Dimebon Reduces the Levels of Aggregated Amyloidogenic Protein Forms in Detergent-Insoluble Fractions *In Vivo*

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Aggregation of proteins liable to assembling into fibrils with subsequent formation of amyloid incorporations is an important component in the pathogenesis of many neurodegenerative diseases. Dimebon, a Russian drug, reduces the content of detergent-insoluble fibrillar forms of synuclein, the main protein component of pathological incorporations in neurons of transgenic mouse strain used in the study.

Key Words: *dimebon; neurodegeneration; synucleins; transgenic animals*

Disorders in the metabolism and functions of some key proteins often underlie the development of a neurodegenerative process. Aggregation of these proteins and subsequent formation of pathological depositions is assumed to be the key events in the pathogenesis of many neurodegenerative diseases, known as proteinopathies [8]. Protein aggregation is a multi-staged process, and all components of this cascade, as well as the intracellular systems for elimination of forming and already formed aggregations, are prospective therapeutic targets for future new generation drugs directly modulating the disease pathogenesis. Dimebon, a Russian drug, has exhibited high efficiency in clinical trials when used for the treatment of phase 2B proteinopathies (Alzheimer's disease and Huntington's chorea) [4,5]. It modulated the molecular cellular processes of formation and/or elimination of pathological protein depositions in the nervous system structures [1,11]. However, precise molecular cellular mechanism of dimebon effects remains unclear.

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We studied the effects of dimebon on the formation of terminal products of protein aggregation in tissues of transgenic animals simulating proteinopathy.

MATERIALS AND METHODS

Experiments were carried out on transgenic Thy1mγSN mice characterized by high pan-neuronal expression of exogenic murine gamma-synuclein (GS) [3] and formation of pathological protein incorporations in the nervous tissues [7]. The transgenic strain was derived from C57Bl/6J (genetic base). Experimental transgenic homozygotic males received dimebon (3,6-dimethyl-9-(2-methyl-pyridyl-5)-ethyl-1,2,3,4-tetrahydro-γ-carboline dihydrochloride, water solution 10 μg/ml; Organika) in a concentration of 10 μg/ml with drinking water *ad libitum* for 9 months starting from the age of 3 months. Control transgenic homozygotic males of the same age receiving no drug were kept under similar conditions. Spinal cord tissues from the thoracic portion were used for biochemical analysis.

Total RNA was isolated using RNAEasy plus kit (Qiagen); 1 μg RNA was used for reverse transcription for the synthesis of complementary DNA chain in the presence of degenerated hexamer primers (Promega) and reverse transcriptase SuperScriptIII (In-

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vitrogen) [7]. Quantitative real-time PCR was carried out on a StepOne Real-Time PCR System (Applied Biosystems) using DyNAmo HS SYBR Green supermix (Finnzymes) and primers for GS amplification 5'-CCATGGACGTCTTCAAGAAAGG-3' and 5'-CGTTCTCCTTGGTTTTGGTG-3'. The level of glyceraldehyde-3-phosphate-dehydrogenase gene amplified with primers 5'-CACRGAGCATCTCCCTCACA-3' and 5'-GTGGGTGCAGCGAACTTTAT-3' [7] served as internal control. The data were processed using StepOne v2.0 software (Applied Biosystems).

Aggregated protein forms were extracted by partial differential fractionation in buffer solutions with or without detergents. Spinal cord tissues were homogenized in high saline buffer: 800 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA with protease inhibitors Complete Mini (Roche). The postmitochondrial fraction was separated by centrifugation at 10,000*g* for 20 min at 4°C and the insoluble protein aggregations present in the postmitochondrial fraction were precipitated by centrifugation at 100,000*g* for 20 min at 4°C. The precipitate was resuspended in the same buffer with 1% Triton X-100 and centrifuged again at 100,000*g*. The terminal detergent-insoluble fraction was resuspended in buffer for application of samples onto PAAG with 10% sodium dodecylsulfate for Laemmli denaturing electrophoresis and boiled for 5 min. Proteins were detected by immunoblotting with SK23 polyclonal antibodies (1:500) [2] specifically recognizing mouse GS and monoclonal antibodies to ubiquitin (Santa Cruz Biotechnology; 1:1000). Protein content in the samples was normalized by β-actin using monoclonal antibodies (Sigma; 1:3000).

RESULTS

Formation of pathological incorporations in the nervous tissues is an important pathogenetic component of many neurodegenerative diseases. These incorporations often acquire the characteristics of amyloid and are based on the terminal products of aggregation of certain proteins. Dimebon effects [10] on the formation of fibrillar detergent-insoluble structures formed by GS protein (the main component of pathological intracellular incorporations in the neurons of Thy1mγSN mice) were studied. Experimental animals received dimebon (1.75 mg/kg/day) permanently from the age of 3 months, when signs of a neurodegenerative process were not yet diagnosed. The study revealed a significant reduction of aggregated synuclein forms in specimens of spinal cord tissue from mice treated with dimebon for 9 months in comparison with the levels of these forms in protein specimens from control animals receiving no dimebon (Fig. 1). The levels of intermediate protein aggregation products,

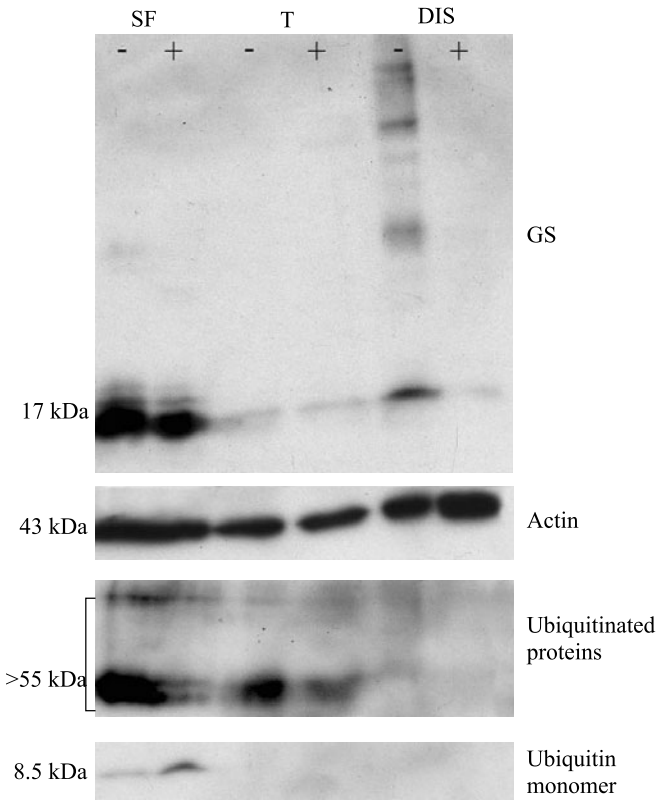

Fig. 1. Immunoblotting analysis of proteins in specimens of the spinal cord from experimental and control transgenic animals. Fractions of soluble (SF), soluble only in the presence of detergent – Triton X-100 (T), and detergent-insoluble (DIS) proteins in preparations from transgenic animals treated (+) and not (-) with dimebon. Control: β-actin (cytoskeleton protein normally always detected in soluble and insoluble fractions).

oligomers and protofibrils (detergent-soluble; released from precipitate by resuspending with Triton X-100 detergent) were the same in protein preparations from experimental and control groups. The effect of appreciable reduction of the levels of insoluble GS forms under the effect of dimebon was in line with the reports demonstrating that chronic dimebon treatment of transgenic animals led to reduction of the number of amyloid incorporations in spinal cord tissues [1]. One of the probable mechanisms of this effect could be stimulation of intracellular systems for elimination of pathological (often toxic) protein forms and their aggregations, including the ubiquitin proteosome (UPS) and autophagosome systems in the presence of dimebon. The level of monomeric ubiquitin in the soluble protein fraction from mice treated with dimebon was higher than in controls, which reflects total activity of UPS. The level of ubiquitinated proteins (55-100 kDa) in preparations from animals treated with dimebon was significantly lower in the soluble protein fraction and in detergent-soluble (1% Triton X-100) protein fraction, this indicating UPS efficiency. No ubiquitinated

proteins were detected in the detergent-insoluble fractions, this presumably indicating their absence in the fibrillar and compactly packed structures, *e.g.* amyloid incorporations.

Analysis of GS mRNA expression in spinal cord tissues of experimental and control animals indicated that it was invariably high, not changing under the effect of dimebon (Fig. 2). Hence, dimebon specifically modulated the content of detergent-insoluble structures, the main components of pathological incorporations in the neurons of transgenic Thy1mγSN mice. According to published data, a single dose of dimebon increased the levels of soluble amyloid-β peptide forms in the extracellular space of model systems *in vitro* and *in vivo* [10]. Though a direct comparison of the results of acute and chronic long-term treatment with dimebon is not correct and the effects of dimebon on aggregated amyloid-β peptide have not been studied [10], we think that the effect could be due to the prevention or inhibition of the formation of insoluble amyloidogenic protein forms. That is why a positive effect of dimebon could be expected for amyloidogenic proteins located in a cell having systems for their programmed elimination, primarily UPS actively utilizing soluble forms by inhibiting the formation of compact incorporations. To be sure, the positive effect of dimebon would depend on the physiological status of these systems and their potentialities to work in a forced mode, as a sharp shift of the soluble amyloidogenic proteins proportion towards an increase of their intracellular concentration is fraught with a cytotoxic effect and can lead to the neuron death [6].

Since abnormal protein aggregation plays an important role in the pathogenesis of Alzheimer's disease and Huntington's chorea, the neuroprotective effect of dimebon [4,5] can be explained by its direct modulation of the pathological aggregation of proteins. In cell cultures dimebon inhibited accumulation of aggregations of one more protein liable to fibrillation, TDP-43; this also suggested protein aggregation as a possible target for dimebon effect [11]. Chronic dimebon therapy inhibits significantly the development of proteinopathy in transgenic mice with high GS expression; a significant reduction of the level of amyloid incorporations was found in spinal cord tissues of model animals treated with dimebon at the early stages of neurodegeneration development and receiving it for manifest forms of the neurodegenerative process in comparison with control transgenic animals receiving no dimebon [1]. However, the molecular mechanism

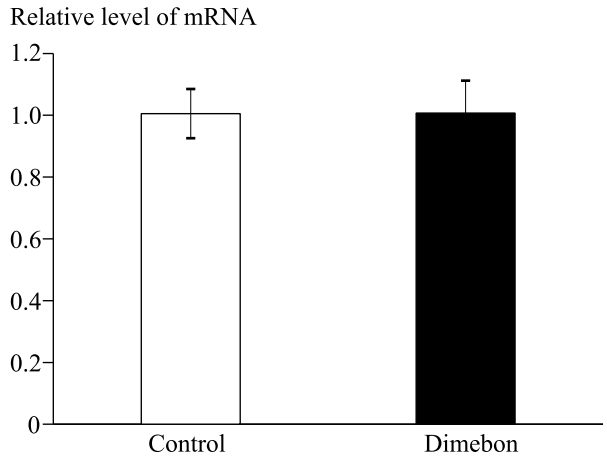

Fig. 2. Expression of GS mRNA in spinal cord tissues of control and experimental transgenic animals treated with dimebon.

of this effect is still to be studied. Our present results once more demonstrate dimebon effects on the formation and/or stability of the terminal products of protein aggregation, highly fibrillar detergent-insoluble structures forming the basis of amyloid incorporations in various proteinopathies.

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REFERENCES

1. S. O. Bachurin, A. A. Ustyugov, O. Peters, *et al.*, *Dokl. Ross. Akad. Nauk*, *Biochim. Biophys.*, **428**, No. 2, 262-265 (2009).
2. V. L. Buchman, J. Adu, L. G. Pinõn, *et al.*, *Nat. Neurosci.*, **1**, No. 2, 101-103 (1998).
3. V. L. Buchman, H. J. Hunter, L. G. Pinõn, *et al.*, *J. Neurosci.*, **15**, No. 18, 9335-9341 (1998).
4. R. S. Doody, S. I. Gavrilova, M. Sano, *et al.*, *Lancet*, **372**, 207-215 (2008).
5. K. Kieburtz, M. P. McDermott, T. S. Voss, *et al.*, *Arch. Neurol.*, **67**, No. 2, 154-160 (2010).
6. P. T. Lansbury and H. A. Lashuel, *Nature*, **443**, 774-779 (2006).
7. N. Ninkina, O. Peters, S. Millership, *et al.*, *Hum. Mol. Genet.*, **18**, No. 10, 1779-1794 (2009).
8. D. M. Skovronsky, V. M. Lee, and J. Q. Trojanowski, *Ann. Rev. Pathol.*, **1**, 151-170 (2006).
9. J. W. Steele, S. H. Kim, J. R. Cirrito, *et al.*, *Mol. Neurodegener.*, **4**, 51 (2009).
10. J. Wu, Q. Li, and I. Bezprozvanny, *Ibid.*, **3**, 15 (2008).
11. M. Yamashita, T. Nonaka, T. Arai, *et al.*, *FEBS Lett.*, **583**, No. 14, 2419-2424 (2009).