motor neurons of dissociated spinal cord cultures, prolonging viability and reducing the proportion containing SOD1 (G93A) inclusions. These data support the central role of calcium dysregulation in ALS (Tradewell and Durham, 2010).

Evidence presented in this work supports the hypothesis that the damage mediated by mutant SOD1, which is provoked on the vasculature, may contribute to the initiation of non-cell autonomous killing of the motor neurons inherent to ALS. It should be stressed that neuromuscular junctions are the first to be lost in ALS, followed by the loss of ventral root axons, while motor neurons are the last to die (Fischer et al., 2004). Pertinent to this, the "dying back" pattern has been proposed (Dadon-Nachum et al., 2011).

Our results imply that mutant SOD1 may provoke a mechanical damage to the muscles and neuromuscular junctions indirectly, by being unable to perform the relaxatory function of the WT SOD1. Such unregulated activity of the muscles may have pathogenic feedback effects on motor neurons as

well as a potentially important role in FALS initiation. Animals overexpressing the mutant gene exhibit a progressive, ALS-like neurodegenerative state, characterized by motor neuron degeneration and paralysis (Fischer et al., 2004). The lack of relaxatory effects on smooth muscle in the presence of G93A SOD1 compared to WT SOD1 may have negative feedback effects on motor neurons. It may provoke increased and uncontrolled activity of the motor neurons, leading to impaired neuromuscular junctions and neuron damage.

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Conflict of interest disclosure

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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