

## THE EFFECTS OF HUMAN WILD-TYPE AND FALS MUTANT L144P SOD1 ON NON-VASCULAR SMOOTH MUSCLE CONTRACTIONS

### EFEKTI HUMANE NORMALNE I FALS MUTIRANE L144P SOD1 NA NEVASKULARNE KONTRAKCIJE GLATKIH MIŠIĆA

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#### Summary

**Background:** Mutated copper, zinc-containing superoxide dismutase (SOD1) may self-aggregate, an event that could also be an initial cause of motor neuron malfunction leading to disease onset. The effects of human mutated SOD1 protein from the blood of familial amyotrophic lateral sclerosis (FALS) patients bearing Leu144Phe (L144F) mutation were compared to wild-type (WT) human SOD1 derived from healthy examinees, for enzymatic activity and the effects on isometric contractions of non-vascular smooth muscle.

**Methods:** We isolated WT and L144F SOD1 enzymes from eight patients with FALS, L144F mutation in exon 5 and eight healthy controls. We then investigated SOD1 activities in the obtained samples by the adrenaline method and profiled them electrophoretically. Finally, we applied WT and L144F SOD1 on the isolated rat uterus.

**Results:** L144F SOD1 showed lower superoxide-dismutating activity compared to WT human SOD1. We found that, in contrast to WT human SOD1, mutated L144F does not induce smooth muscle relaxation.

**Conclusions:** Our data suggest that the lack of relaxation of muscle tonus in the presence of mutated SOD1 may have pathogenic feedback effects in FALS.

**Keywords:** FALS, L144F SOD1, WT SOD1, relaxation

#### Kratak sadržaj

**Uvod:** Mutirana bakar, cink superoksid-dizmutaza (SOD1) može da pravi agregate, što predstavlja početni uzrok oštećenja motornog neurona može da izazove nastanak bolesti. U ovom radu su pokazani efekti humane bakar, cink superoksid dizmutaze iz krvi pacijenata obolelih od familijarne amiotrofične lateralne skleroze (FALS) sa Leu144Phe (L144F) mutacijom i normalne (wild-type – WT) humane SOD1, iz krvi zdravih kontrola, na glatkom mišiću.

**Metode:** Izolovali smo WT i L144F SOD1 enzime kod osam odabranih FALS pacijenata sa L144F mutacijom na egzonu 5 i pet zdravih kontrola. Dalje smo ispitivali aktivnost SOD1 u dobijenim uzorcima adrenalinskom metodom i elektroforetski ih profilisali. Konačno, izolovanu WT i L144F SOD1 aplicirali smo na izolovani uterus pacova.

**Rezultati:** Aktivnost L144F SOD1 je statistički značajno manja ( $p < 0,05$ ) u poređenju sa aktivnosti WT SOD1 zdravih kontrola. L144F ne izaziva relaksaciju glatkog mišića, kao što je to slučaj sa WT SOD1.

**Zaključak:** Naši rezultati pokazuju da izostanak relaksacije mišićnog tonusa u prisustvu mutirane SOD1 može imati štetni povratni efekat kod FALS pacijenata.

**Ključne reči:** FALS, L144F SOD1, WT SOD1, relaksacija

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## Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal progressive disorder clinically characterized by muscle wasting and weakness and pathologically characterized by the relatively selective degeneration of upper motor neurons in the motor cortex and lower motor neurons in the brain stem and spinal cord (1). The discovery of a mutation in the SOD1 gene prompted the hypothesis that oxidative stress may play a role in the pathogenesis of ALS (2). About 10% of ALS cases are familial ALS (FALS), which has been associated with more than 150 different point mutations on superoxide dismutase-1 (SOD1) locus (3). Wild-type and mutated SOD1 (WT SOD) can exhibit additional enzymatic activities, including superoxide reductase and/or oxidase activities, may self-aggregate, an event that could also be an initial cause of motor neuron malfunction leading to disease onset (4). An increasing body of evidence implies that SOD1 mutation may show ALS-related effects by affecting muscles and neuromuscular junction. In line with recent evidence of SOD1 secretion in diverse cell lines, Turner et al. (5) have proposed that mutant SOD1 engages in ALS pathogenesis by modulating different secretory pathways. In other words, mutated SOD1 may be involved in extra-cellular events directly affecting other cells, as previously shown on microglia (6). Growing evidence in support of a toxic channel hypothesis also for Alzheimer's disease and other neurodegenerative diseases lends credence to a common disease mechanism in protein misfolding diseases (7, 8). Consistent with this scenario, altered levels of intracellular calcium ( $Ca^{2+}$ ) are reported to contribute to the motor neuron degeneration and mitochondrial dysfunction associated with ALS (9–12). Morikawa et al. (13) showed that SOD1 beside dismutation had some other functions (13). WT SOD1 could provoke endothelium-dependent relaxation, implying a role of extracellular SOD in smooth muscle function.

This prompted us to compare the effects of WT SOD1 isolated from the blood of healthy volunteers with mutated L144F human SOD1 obtained from the blood of FALS patients on isometric contractions of uteri taken from healthy virgin rats. The isolated uterus is a very suitable experimental model for this kind of study due to the presence of complex signal transduction systems, most of up-to-date identified ion channels and the redox sensitivity of this non-vascular smooth muscle (14).

## Material and Methods

### Material

In this paper, we have selected patients with FALS, L144F mutation in exon 5, because this is the most common SOD1 mutation so far registered in Serbia. Eight patients diagnosed for L144F FALS were

informed that their blood was to be used for both routine medical analyses and our laboratory research. Patient recruitment, counseling, sample collection and handling were conducted according to internationally recognized ethical standards (The Helsinki Declaration of 1964, as revised in 1975, 1983, and 1989). Institutional approval for the study was granted by The Clinic Ethics Committee which followed international guidelines. All participants provided their written consent.

In all the examined FALS patients, molecular genetic analyses were performed according to the following procedures: DNA samples of FALS patients were collected at the PCR Center, Faculty of Biology, University of Belgrade. Coding regions and exon-intron boundaries of SOD1 (5 exons) genes were amplified and analyzed using the Big Dye terminator v.1.1 sequencing kit on an ABI 3130 genetic analyzer. Sequence of the primers and amplification conditions are available upon request. In our FALS patients with L144F SOD1 mutation, direct sequencing analysis of all five exons of SOD 1 gene revealed presence of heterozygous c.435G>C (L144F) point mutation in exon5.

### Isolation of wild-type and mutated SOD1

We used leftovers of blood samples collected for standard biochemical analyses from 8 FALS patients (q/q) with L144F mutation as well as the blood from 8 healthy controls for isolation of SOD 1. Heparinized blood samples were centrifuged at 3000 rpm for 15 min at 4 °C, and the separated erythrocytes were washed three times with 0.9% NaCl, and then lysed by adding 3 mL of ice-cold distilled water. Proteins in the lysates were denatured by heat (60 °C) with constant stirring (SOD1 is stable up to 70 °C). Residue was separated by centrifugation at 5000 rpm at 5 °C. All other proteins and hemoglobin (Hb) residues were removed by the repeated Tsuchihashi procedure (15). The activity of SOD1 and the purity of preparations were determined by electrophoresis, showing no other protein band except for SOD1 bands.

### Biochemical procedures

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The activity of SOD1 was assayed using superoxide anion radical-mediated oxidation of epinephrine to adrenochrome at pH 10.2 (16), and expressed in U/g of Hb. Hb was estimated by the method of Drabkin and Austin (17). Native polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (18), using 12% acrylamide. SOD was diluted to 2 mg proteins/mL of a solution containing 12% glycerol, 0.5 mmol/L Tris-HCl (pH 6.8), and 0.2 EDTA before loading 50 mL wells. For detection of 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 (19). The gel was first soaked in 25 mL of 1.23 mmol/L NBT for 15 min, briefly washed, then soaked in the dark in 30 mL of 1 mol/L potassium phosphate buffer (pH 7.0) containing 20 mmol/L TEMED and  $2.8 \times 10^{-2}$  mmol/L riboflavin for another 15 min. The gel was briefly washed again, and then illuminated on a light box with a light intensity of  $30 \text{ mEm}^{-2}\text{s}^{-1}$  (measured by LI-COR LI 1000) for 15 min to initiate the photochemical reaction.

#### Uterine contraction experimental system

Uteri were isolated from virgin Wistar rats (200–250 g) in estrous determined by examination of daily vaginal lavage (20). 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  $\text{Ca}^{2+}$  induced active uteri and treated with SODs. Isometric contractions were recorded by isometric force transducer (Experimetria, Budapest, Hungary). Uteri were exposed to SOD1 showing total activity of ~ 200 U. Reduction of peak represents relaxation.

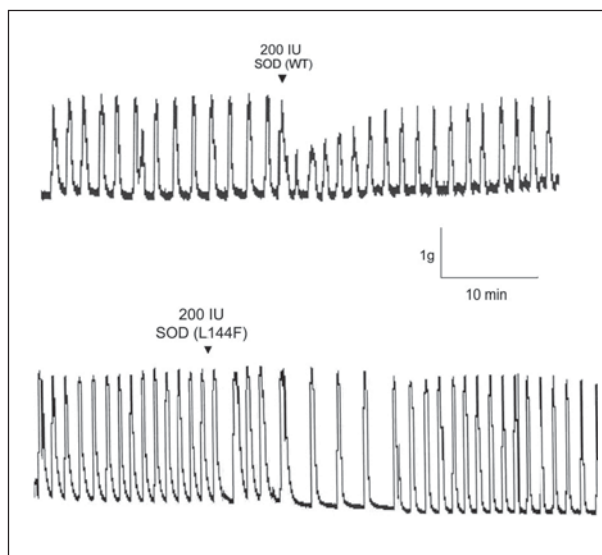
#### Statistical analysis

All experiments using human SOD1 were repeated 8 times. Statistical difference was determined by the means of the non-parametric two-tailed Mann-Whitney test using Statistica 6.0 (StatSoft Inc, Tulsa, OK, USA). Results are presented as means  $\pm$  S.D. and were taken to be statistically different if  $p < 0.05$ .

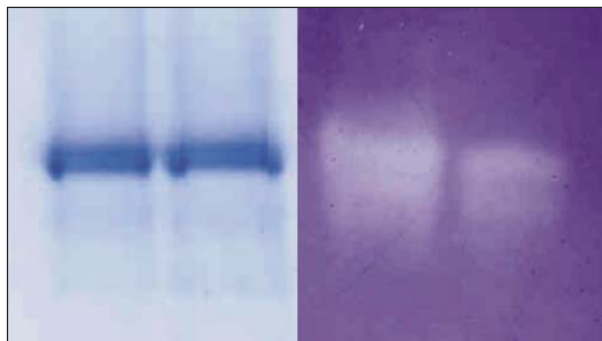
## Results and Discussion

The effects of SODs on the  $\text{Ca}^{2+}$  induced activation of uteri are presented in *Figure 1*. Human WT SOD1 had a relaxing effect on the isolated smooth muscle, while human L144F mutated SOD1 did not show any effects on isolated smooth muscle contractions.

L144F SOD1 showed significantly ( $p < 0.05$ ) lower enzyme activity compared to WT SOD1. The activity of human WT SOD1 isolated from the erythrocytes was  $2791 \pm 22$  U/g of Hb, while the activity of human L144F SOD1 isolated from the erythrocytes of FALS patient was  $1342 \pm 37$  U/g of Hb. The decreased activities of L144F SOD1 biochemical assay were verified by the results of electrophoresis, as shown in *Figure 2*. Although the same amount of proteins was loaded on the gels (*Figure 2* left), visu-



**Figure 1** Characteristic trace of  $\text{Ca}^{2+}$  induced uterine contractions monitoring. WT SOD1 (200 U); ▼ – The points of SOD1 and L144F SOD1 application (200 U).



**Figure 2** Non-reducing PAGE of SOD1 electrophoresis. Left: proteins stained with Coomassie blue; WT SOD1 and L144F SOD1; right: staining for SOD activity; WT SOD1 and L144F SOD1.

alized L144F SOD 1, according to the resulting bands (*Figure 2* right), showed decreasing SOD 1 activity.

We propose that the changes in the structure of SOD1 mutated proteins force it to bind the muscle surface, leading to increased  $\text{Ca}^{2+}$  permeability and thus eliminating the relaxation effect which was observed for WT SOD1. Similar to the finding of Miler et al, that mutated SOD1 induce channel-like ionic conductance of pore-like structures they form, the cellular  $\text{Ca}^{2+}$  uptake and cell membrane depolarization is affected by the mutant SOD1. The lack of relaxing effects in the presence of L144F SOD1 compared to WT SOD1 may have negative feedback effects on motor neurons. It has been shown that mutations may cause SOD1 to have an increased propensity to misfold or aggregate (21). Pertinent to this, Zhong et al. (22) have proposed that the failure

of mutated SOD1 to initiate smooth muscle relaxation may be involved in ALS pathogenesis.

Recent findings link abnormalities in ubiquitin 2 in juvenile and adult-onset ALS to defects in the protein degradation pathway, abnormal protein aggregation and neurodegeneration, indicating a common pathogenic mechanism that can be exploited for therapeutic intervention (23, 24). WT and mutant (G41R, G93A, or N139K) human SOD1 have been expressed in motor neurons of dissociated cultures of murine spinal cord by intranuclear microinjection of plasmid expression vector. Both the general antagonist of AMPA/kainate receptors (CNQX) and specific antagonist of Ca<sup>2+</sup>-permeable AMPA receptors (joro spider toxin) have reduced formation of SOD1 proteinaceous aggregates and prevented death of motor neurons expressing SOD1 mutants. Partial protection has been obtained by treatment with nifedipine, implicating Ca<sup>2+</sup> entry through voltage-gated Ca<sup>2+</sup> channels, as well as glutamate receptors in potentiating the toxicity of mutant SOD1 in motor neurons. Dramatic neuroprotection has been obtained by coexpressing the Ca<sup>2+</sup>-binding protein calbindin-D28k, but not by increasing intracellular glutathione levels or treatment with a free radical spin trap agent, N-tert-butyl-alpha-phenylnitron. Therefore, the generalized oxidative stress could have contributed to the death of motor neurons expressing the mutant SOD1

only in a minor way. These studies have demonstrated that the toxicity of these mutants is Ca<sup>2+</sup>-dependent and they provide direct evidence that Ca<sup>2+</sup> entry during neurotransmission, coupled with deficiency of cytosolic Ca<sup>2+</sup>-binding proteins, is a major factor in the preferential vulnerability of motor neurons to disease (25).

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 (26). Pertinent to this, the »dying back« pattern has been proposed (27). Our results imply that mutant SOD1 may provoke mechanical damage to the muscles and neuromuscular junctions indirectly, by being unable to perform the relaxing function of the WT. Such unregulated activity of the muscles may have pathogenic feedback effects on neurons and potentially an important role in FALS initiation.

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### Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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