Trekrezan as a stimulator of muscle strength and endurance. Possible molecular mechanisms of action

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Trekrezan activated the *in vivo* expression of the PGC-1α gene in rat muscle tissues. Quantification of mitochondria in tissues and gene expression analysis for genes involved in regulatory energy metabolism cascades revealed that the mechanisms of the energy metabolism regulation are probably coupled with protein biosynthesis as well as with innate immunity. The immunostimulatory and adaptogenic drug trekrezan was found to induce concerted *in vivo* stimulation of the expression of the gene coding tryptophanyl-tRNA synthetase and its activity in the expression of the PGC-1α gene.

Key words: trekrezan, coactivator PGC-1α, myocytes, biomarkers.

Elucidation of the maintenance mechanism of cellular homeostasis is currently of particular importance because of a great body of evidence has been accumulated regarding the effects of metabolic pathways and gene complexes on the vital functions of cells. One of the key genes that govern metabolic pathways by regulating the oxidative stress level, mitochondrial biogenesis, and an adaptive increase in the energy supply to stressed tissues is the gene of peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α). A change in the activity of the tissue-specific expression of the PGC-1α gene can serve as a criterion of the efficacy of adaptogenic agents. Therefore, the PGC-1α gene is a promising therapeutic target,[1] also when local and general adaptation syndromes (according to Selye´s classification[2]) are formed. A search for new highly specific PGC-1α stimulators having optimum efficiency and no side effects becomes of current interest. In this respect, triethanolammonium aroxyacetates of the general formula $X^-N^+H(CH_2CH_2OH)_3$, seem to be attractive. These protatrane structures act by diminishing the damage to elastic fibers and by stabilizing cell membranes.[3] An example of such drugs is trekrezan, tris(2-hydroxyethyl)ammonium 2-methylphenoxyacetate. This compound has been included in the State Register of Drugs of the Russian Federation as an adaptogen; its immunomodulating effect is also worth noting.

Earlier, we have demonstrated that trekrezan stimulates the development of the muscle systems in underweight girls having protein deficiency.[4] However, the mechanism of trekrezan action under these conditions remains unclear. We have also found that trekrezan stimulates the expression of the matrix RNA of tryptophanyl-tRNA synthetase (TRSase).[5] Continuing those investigations dealing with the effect of trekrezan on some units of the very complex chain in the myocytic machinery of protein biosynthesis (especially under stressful conditions), we found it interesting to study its effect on the expression of the PGC-1α gene.

Experimental

Procedure. All experiments with Wistar male and female rats (body weight 180—200 g) were carried out in concordance with the ethical principles and guidelines recommended by the Euro-

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pean Science Foundation (ESF) and the Declaration of Humane Animal Treatment. Animals were divided into three groups of 10 rats. The rats of two groups intraperitoneally received a solution of trekrezan for seven days. The doses were 10 (first group) and 25 mg kg^{-1} of body weight (second group). The rats of the third group (control) received an injection of saline (placebo) of the same volume for seven days. The rats were monitored diurnally (from 11.00 till 15.00). After the seven-day course of either trekrezan or placebo administration was completed, the rats were decapitated for tissue sampling.

Rat muscle strength was assessed in an inverted screen test. Each rat was placed on a horizontal wire grid, which was then carefully inverted. The rat tried to hold on, counteracting gravity, by grasping the grid with its paws. The latency to fall was recorded. If the rat fell down within three minutes, it was placed back on the grid (this was repeated three times). The latency periods in all three trials were added up, with the latency to the first fall being specially noted. Rat muscle endurance was assessed in a forced swim test. The swimming time of loaded rats (the load was 8% of the rat body weight) was measured.[6]

Extraction of RNA and the reverse transcription synthesis of cDNA. Tissue samples for RNA extraction were promptly cut from decapitated rats and placed in RNA-fresh solution. Total RNA was extracted with guanidinium isothiocyanate. A thigh muscle sample (100 mg) was rapidly frozen in liquid nitrogen in the presence of Trizol®Reagent (1 mL) (Invitrogen, USA) according to the manufacturer´s protocol and then homogenized by grinding. The precipitate of RNA was washed with 75% ethanol, dried in air, and dissolved in twice-distilled water containing sodium dodecyl sulfate (0.5%). The solution was incubated at 55 °C for 10 min and frozen at −70 °C. An aliquot (2.5 μL) of mRNA extracted and treated with diethyl pyrocarbonate was dissolved in twice-distilled water. Spectrophotometric measurements of the absorption at $\lambda = 260$ nm were used to identify and quantify mRNA. The quality of mRNA samples was evaluated from the ratio of their absorbances at $\lambda = 260$ and 280 nm. An RNA sample was considered suitable for further analysis if the ratio exceeded 1.6. RNA samples were stored at −20 °C and/or below. The synthesis of cDNA from total RNA pretreated with DNase I (1 unit of the enzyme per microgram of RNA) was carried out using RT-PCR reagent kits (Silex, Russia) on a DT-322 instrument (DNK-Tekhnologii, Russia) according to the manufacturer´s protocol.

Real-time polymerase chain reaction (PCR). The specific expressions of the PGC-1α gene and the TPCase gene were studied using reverse transcription polymerase chain reactions (RT-PCR) on a DT-322 instrument (DNK-Tekhnologii, Russia); amplification kits (Sintol, Russia) were employed. The expression of the PGC-1α and TPCase genes was quantified by evaluation of the level of specific mRNA using TaqMan probes (see below). The mRNA level was determined by RT-PCR quantification of total cDNA (cDNA served as the input PCR template). The reaction mixture consisted of 5´- and 3´-primers (10 pmol), 1.6 m*M* $MgCl_2$, 0.25 μ*M* deoxynucleotide triphosphate, 10x PCR buffer with a TaqMan fluorescent probe, and the DNA polymerase Taq (1 unit).

The preheated (95 °C, 10 min) reaction mixture was denatured, annealed at 60 °C, and polymerized at 72 °C in a total of 40 cycles (the duration of each step was 15 s). Data were collected during the polymerization.

Each reaction was carried out in triplicate. Primers were designed with the Primer Express 3.0 program and the Primer-BLAST software. The β-actin gene, a housekeeping gene, was used as a reference for equalizing the amounts of the transcripts added to different probes. The primer annealing temperature and the number of cycles at which each fragment was amplified were selected experimentally. When analyzing the expression of PGC-1α and TPCase, the primers at the ends of two exons flanking an intron were used to prevent PCR of genomic DNA impurities in total RNA. A designed fluorescent TaqMan probe was located within the amplification region of the gene and the corresponding transcribed mRNA. In the case of TPCase, the nucleotide and amino acid sequences of PGC-1α and TPCase were highly homologous for rats, humans, and mammals in general, at least in the regions of the gene sequences for which the probes had been designed. The data obtained were analyzed with the SPSS 13.0 program package (SPSS Inc., USA).

Quantification of mtDNA in muscle tissue was carried out by RT-PCR with tissue DNA samples extracted using Silex reagent kits according to the manufacturer´s protocols. For PCR, amplification kits (Sintol, Russia) were employed. An analytical amount of a DNA sample was 10 ng per reaction. The relative amounts of nuclear DNA and mtDNA were determined by comparing the replication kinetics of the selected fragment of the β-actin gene localized in the nuclear genome with that of the mitochondrial gene of cytochrome b followed by estimation of the copy number of these genes. The reaction mixture ($V = 25$ μL) contained the same components as in the analysis of the specific expression of the PGC-1α gene. The amounts of the DNA sample and the primers were 10 ng and 50 nmol, respectively. The mixture was incubated at 50 °C for 2 min and then at 95 °C for 10 min. The amplification was repeated for 40 cycles, each cycle including denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 15 s (the data were recorded during the polymerization). Primers were designed with the Primer Express 3.0 program using an ABI Prism 7900HT attachment to the RT-PCR instrument. For mtDNA of TPCase, the forward primer is

GAAAGGCATTTTCGGCTTCA,

while the reverse primer is

CAGCCTGGATGGCAGGAA.

Each reaction was carried out in triplicate. The data obtained were analyzed with the SPSS 13.0 program package (SPSS Inc., USA) providing for the calculation of the average mRNA level changes for genes and standard errors. The data were considered reliable for $p < 0.05$.

To quantify the mitochondria in muscle tissues from mtDNA, the C_T values (the number of cycles required to achieve a given reporter fluorescence level (threshold fluorescence)) for the nuclear gene of β-actin and the mitochondrial gene of cytochrome b were determined in the same amplification experiment. The experiments showed a good reproducibility both within a single run and between different repeated runs. The C_T values were employed as a measure of the copy number of the DNA sequence under analysis in early amplification; the difference between the C_T values were used to quantify the copies (Rc) of mtDNA

with respect to the copy number of the β-actin gene according to the formula:

$$Rc = 2\Delta C_T,$$

where ΔC_T is the difference between the C_T values for β-actin and cytochrome b.

Results and Discussion

We found that injections of trekrezan (10 mg kg^{-1}) into rats increase their muscle strength and endurance. At a higher dose of trekrezan (25 mg kg^{-1}), the effect becomes stronger (Table 1).

At the same time, the number of mitochondria in tissues increased in proportion to the activation of the expression of mRNA of PGC-1α. The results obtained for the target PGC-1α gene and mRNA of β-actin are compared in Table 2. It can be seen that trekrezan in both doses (10 and 25 mg kg^{-1}) nearly doubles the expression of the PGC-1α gene in muscle fibers. Correlation analysis revealed that the changes observed in the testing of rat strength and endurance are highly correlated with the gene expression data.

The data obtained for the expression of the PGC-1α and TPCase genes in rat muscles (Fig. 1) show that a solution of trekrezan injected intraperitoneally for 7 days in a daily dose of 25 mg kg^{-1} causes maximum activation of the expression of the PGC-1α gene in muscle fibers. For both control and test samples, the gene expression was analyzed using a combined sample containing cDNA synthesized from the RNA template. The relative increase

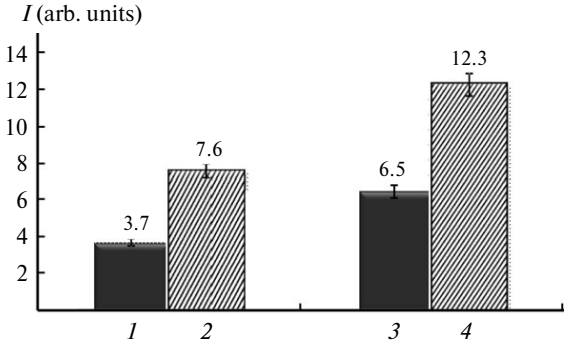

Fig. 1. *In vivo* expression of (*I*) the PGC-1α (*1*, *2*) and TPCase genes (*3*, *4*) in the absence (*1*, *3*) and in the presence (*2*, *4*) of trekrezan; *I* = [mRNA of PGC-1α or TPCase]/[mRNA of β-actin].

in the expression of the PGC-1α gene agrees with the previous data. A similar stimulating effect was achieved for the expression of the TPCase gene. Earlier, our *in vitro* tests have revealed the tendency of trekrezan to activate mRNA.[4] This suggests that the coactivator of PGC-1α and TPCase plays an important role in a complex system of reactions responsible for reaching and maintaining homeostasis at the cellular, tissue, and organism levels. Their concerted activation and interrelation seem to be associated with sophisticated regulation of canonical and noncanonical activities of TPCase. To verify this assumption, we conducted a special series of tests to study the effect of trekrezan on the *in vivo* expression of mRNA of TPCase in muscle tissues. We compared the expression

Table 1. Effect of trekrezan on the muscle strength and endurance in rats

Group	Total latency (in three successive trials), min	Swimming to fatigue, min
Control (placebo)	11.45±0.85	33.50±1.7
Entry *1* (10 mg kg^{-1} TK)	15.45±0.97 ($r = 0.57$)[a]	48.00±2.4 ($r = 0.61$)[a]
Entry *2* (25 mg kg^{-1} TK)	19.35±1.02 ($r = 0.72$)[a]	59.30±2.8 ($r = 0.75$)[a]

[a] The doses of trekrezan are 10 (for entry *1*) and 25 mg kg^{-1} (for entry *2*).
[b] $p < 0.05$ with respect to the control entry; *r* is the correlation coefficient.

Table 2. Comparison of the target PGC-1α gene with mRNA of β-actin

Group	Relative induction[a]	Number of mitochondria in myocytes from the copy number[b]
Control (placebo)	0.9±0.1	0.9±0.1
Entry *1* (10 mg kg^{-1} TK)	1.72±0.15 ($r = 0.56$)[c]	1.25±0.1 ($r = 0.6$)[c]
Entry *2* (25 mg kg^{-1} TK)	1.9±0.2 ($r = 0.62$)[c]	2.75±0.2 ($r = 0.69$)[c]

[a] The ratio [mRNA of PGC-1α]/[mRNA of β-actin].
[b] The ratio [mtDNA (cytochrome b)]/[diploid genome (β-actin gene)].
[c] The doses of trekrezan are 10 (for entry *1*) and 25 mg kg^{-1} (for entry *2*).
[d] $p < 0.05$ with respect to the control entry.

levels for the PGC-1α and TPCase genes in this system and went into the mechanism by which they are mutually related. The test conditions were similar to those in separate tests; however, the dose of trekrezan was 25 mg kg^{-1} for seven days because of its maximum stimulating effect (see Table 2). Analysis of the expression of PGC-1α and TPCase was performed in the same sample using specific primers and probes. The stably expressing gene of β-actin was used as a reference.

According to the results obtained, trekrezan greatly stimulates the expression of the PGC-1α gene, being roughly as efficient as natural stimulators.[7,8] For this reason, we found it important to compare the *in vivo* expression of the PGC-1α and TPCase genes in a joint test. Comparison of the expression of the PGC-1α gene in rat muscles and the *in vitro* expression of TPCase revealed that their activation is comparable with the previous[4] data (by factors of 1.9 for PGC-1α and of 2.5 for mRNA of TPCase), which suggests a relationship between these processes. Comparison of the absolute numbers of detected mRNA of TPCase and PGC-1α (from the corresponding C_T values) revealed earlier amplification of mRNA of TPCase in both the control (no trekrezan) and test systems (in the presence of trekrezan), which reflects the quantitative ratio of mRNA of these genes.

To sum up, we were the first to demonstrate that the use of trekrezan promotes the expression of the gene of the coactivator PGC-1α as well as a cascade of reactions involved in the biogenesis of mitochondria, thus increasing their number. In addition, we should note active replication of the mitochondrial genome and, accordingly, good myocytic bioenergetics. The results obtained (1) provide evidence for the efficiency of the mtDNA level analysis we used to check the number of mitochondria in myocytes and (2) confirm the mechanism of action of trekrezan. Comparison of these data with the results of the inverted screen and forced swim tests revealed a substantial increase in the muscle strength and endurance of test rats, in agreement with the expression data.

Based on the data presented above, we can propose the TPCase and PGC-1α genes and their products as therapeutic targets and biological markers when selecting a set of therapeutic agents and optimizing the hormesis processes involving these genes. In addition, we can hypothesize the mechanisms coupling the energy metabolism regulation with protein biosynthesis and innate immunity on the basis of the principles of person-oriented medicine, which agrees with the literature data.[9,10]

Moreover, the results obtained show a good outlook for further investigations in this field. We believe that the next important aspect of the study of molecular mechanisms behind the stimulating effect of trekrezan is a discovery of tissue-specific regulation of the metabolic pathways involving the PGC-1α gene and its product, which is an important target for drugs and modulators as well as a target for regulation of the interplay between genes and signaling pathways in the cell.

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References

1. M. W. Laupheimer, P. M. Malliaris, P. Berdejo-del-Fresno, D. Maffulli, N. S. Hemmings, *J. Sport Health Res.*, 2013, **5**, 237.
2. H. Selye, *Stress without Distress*, New American Library, New York, 1975.
3. M. G. Voronkov, M. M. Rasulov, *Khim.-Farm. Zh.*, 2007, **41**, 3 [*Pharm. Chem. J.* (*Engl. Transl.*), 2007, **41**].
4. M. M. Rasulov, M. K. Nurbekov, V. V. Bulanova, N. D. Anokhina, R. N. Arkhipov, S. A. Golovanov, Yu. M. Sikacheva, M. I. Susova, M. G. Voronkov, RF Pat. 2407525 S1, 27.12.2010 (in Russian).
5. M. M. Rasulov, M. K. Nurbekov, M. G. Voronkov, S. N. Bobkova, O. A. Belikova, I. G. Kizlikov, RF Pat. 2429836 S1, 27.09.2011 (in Russian).
6. R. U. Ostrovskaya, N. N. Kleimenova, V. A. Kamysheva, G. M. Molodavkin, A. N. Yavorskii, S. S. Boiko, in *Farmakologicheskaya korrektsiya utomleniya* [*Pharmacological Correction of Fatigue*], Ed. Yu. G. Bobkov, Meditsina, Moscow, 1982, 39 (in Russian).
7. M. K. Nurbekov, M. M. Rasulov, M. G. Voronkov, S. N. Bobkova, O. A. Belikova, *Dokl. Biochem. Biophys.*, 2011, **438**, 131.
8. K. Higashida, S. H. Kim, S. R. Jung, *PLOS Biol.*, 2013, **7**, 11, e1001603.
9. L. Mao, J. Franke, *Int. J. Mol. Sci.*, 2013, **14**, 13109.
10. C. Cornelius, R. Perrotta, *Immun. Ageing*, 2013, **10**, 1.

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