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SkQ1 Regulates Expression of *Nrf2*, ARE-Controlled Genes Encoding Antioxidant Enzymes, and Their Activity in Cerebral Cortex under Oxidative Stress

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Abstract—The administration of SkQ1 to rats at the dose of 50 nmol/kg for five days significantly increased the mRNA levels of transcription factor Nrf2 and of Nrf2-controlled genes encoding antioxidant enzymes *SOD1*, *SOD2*, *CAT*, and *GPx4*, whereas changes in the level of mRNA of *SOD3* in the cerebral cortex of the rat brain were not significant. This was accompanied by activation of antioxidant enzymes (SOD, CAT, GPx, and GST) and increase in reduced glutathione concentration. Under oxidative stress induced by hyperoxia (0.5 MPa for 90 min), the mRNA level of transcription factor Nrf2 decreased, whereas changes in the transcriptional activity of Nrf2-induced genes (*SOD1-3*, *CAT*, *GPx4*) encoding antioxidant enzymes in the cortex of the rat brain hemispheres were insignificant. Under conditions of hyperoxia, lipid peroxidation intensity was increased, CAT was inhibited, and GST activity was moderately increased, whereas SOD and GPx activities in the rat brain cerebral cortex remained at the stationary level. Pretreatment with SkQ1 before the exposure to hyperbaric oxygenation led to an increase in mRNA level of transcription factor Nrf2 and of Nrf2-induced genes (*SOD1-2*, *CAT*, and *GPx4*) encoding antioxidant enzymes, whereas *SOD3* expression in the cerebral cortex of the rat brain under oxidative stress was not changed. Concurrently, we observed an increase in activities of these antioxidant enzymes (SOD, CAT, GPx, and GST) and in level of reduced glutathione. We hypothesize that the protective effect of SkQ1 under hyperoxia-induced oxidative stress could be realized via direct antioxidant activity and through stimulation of the signaling defense system Keap1/Nrf2/ARE.

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Oxidative stress, which damages brain cells, contributes significantly to the pathogenesis of many diseases of the nervous system. The signaling pathway Nrf2/Keap1/ARE involved in cytoprotective mechanisms in various pathological processes is now considered as a sensitive sensor and regulator of oxidative stress [1, 2]. It is established that transcription factor Nrf2 and its repressor Keap1 regulate a network of cytoprotective genes that includes about 1% of the genome [3].

Transcription factor Nrf2 is expressed in the central nervous system and is an important component of response in both acute and chronic nervous system disease associated with oxidative stress [4, 5]. Hyperbaric oxygenation (HBO) is a useful model of oxidative stress, and it is used to study disorders in free radical homeostasis in nervous tissue, as well as in other tissues of the body [6].

Many studies have demonstrated high efficiency and neuroprotective action of mitochondria-targeted antioxidants of the SkQ1 family in ischemia/reperfusion, brain trauma, and neurodegenerative diseases [7-9]. Therefore, studies on mechanisms of neuroprotective action of SkQ family compounds under oxidative stress of different etiology are very important.

The purpose of this work was to study the effect of SkQ1 on the expression of the transcription factor gene *Nrf2* and of Nrf2-dependent genes of antioxidant enzymes and on their activities in the cerebral cortex of rat brain under conditions of hyperoxia-induced oxidative stress.

Abbreviations: ARE, antioxidant-responsive element; HBO, hyperbaric oxygenation; LPO, lipid peroxidation; Nrf2, NF-E2-related factor 2; ROS, reactive oxygen species.

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MATERIALS AND METHODS

Experiments were performed on male mongrel *Rattus norvegicus* rats with body weight of 180-200 g. The rats were divided into four groups: the first group (control) included intact animals that were maintained under standard vivarium conditions; the second group included rats that were injected with SkQ1 at the dose of 50 nmol/kg ("SkQ1" group) for five days; the third group included animals that were subjected to HBO (0.5 MPa for 90 min) and decapitated 12 h after HBO ("HBO" group). The rats were placed into a 60-liter altitude chamber equipped with an alkaline absorber of CO_2. After ventilation for 3 min with pure oxygen, the pressure in the chamber was raised to 0.5 MPa. Compression and decompression were performed at the rate of 0.2 MPa/min, and isopression was for 90 min. The described regimen of HBO (0.5 MPa for 90 min) caused acute oxidative stress [10]. The fourth group included rats that were injected for five days with SkQ1 at the dose of 50 nmol/kg and 1 h after the last injection were subjected to HBO (0.5 MPa for 90 min) and decapitated 12 h after the HBO ("SkQ1 + HBO" group). The preparation of SkQ1 was injected into cheek pouches of the animals in the calculated dose dissolved in 100 μl of 0.2% ethanol. The SkQ1 dose and injection scheme were chosen according to published recommendations [11] and our own investigations.

In studies on the gene expression, the number of animals in each of the four groups was as follows: 20 (*Nrf2*), 18 (*SOD1-3*), 22 (*CAT*), 19 (*GPx4*); in biochemical studies, every group included 12 animals.

All procedures with the animals were performed in accordance with principles of the European Convention for protecting vertebrates used in experiments or for scientific purpose (Strasburg, March 18, 1986).

Expression of mRNA was analyzed using the reverse transcription with subsequent polymerase chain reaction (RT-PCR) with specific primers. For investigation, 30 mg of the brain hemisphere cortex was taken. Total RNA was extracted with guanidine–thiocyanate–phenol–chloroform using a commercial RIBO-sol-B kit (InterLabServis, Russia). The quality of the isolated RNA was assessed by electrophoresis in 1.2% agarose gel, and its quantity was determined by optical density at 260 nm. Synthesis of cDNA was performed with a kit of reagents for reverse transcription (SYNTOL, Russia) containing M-MLV-RT (Moloney murine leukemia virus reverse transcriptase), the Random-6 primer, a mixture of deoxyribonucleotidyl triphosphates (dNTP), and an inhibitor of RNases.

Expression levels of genes encoding the transcription factor Nrf2 (*Nrf2*), the superoxide dismutase isoenzymes Cu,Zn-SOD (*SOD1*), Mn-SOD (*SOD2*), extracellular SOD, E-SOD (*SOD3*), catalase (*CAT*), and glutathione peroxidase 4 (*GPx4*) were studied by real-time PCR in the presence of intercalation dye EVA Green (Molecular Probes, USA) using a kit for Real-Time PCR from SYNTOL. Real-time PCR was performed using an iQ5 real-time PCR detection system (Bio-Rad Laboratories, USA). As a reference gene, β-actin (*BACT*) was used. Specific primers were chosen with the Primer BLAST and Primer 3 program. Sequences of the specific primers are presented in the table.

To assess the efficiency (E) of each pair of primers, PCR was performed using different dilutions of cDNA (1 : 1, 1 : 2, 1 : 4, 1 : 8) with a subsequent calculation of averaged ΔCt. The table presents the efficiency of the primers.

Real-time PCR was performed under the following conditions: 1st stage – 95°C 300 s; 2nd stage – 58(60)°C 50 s (fluorescence detection); 3rd stage – 95°C 15 s with transition to the 2nd stage. The 2nd and 3rd stages were repeated 40 times, and after this operation was terminated we obtained plots of the fluorescence changes with time. Specificity of the amplification products was tested by melting curves of the PCR products.

Plots of accumulation of PCR products were analyzed with Bio-Rad IQ5 Optical System Software version 2.0 program. Results of real-time PCR analysis were treated with iCycler IQ5 software (BioRad, USA). The relative level of gene expression was calculated with the ΔCt method using the reference gene.

For biochemical studies, the animals were decapitated, the brain was isolated, washed in 0.9% NaCl ice-cold solution, and the cerebral cortex was isolated (tissues from two animals were combined into one sample). The tissue was homogenized at ratio of 1 : 10 (w/v) in buffer consisting of 100 mM Tris-HCl, 150 mM NaCl (pH 7.4), and 0.5 mM EDTA in a Potter S homogenizer (Teflon–glass) with a rate of 1500 rpm. Some of the resulting homogenates were treated with Triton X-100 (final concentration 0.1%), incubated for 10 min at 37°C, and then centrifuged for 15 min at 3000 rpm. The resulting supernatant was used for determination of the enzyme activities.

Activity of superoxide dismutase (SOD) was evaluated by inhibition of Nitro Blue Tetrazolium (NBT) reduction by superoxide generated during autooxidation of epinephrine [12]. Activity of catalase (CAT) was determined by the reaction of hydrogen peroxide with ammonium molybdate [13]. Activity of glutathione peroxidase (GPx) was determined by the oxidation rate of reduced glutathione in the presence of tertiary butyl hydroperoxide [14], and activity of glutathione-S-transferase (GST) was evaluated by the rate of enzymatic production of reduced glutathione conjugates with 1-chloro-2,4-dinitrobenzene [15]. Activity of xanthine oxidoreductase (XOR) was determined with a spectrophotometer by increase in uric acid level [16] determined with a standard kit from Olvex Diagnosticum (Russia).

In homogenates that were not treated with detergent, the intensity of lipid peroxidation (LPO) was deter-

Primers for real-time PCR and efficiency of primer pairs used

Gene	Nucleotide sequence of forward (f) and reverse (r) primers	Primer pairs	Efficiency, E
BACT	f: 5'-agccatgtacgtagccatcc-3' r: 5'-tcggaaccgctcattgccg-3'	*BACT-f, BACT-r*	0.953
Nrf2	f: 5'-atgtcaccagctcaagggcacagtgc-3' r: 5'-ccatcctccccgaacctagtt-3'	*Nrf2-f, Nrf2-r*	0.970
SOD1	f: 5'-aaccagttgtggtgtcagga-3' r: 5'-ctcctgagagtgagatcaca-3'	*SOD1-f, SOD1-r*	0.930
SOD2	f: 5'-taacgcgcagatcatgcag-3' r: 5'-gtcacgcttgatagcctcca-3'	*SOD2-f, SOD2-r*	0.873
SOD3	f: 5'-aggctctttctcaggcctc-3' r: 5'-agatctccaggtctttggag-3'	*SOD3-f, SOD3-r*	0.933
CAT	f: 5'-ttctacactgaagatggtaactg-3' r:5'-gaaagtaacctgatggagagac-3'	*CAT-f, CAT-r*	0.903
GPx4	f: 5'-ggctacaatgtcaggtt-3' r: 5'-ttatcaatgagaaacttggtaa-3'	*GPx4-f, GPx4-r*	0.780

mined by accumulation of LPO products. The level of diene conjugates (DC) was determined in chloroform extract by spectrophotometry at 233 nm [17]. The malondialdehyde level was assessed by reaction with thiobarbituric acid [18]. The content of Schiff bases was determined by spectrofluorometry at excitation wavelength 360 nm and emission wavelength 440 nm [19]. The chloroform extract was prepared as described by Bligh and Dyer [20].

The statistical analyses were performed using the Statistica 6.1 software package. The normal distribution was checked using the Shapiro–Wilk test. For comparison of the groups, one-way and two-way ANOVA with pairwise comparisons of the group means (Newman–Keuls test) were used. Differences were considered as significant at $p < 0.05$, and $0.05 < p < 0.1$ was considered as a trend towards significance.

RESULTS

Under physiological conditions, injections of SkQ1 (50 nmol/kg for five days) led to increase in the level of mRNA of transcription factor Nrf2 in the rat brain cerebral cortex (Fig. 1). *Post hoc* pairwise comparisons (Newman–Keuls test) revealed that the influence of SkQ1 on mRNA level of the transcription factor under normal conditions was statistically significant and equal to 121% ($F = 13.95$; $p = 0.001$). This indicated an increase in expression of the *Nrf2* gene capable of regulating its own transcription and the transcription of Nrf2-dependent genes of antioxidant enzymes. Under the same conditions, increase was observed in the level of mRNA of antioxidant genes: *SOD1* – by 41% ($F = 13.95$; $p = 0.0004$), *SOD2* – by 50% ($F = 9.7$; $p < 0.003$), *CAT* – by 62% ($F = 10.66$; $p < 0.002$), *GPx4* – by 58% ($F = 7.81$; $p = 0.007$) on the background of the stationary level of mRNA of the *SOD3* gene (Fig. 1).

The ANOVA with *post hoc* pairwise comparisons indicated that after HBO (0.5 MPa for 90 min) the level of mRNA of the transcription factor Nrf2 in the brain hemisphere cortex significantly decreased by 23% ($F = 11.83$; $p < 0.001$), whereas levels of mRNAs of antioxidant enzyme genes (*SOD1-3*, *CAT*, *GPx4*) changed insignificantly with respect to the control.

Pretreatment with SkQ1 (50 nmol/kg) for five days before the exposure to HBO resulted in a significant increase in mRNA level of the *Nrf2* gene and of *Nrf2*-dependent genes of antioxidant enzymes in the rat brain cerebral cortex (Fig. 1). In the "SkQ1 + HBO" group, the level of mRNA of *Nrf2* increased by 41% ($F = 16.14$; $p < 0.0003$) compared to the control, and two-fold compared to its level in the "HBO" group ($F = 7.12$; $p = 0.0012$) in which the animals were not pretreated with SkQ1 before the exposure to hypoxia. The increase in *Nrf2* expression after exposure to hyperoxia was accompanied by a significant increase in the level of mRNAs of the Nrf2-controlled antioxidant enzyme genes: *SOD1* – by 54% ($F = 9.04$; $p = 0.005$), *SOD2* – by 48% ($F = 5.12$; $p = 0.03$), *CAT* – by 39% ($F = 3.08$; $0.05 < p < 0.1$), *GPx4* – by 44%

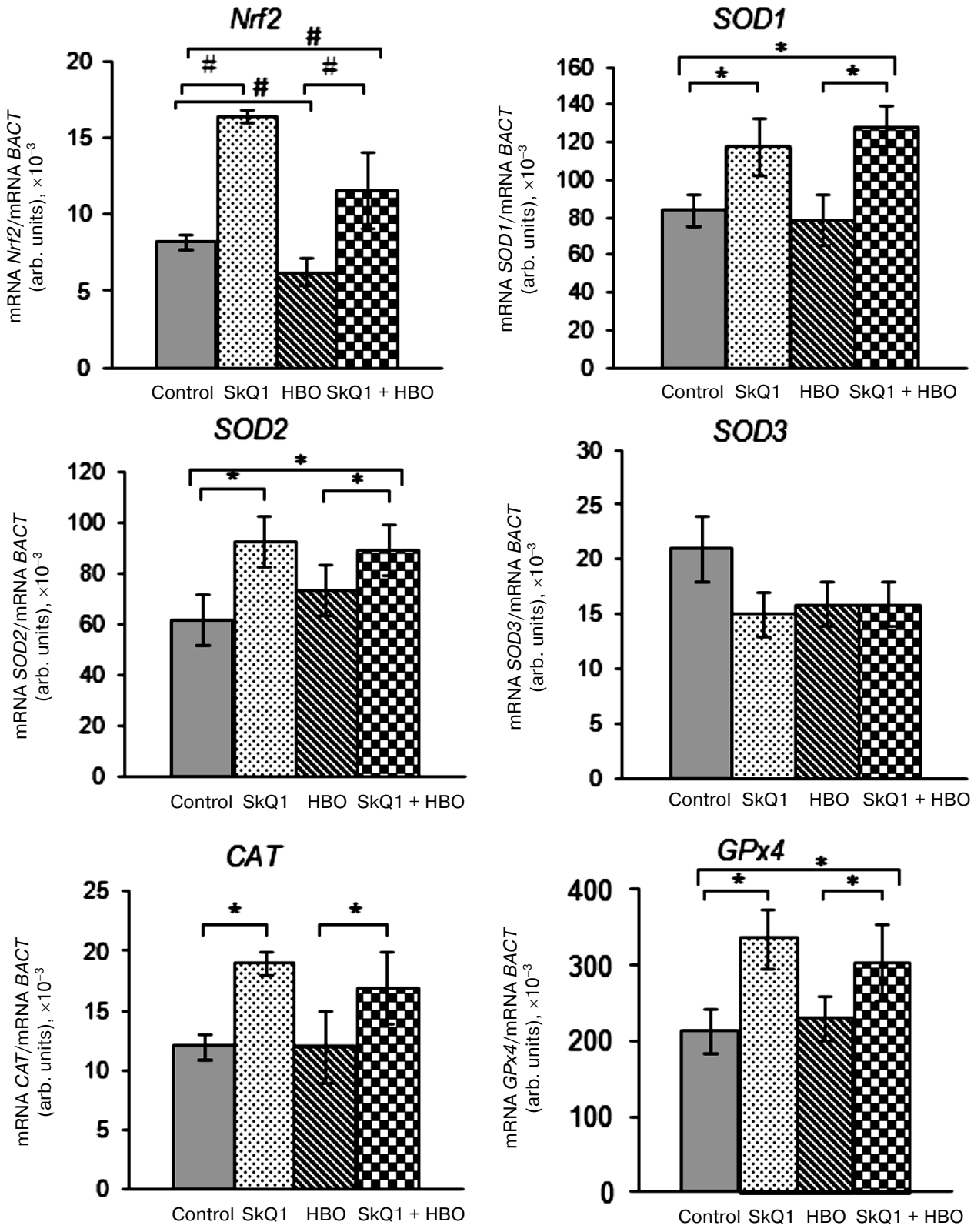

Fig. 1. Influence of SkQ1 on mRNA level of the *Nrf2* gene and of genes of antioxidant enzymes in rat brain cerebral cortex under normal conditions and after HBO-induced oxidative stress (M ± m). The number of animals in groups was 18-22. The comparison was performed using two-way (ANOVA) with *post hoc* pairwise tests of group means; * $p < 0.05$; # $p < 0.001$.

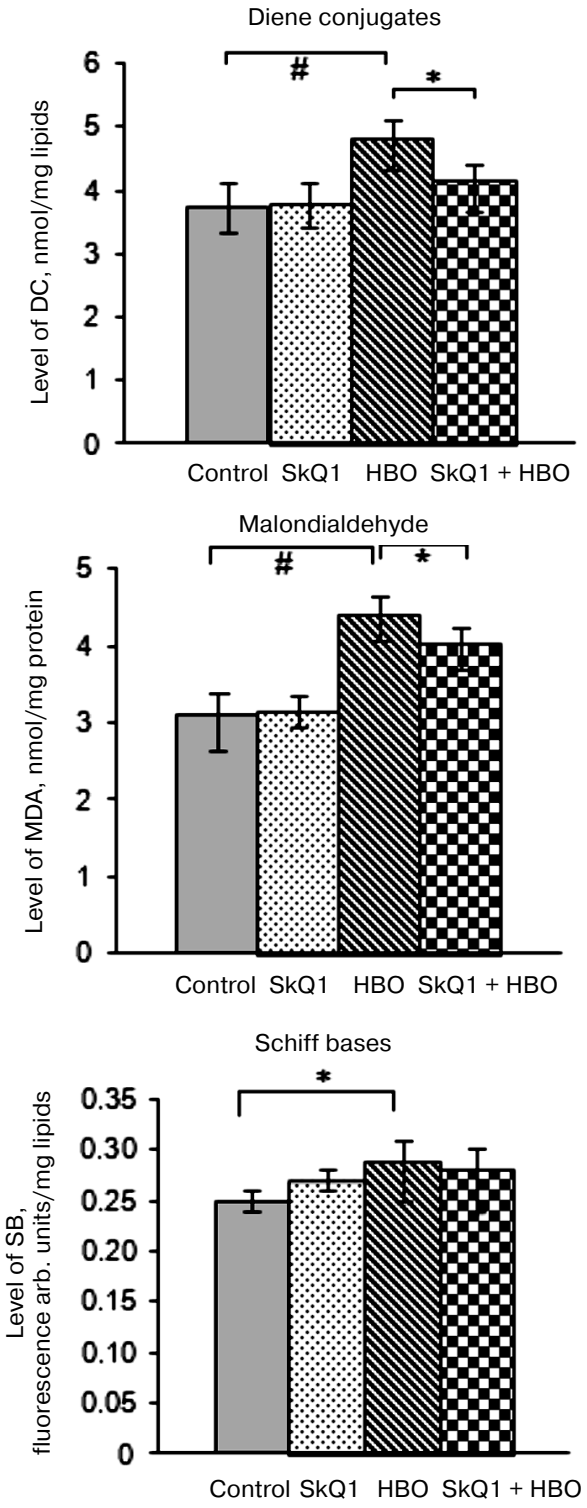

Fig. 2. Influence of SkQ1 on lipid peroxidation intensity in rat brain cerebral cortex under normal conditions and after HBO-induced oxidative stress (M ± m). There were 12 animals in the groups. The comparison was performed using two-way ANOVA with *post hoc* pairwise tests of group means; * $p < 0.05$; # $p < 0.001$.

(F = 3.97; $p = 0.05$) with respect to the control. It should be noted that, according to the results of ANOVA, the level of mRNAs of the antioxidant enzyme genes *SOD1*, *SOD2*, *CAT*, and *GPx4* significantly increased by 29-72% compared to the group of animals that were not pretreated with SkQ1 before the exposure to hypoxia ("HBO" group), and this indicated a stimulatory effect of the compound. The level of *SOD3* mRNA under these conditions changed insignificantly.

The results show that administration of SkQ1 for five days under normal conditions does not cause changes in LPO intensity in the rat brain cerebral cortex. After the HBO-induced oxidative stress, LPO in the rat brain was intensified (Fig. 2). According to *post hoc* pairwise comparisons (Newman–Keuls test), the level of LPO primary molecular products, diene conjugates (DC), was increased by 30% (F = 18.93; $p < 0.0001$), of LPO secondary product, malondialdehyde (MDA), was increased by 42% (F = 12.95; $p < 0.001$), and of LPO end-products, Schiff bases (SB), was increased by 18% (F = 7.02; $p = 0.015$) in the "HBO" group with respect to the control. In the group pretreated with SkQ1, the LPO intensity in the brain cerebral cortex was maintained at the stationary level, but it was significantly decreased with respect to the "HBO" group, i.e. in comparison with the animals that were not pretreated with SkQ1 before the exposure to hyperoxia. The level of DC was decreased by 24% (F = 6.46; $p = 0.015$), of MDA – by 25% (F = 6.39; $p = 0.015$), and the level of SB demonstrated a tendency for decrease ($0.05 < p < 0.1$) compared to the "HBO" group. Thus, ANOVA revealed significant differences in the levels of LPO products, increase in their contents with respect to the control after hyperoxia and decrease in their levels in the "SkQ1 + HBO" group with respect to the "HBO" group, which indicates a significant effect of SkQ1.

As the ANOVA revealed, injection of SkQ1 for five days activated antioxidant enzymes in the rat brain cerebral cortex. The activity of SOD with respect to the control was increased by 50% (F = 32.86; $p < 0.0001$), of catalase by 53% (F = 41.2; $p < 0.0001$), of GPx by 63% (F = 23.6; $p < 0.0001$), and of GST by 55% (F = 23.0; $p < 0.0001$), whereas the level of reduced glutathione (GSH) was increased by 39% (F = 10.77; $p = 0.002$) (Fig. 3). After HBO-induced oxidative stress (12 h after exposure) on the background of insignificant changes in the activity of SOD, the activity of catalase was decreased by 18% (F = 11.49; $p < 0.001$) and the activity of GST was increased by 18% (F = 8.06; $p < 0.01$) with respect to the control group. The pretreatment with SkQ1 for five days before the exposure to HBO resulted in increase in activities of antioxidant enzymes: SOD by 43% (F = 22.72; $p < 0.0001$), catalase by 27% (F = 7.95; $p = 0.01$), GPx by 65% (F = 29.78; $p < 0.0001$), and of glutathione-S-transferase by 25% (F = 13.76; $p < 0.001$); the level of GSH in the rat brain hemisphere cortex increased by 35% (F = 10.76; $p = 0.003$) with respect to the control. In this group

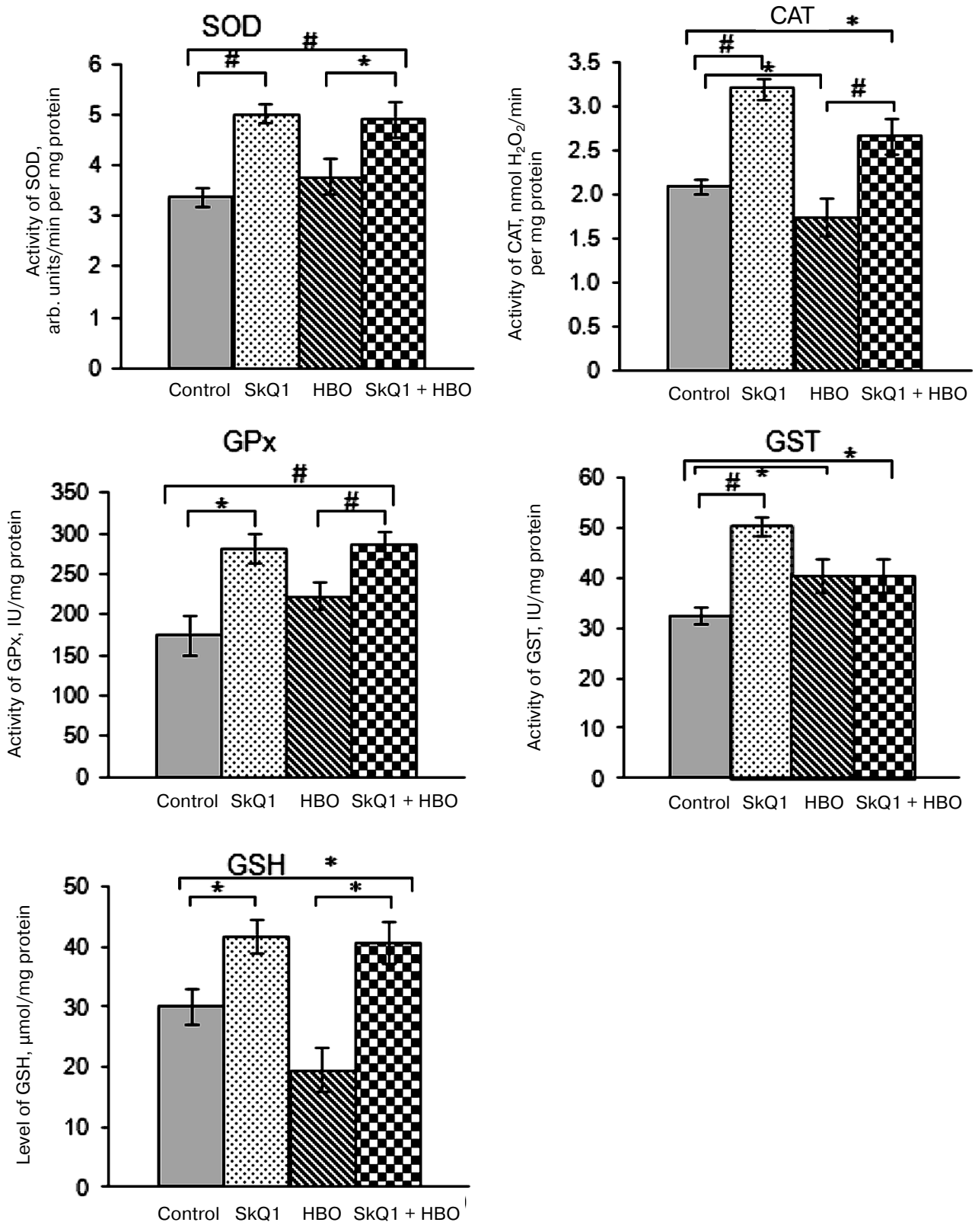

Fig. 3. Influence of SkQ1 on activities of antioxidant enzymes and on the level of GSH in rat brain hemisphere cortex under normal conditions and after HBO-induced oxidative stress (M ± m). There were 12 animals in each group. The comparison was performed using two-way ANOVA with *post hoc* pairwise tests of group means; * $p < 0.05$; # $p < 0.001$.

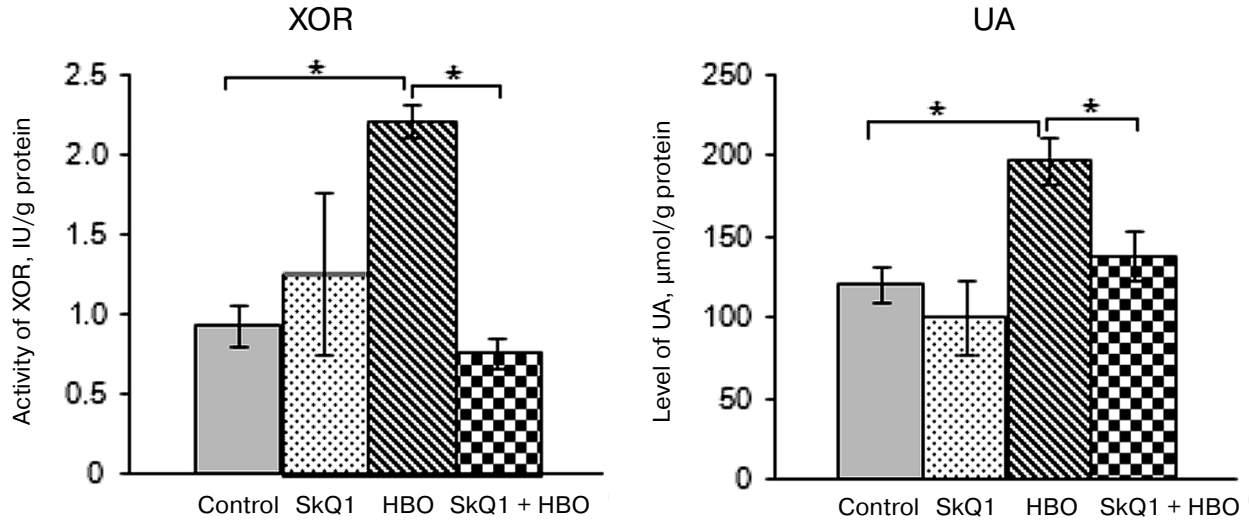

Fig. 4. Effect of SkQ1 on activity of xanthine oxidoreductase (XOR) and content of uric acid (UA) in rat brain hemisphere cortex under normal conditions and after HBO-induced oxidative stress (M ± m). There were 12 animals in each group. The comparison was performed using two-way ANOVA with *post hoc* tests of group means; * $p < 0.05$; # $p < 0.001$.

of animals, activities of the antioxidant enzymes (SOD, CAT, GPx) were significantly increased by 27-55% and the GSH level was increased twofold compared with the "HBO" group animals which were not pretreated with SkQ1; obviously, this is an evidence of the influence of SkQ1.

Under normal condition, injection of SkQ1 did not cause significant changes in xanthine oxidoreductase (XOR) activity in rat brain hemisphere cortex (Fig. 4). *Post hoc* pairwise comparisons (Newman–Keuls test) revealed 124% increase in XOR activity in the "HBO" group with respect to the control ($F = 4.85$; $p = 0.033$), whereas the injections of SkQ1 before the exposure to hyperoxia promoted normalization of the XOR activity after oxidative stress. In the "SkQ1 + HBO" group, the enzyme activity was decreased by 63% ($F = 10.26$; $p = 0.003$) compared to the "HBO" group. Note that the XOR activation after hyperoxia correlated with a 76% increase ($F = 13.93$; $p = 0.003$) in the content of the enzymatic reaction product, uric acid, whereas the injection of SkQ1 under physiological conditions and before the exposure to HBO did not induce significant changes in the level of uric acid in rat brain hemisphere cortex (Fig. 4). Thus, the ANOVA shows significant increase in XOR activity and in level of uric acid after oxidative stress and normalization of these parameters in the "SkQ1 + HBO" group.

DISCUSSION

This study has shown that under physiological conditions administration of SkQ1 results in twofold increase in *Nrf2* gene transcriptional activity in rat brain hemisphere cortex. As shown earlier [21, 22], SkQ1 increases *Nrf2* gene expression in peripheral blood leukocytes of rats under physiological conditions and under hyperoxia-induced oxidative stress, and it thus stimulates the Keap1/Nrf2/ARE signaling system.

Various inducers of Nrf2 are known, including the most important group of natural quinones (flavonoids, curcumin, resveratrol, plastoquinone, etc.) [4, 23]. Many authors have reported that the presence of OH-groups in the *ortho*- and *para*-positions in phenolic antioxidants is responsible for their stimulating action on Nrf2 [23]. Just such position of OH-groups is specific for SkQ1, which contains a derivative of 1,4-benzoquinone, plastoquinone [24]. It is known that the antioxidant features of phenolic compounds are first of all due to the presence of OH-groups in their structure.

It is reasonable to suppose that due to the presence of quinone in the SkQ1 molecule, this compound could induce Nrf2. It should be emphasized that in the promoter region of the *Nrf2* gene there are two ARE-like sequences. That is why *Nrf2* can regulate its own transcription and transcription of *Nrf2*-dependent genes of antioxidant enzymes through antioxidant-responsive elements (ARE), which are present in promoters of both *Nrf2* and ARE-controlled genes [25, 26]. The presence of a positive feedback loop in the Keap1/Nrf2/ARE signaling pathway significantly increases the system sensitivity and efficiency of cell defense mechanisms [27, 28].

It is known that Nrf2 (NF-E2-related factor 2) belongs to the Cap'n'Collar (CNC) family, is a member of the large group of DNA-binding proteins with the "leucine zip", bZIP [1], and is widely expressed in various tissues including the brain [5]. Nrf2 controls both basal gene expression under conditions of homeostasis

and inducible expression of a multiplicity of genes in disorders of redox-balance and on the development of oxidative/electrophilic stress [4].

In addition, it has been shown that the SkQ1-induced increase in *Nrf2* expression in the brain hemisphere cortex under physiological conditions stimulates an increase in the level of mRNA of antioxidant enzyme genes (*SOD1*, *SOD2*, *CAT*, *GPx4*) that can be favorable for increasing the antioxidant potential of nervous-system cells. This is especially important because the brain is very sensitive to oxidative stress, and the oxidative modification of nervous tissue components develops under both acute damage and chronic disorders of the central nervous system [2, 5]. The brain has a high rate of oxygen consumption, which leads to high rate of superoxide production [28] and increased rate of metabolism that is associated, in particular, with high contents of easily oxidized ω-3 polyunsaturated fatty acids [29]. However, notwithstanding the variety of sources and the increased rate of reactive oxygen species (ROS) generation, no endogenous antioxidant system with high efficiency has been detected in the brain [30], and this emphasizes the key role of the transcription factor Nrf2 and its inducers in the defense of the nervous system against oxidative stress. Note that the administration of SkQ1 under physiological conditions does not cause changes in the stationary levels of LPO products in brain cells. It should be noted that maintenance of free-radical homeostasis under physiological conditions on the background of a mitochondria-targeted antioxidant can be important for nervous and for other tissues due to various positive effects of physiological concentrations of oxidized phospholipids: they are involved in the most important signaling pathways, restrict inflammation, etc. [31].

In the present study, a decrease in the *Nrf2* gene transcriptional activity has been found in rat brain after oxidative stress. In our previous work [22], inhibition of *Nrf2* gene expression was also shown in rat blood leukocytes after HBO.

In a study [32] on chromosome 2, a hyperoxia-sensitive locus 1 containing the gene-candidate *Nrf2* encoding transcription factor Nrf2 was identified. Mice with *Nrf2* gene knockout were shown to be more sensitive to hyperoxia-induced damage to their lungs and to cell death compared to wild-type animals [33].

It should be noted that under conditions of homeostasis, Nrf2 is strictly controlled by the ubiquitin-proteasomal degradation system that is responsible for low level of the transcription factor. The Nrf2 level is regulated through Keap1-dependent and -independent pathways [26]. The Keap1-dependent pathway of Nrf2 degradation is considered to be canonical and redox-dependent, it is represented by the Keap1–Cul3–Rbx1 complex of ubiquitin ligase E3, which is sensitive to redox-state of the cell due to exceptionally high content of SH-groups (up to 25 cysteine residues in the structure of Keap1 and 7 residues in Nrf2) [34]. It is very likely that oxidative modification of reactive cysteine residues in the Keap1–Nrf2 complex after hyperoxia is caused by an increased production of ROS [11] and that accumulation of LPO products can induce conformational changes in Nrf2 and Keap1 that, in turn, can accelerate ubiquitinylation of Nrf2 and its proteasomal degradation. This prevents the nuclear translocation of Nrf2 and subsequent activation of expression of *Nrf2* and *Nrf2*-dependent genes of antioxidant enzymes.

However, a redox-independent pathway of the transcription factor degradation has been detected. This pathway is connected with phosphodegron in the Neh6 domain of Nrf2, which contains serine residues that can be phosphorylated by serine-threonine kinase-3β of glycogen synthase. Phosphorylation in the Neh6 domain leads to formation of a destructive motif (phosphodegron) that is later recognized by β-TrCP–Skp1–Cul1–Rbx E3 ubiquitin ligase complex that promotes degradation of Nrf2. It is important to note that activation of kinase-3β of glycogen synthase (GSK-3β) was found after hyperoxia [35]. This was accompanied by inflammation and damage to lungs of neonatal rats, whereas inhibition of GSK-3β protected the lung tissue against the HBO-induced damage. Thus, it was supposed that a decrease in Nrf2 level in the rat brain cortex after hyperoxia could be due to both redox-dependent and redox-independent mechanisms of transcription factor degradation. In turn, this can result in a decrease in transcriptional activity of the *Nrf2* gene. The decrease in expression of the transcription factor gene after hyperoxia because of increased degradation of Nrf2, in turn, will inevitably lower its ability for autoactivation and autoregulation associated with the presence of ARE elements in the region of its promoter [25].

In the present study, no significant changes were found in the transcriptional activity of the ARE-controlled genes on the background of decreased expression of the *Nrf2* gene in rat brain cortex after HBO. Note that in another study [36] under normobaric hyperoxia (95% O_2 for 18 h) a significant decrease was also found in *Nrf2* gene expression, whereas no changes were observed in the transcriptional activities of antioxidant enzyme genes (*SOD1*, *SOD2*, *CAT*, *GPx1*, *HO1*) in the brain of male CBA/H mice. In another work [37], it was shown that exposure of mice to prolonged normobaric hyperoxia (95% O_2 for 24 h) did not cause changes in the expression of the key antioxidant enzyme genes – *SOD1*, *SOD2*, *CAT*, *GPx1*, and some GST isoforms, whereas a significant increase was observed in the transcriptional activity of the genes of heme oxygenase-1 (*HO-1*) and *GPx 2,3* in lung tissue. Obviously, the expression level of genes regulating the state of the antioxidant system significantly depends on the rate of oxidative stress, type of the tissue, its metabolic status, interaction of signaling pathways, and many other factors. It seems that the decrease in *Nrf2*

expression after HBO can be an important cause of slight changes in the transcriptional activities of the antioxidant enzyme genes, which are Nrf2-dependent, and their expression is markedly controlled by this transcription factor.

The decrease in the *Nrf2* expression can lead to various negative consequences, such as increase in LPO and oxidative damage of nervous tissue components. We observed an increase in LPO intensity and accumulation of its molecular products in the brain after hyperoxia.

During the post-hyperoxia period, in the rat brain hemispheres there is a misbalance in the oxidative system components: slight changes in activity of *SOD*, inhibition of *CAT* activity, and moderate activation of GST. As a rule, the misbalance of antioxidant enzyme activities in brain and other tissues is expressed much more strongly immediately after exposure to hyperoxia than during the post-hyperoxia period [11, 38].

In the present work, significant increase in XOR activity and in the level of its product, uric acid, was observed in the brain under conditions of the HBO-induced oxidative stress, which could be associated with an increased production of proinflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor (TNF-α), which are effective inducers of XOR, and their increased production was shown after hyperoxia [39, 40]. Normalization of XOR activity in the brain of animals after HBO-induced oxidative stress on the background of SkQ1 administration is probably associated with stimulation of *Nrf2*, which could inhibit inflammation and production of proinflammatory cytokines [41].

The administration of SkQ1 for five days before exposure to hyperoxia led to an increase in transcriptional activity of the *Nrf2* gene and of Nrf2-controlled genes (*SOD1*, *SOD2*, *CAT*, *GPx4*) in the hemisphere cortex of rat brain, and this correlated with the activation of SOD, CAT, GPx, and GST and with increase in reduced glutathione level. This significantly enhanced the antioxidant potential of the brain during the post-hyperoxia period and promoted the stationary level of LPO, which confirmed the efficiency of SkQ1 administration under conditions of hyperoxia-induced oxidative stress.

However, it should be noted that under hyperoxic conditions, SkQ1 could act on the brain also indirectly. Studies by Zorov et al. [42, 43] revealed a stimulating effect of SkQR1 on the production of erythropoietin in kidneys [42, 43]. The neuroprotective effect of SkQR1 under cerebral ischemia associated with the development of oxidative stress was mediated through secretion of erythropoietin by the kidneys [43]. These results are especially interesting because the injection of recombinant erythropoietin under hyperoxia displayed a protective effect due to decrease in oxidative stress markers in rat brain [44]. Moreover, in *in vitro* experiments on cortical neurons on the background of sevoflurane-induced neurotoxicity, administration of erythropoietin promoted an increase in the Nrf2/Bach1 ratio in the nucleus due to activation of the erythropoietin receptor and of the Erk1/2 signaling pathway [45].

On consideration of the protective effect of SkQ1 in the brain, there is the problem of its penetration across the blood–brain barrier. A work using a fluorescent derivative of SkQ1 (SkQR1) [46] revealed the accumulation of this compound in mitochondria of the brain cortex and in its other structures when OXYS rats were given SkQR1 *per os* for 7-14 days.

Thus, the administration of SkQ1 at the dose 50 nmol/kg for five days under physiological conditions and before exposure to HBO stimulates the Keap1/Nrf2/ARE defense signaling system through the stimulation of expression of transcription factor gene *Nrf2* and of *Nrf2*-dependent genes of antioxidant enzymes and the increase in their activities.

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