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Intracellular Location and Function of Nuclear Factor of Erythroid Origin 2 (Nrf2) in Modeling Oxidative Stress *in vitro*

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ABSTRACT

INTRODUCTION: Nuclear factor E2-related factor 2 (Nrf2) is a member of cap'n'collar (CNC) family of subfamily of leucine zipper transcription factors that regulates cell protection against toxic substances and oxidants.

AIM: To determine location, mechanism of activation and role of Nrf2 in conditions of oxidative stress *in vitro*.

MATERIALS AND METHODS: The study was performed on human colon adenocarcinoma cell line (*Caco-2*). Oxidative stress (OS) was modeled by adding hydrogen peroxide (H_2O_2) at concentrations of 0.1 μM–100 μM to the nutritive medium and incubation for 24 and 72 hours. In assessment of Nrf2 function, its inhibitor — AEM1 — was added to cells at a concentration of 5 μM. The extent of OS development was determined using photometric methods by the concentration of protein SH-groups and carbonyl derivatives of protein, and the activity of superoxide dismutase (SOD). Viability of cells was assessed by the results of cytotoxic test (MTT assay), the amount of Nrf2 in the cytoplasm and nucleus was determined by heterogenous ELISA method.

RESULTS: Incubation of *Caco-2* cells with H_2O_2 resulted in decrease in the level of protein SH-groups and increase in the concentration of carbonyl derivatives of protein. In incubation with H_2O_2 at concentrations of 0.1 μM–10 μM for 24 hours and 10 μM for 72 hours, the activity of SOD increased. At concentrations of H_2O_2 of 50 μM and 100 μM (24 hour and 72 hour), SOD activity and viability of cells decreased. Exposure to H_2O_2 led to translocation of Nrf2 from the cytoplasm into nucleus. Direct correlation dependence was revealed between concentration of protein SH-groups and the amount of Nrf2 in the cytoplasm in incubation with H_2O_2 for 24 hour ($r = 0.44$, $p = 0.03$), 72 hour ($r = 0.34$, $p = 0.05$). The amount of Nrf2 in the nucleus positively correlated with SOD activity in the cytoplasm on exposure to H_2O_2 for 24 hour ($r = 0.77$, $p = 0.0001$) and 72 hour ($r = 0.36$, $p = 0.06$). In inhibition of Nrf2 in conditions of exposure to H_2O_2, the viability of cells decreased to a larger extent.

CONCLUSION: Hydrogen peroxide induces the nuclear translocation of Nrf2, which promotes activation of antioxidant enzyme SOD and preserves viability of cells of OS conditions *in vitro*.

Keywords: *nuclear factor E2-related factor 2 (Nrf2); superoxide dismutase; oxidative stress; Caco-2 line cells*

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ECO•VECTOR

LIST OF ABBREVIATIONS

ROS — reactive oxygen species
DTNB — 5,5′-dithiobis-(2-nitro)-benzoic acid
MTT — 3-(4,5-dimehylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
OD — optical density
OS — oxidative stress
SOD — superoxide dismutase
ARE — antioxidant respons(iv)e element
Cul3 — cullin 3
keap1 — kelch-like ECH associated protein 1
Maf — musculoaponeurotic fibrosarcoma
Nrf2 — nuclear factor E2-related factor 2
Ub — ubiquitin
AEM1 — antioxidant respons(iv)e element expression modulator

INTRODUCTION

Reactive oxygen species (ROS) are continuously produced in an organism as a result of intracellular metabolism or exogenous action of pro-oxidants. ROS produced in response to physiological changes, act as important signaling molecules regulating such processes as *cell division, inflammation, immune response, autophagy and response to stress* [1]. Overproduction of free radicals accompanied by reduction of antioxidant activity of a cell, leads to oxidative stress (OS) which impairs cell functions and contributes to development of many pathologies [2].

The mechanism of signaling action of ROS is realized through activation of transcription factors which, binding with gene promoters, trigger expression of a number of proteins [1].

The nuclear factor E2-related factor 2 (Nrf2) is a member of cap'n'collar (CNC) family of leucine zipper transcription factors (bZIP) [3]. Nrf2 is a transcription factor that regulates defense of cells against toxic substances and oxidizers [4].

Nrf2 is proven to stimulate induction of enzymes (glutathione S-transferase and NAD(P)H-dehydrogenase, oxidoreductase and others) that participate in metabolism of xenobiotics, which leads to biotransformation and excretion of exogenous and endogenous chemical substances [5]. In this case, Nrf2 functions as xenobiotic-activated receptor (XAR) [4]. Nevertheless, the basic function of Nrf2 is providing resistance of cells to OS.

It is known that Nrf2 knockout in mice increased their sensitivity to a wide spectrum of chemicals and OS-associated pathologies [6]. Increase in the activity of Nrf2 on exposure to chemicals is noted to protect animals against OS [7, 8].

Thus, inducing expression of antioxidant enzymatic and signaling proteins, Nrf2 performs protective functions on exposure to toxic substances and in pathological conditions associated with OS, therefore, elucidation of protective mechanisms of Nrf2 is an important direction of studies, since it will permit to determine the ways of adaptation to OS and to avoid the negative effect of ROS.

The **aim** was to determine location, mechanism of activation and the role of Nrf2 in conditions of oxidative stress in vitro.

MATERIALS AND METHODS

The study was performed on human colon adenocarcinoma cell line (Caco-2, Common Use Center "Collection of Vertebrate Cell Cultures", Saint-Petersburg, Russia). Cells were cultured at 37°C with 5% concentration of CO_2 in WS-189C incubator (World Science, Korea) in Dulbecco's Modified Eagle's Medium (DMEM) with high concentration of glucose (4,500 mg/l) with addition of L-glutamine (4 mM), 15% embryonic bovine serum, 100 U/ml and 100 μg/ml penicillin and streptomycin, respectively (all components of Sigma-Aldrich manufacture, Germany). The cells were cultured for 21 days, since in this period they spontaneously differentiate to enterocyte-like cells [9].

OS was modeled by addition of hydrogen peroxide (H_2O_2, Sigma-Aldrich, Germany) at concentrations of 0.1, 0.5, 1, 10, 50, 100 μM with incubation for 24 and 72 hours. To the control cells, water for injections (H_2O_2 solvent) was added in equivalent quantities.

To evaluate participation of Nrf2 in adaptation mechanisms of cell protection in the conditions of OS, 30 minutes before addition of H_2O_2, inhibitor of Nrf2 — N-(1,3-benzodioxol-5-ilmethyl)-5-(4-fluorophenyl)-thieno[2,3-d]pyrimidine-4-amine (antioxidant respons(iv)e element expression modulator, AEM1, Sigma-Aldrich, Germany) was introduced in the nutrient medium at a concentration of 5 μM [10].

To study the viability of cells, they were inoculated in a 96-well plate (Corning, USA); to study the influence of H_2O_2 on the quantity of Nrf2, concentration of protein SH-groups and carbonyl protein derivatives, activity of Cu, Zn-superoxide dismutase (SOD), the cells were inoculated in 6-well plates (Corning, USA).

Cytotoxic test (MTT test). The cells were inoculated in a 96-well plate at the rate of 104 cells per well and cultured for 21 days, then the nutrient medium with

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H_2O_2 was added. After termination of incubation, 20 µl of 0.5% 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium (MTT) bromide solution were added into each well and incubated for 2 hours, then the MTT solution was removed and 200 µl of 1% dimethyl sulfoxide solution were added (PanEco, Russian Federation) [11]. Absorption was measured after 10 min at 530 nm on Stat Fax 2100 spectrophotometer for plates (Awareness Technology, USA).

Viability of Caco-2 cells in the presence of H_2O_2 was calculated from the formula:

$$\text{Viability} = \frac{\text{OD of experimental wells} - \text{OD of medium}}{\text{OD of control wells} - \text{OD of medium}} \times 100\%$$

where OD — optical density.

Preparation of cytoplasmic and nuclear fractions of cell lysates for analysis. The cells were inoculated in 6-well plates on the basis of 10^5 cells per each well, and cultured for 21 days, then cultural medium with H_2O_2 was added. After termination of exposure, cells were removed from the wells with trypsin-EDTA solution (0.25% trypsin and 0.2% EDTA, Sigma-Aldrich, Germany). The cells in the quantity of 1×10^6 were three times washed with pH 7.4 phosphate buffer (PanEko, Russian Federation) and separated to cytoplasmic and nuclear fractions using commercial Protein extraction kit (cytoplasmic/nuclear), BioRad, USA). The obtained lysates were used for quantitative determination of Nfr2 in the cytoplasm and nucleus.

Quantitative analysis of Nrf2 content in Caco-2 line cells. In lysate of cells, the amount of Nrf2 was determined by heterogenous immunoenzyme assay with a commercial kit (Human Nuclear factor erythroid 2-related factor 2 ELISA Kit, Bluegene, China). Light absorption was measured at 450 nm on Stat Fax 2100 (Awareness Technology, USA) immunoenzyme plate analyzer.

Preparation of total cell lysates for determination of the activity of SOD, concentration of carbonyl protein derivatives and protein SH-groups. After exposure to H_2O_2, cells were removed from 6-well plates with trypsin-EDTA solution (0.25% trypsin and 0.2% EDTA, Sigma-Aldrich, Germany), three times washed with pH 7.4 phosphate buffer (PanEko, Russian Federation) and lyzed with ice buffer for lysis containing tris-HCl (pH 7.4) 50 mM, KCl 150 mM, 0.5% triton and inhibitors of proteinases (4-(2 aminoethyl benzenesulfonyl florida hydrochloride (AEBSF) 2 mM, aprotinin 0.3 µM, bestatin 130 µM, EDTA 1 mM, trans-epoxysuccinyl-L-leucylamido(-guanidino)butane (E-64) 14 µM, leupeptin 1 µM, Sigma-Aldrich, Germany), and were shaken on a shaker. The cells were incubated on ice for 10 minutes, after which they were centrifuged for 10 minutes at 5,000 g (CM-50, Eppendorf, Germany) for sedimentation of the nuclei. Supernatant was used for biochemical analyses.

The ***method for determination of carbonyl protein derivatives*** was based on their interaction with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazones, which were registered at 375 nm wavelength. The concentration of carbonyl protein derivatives was calculated based on the extinction coefficient ε_{375} = 22 mM^{-1} cm^{-1} [12]. The analysis was performed on Stat Fax 2100 immunoenzyme plate analyzer (Awareness Technology, USA).

Determination of the concentration of protein SH-groups. The concentration of protein thiol groups was determined by the difference between the level of the total and low molecular weight SH-groups. The content of total SH-groups in the cell lysate was analyzed by Ellman method with 5.5'-di-thiobis(2-nitro)-benzoic acid (DTNB) [13]. To 100 µl of the sample, 100 µl of 2 mM DTNB (Serva, Germany) in 1 M tris-HCl buffer (pH 8.0) and 1000 µl of distilled water were added. After 30-minute exposure, the content of 5-thio-2-nitro-benzoic acid was assessed at 412 nm on Stat Fax 2100 (Awareness Technology, USA). The concentration of SH-groups was calculated based on the extinction coefficient ε_{412} = 13.6 $mM^{-1}cm^{-1}$ [14]. To determine the content of low molecular weight SH-groups, the sample was pre-mixed with cooled 5% trichloroacetic acid (Khimmed, Russian Federation), incubated on ice for 15 minutes, then centrifuged at 11,000 g (CM450, Eppendorf, Germany) for 5 minutes at 4°C. The resulting supernatant was neutralized with 1% NaOH (Khimmed, Russian Federation) and used to determine low molecular weight SH-groups by reaction with DTNB.

SOD activity was evaluated by the degree of inhibition of quercetin oxidation reaction determined by a change of the optical density of reaction mixture at λ = 406 nm. Percent of inhibition of quercetin oxidation within 3 minutes was found by the formula:

$$\% \text{ of inhibition} = \Delta D_x - \Delta D_0 / \Delta D_x \times 100\%,$$

where ΔDx — change in the optical density at 406 nm within 3 min in the control sample without SOD; ΔD0 — change in the optical density at 406 nm within 3 min in the control sample containing SOD [15].

For one conventional (standard) unit (U) of activity, 50 % inhibition was taken. The enzyme activity was expressed in U/mg of protein.

The amount of protein in samples was analyzed by Bradford method (Pierce Coomassie Plus (Bradford) Assay Kit, ThermoFisher, USA).

The obtained results were analyzed using Statistica 13.0 (Stat Soft Inc., USA) and Excel (Microsoft, USA) programs. The results are presented in the form of mean (M) and standard deviation (SD). To assess the statistical significance of the differences, analysis of variance (ANOVA) was used, pairwise comparisons

were performed using Newman–Keuls test. Correlation analysis was performed using Pearson test. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Evaluation of the extent of oxidative stress and antioxidant protection on exposure to hydrogen peroxide at concentrations of 0.1–100 μM in Caco-2 line cells in vitro. The extent of development of OS was evaluated by the concentration of protein SH-groups and the level of carbonyl derivatives of proteins, and the extent of antioxidant protection — by SOD activity.

In incubation of Caco-2 line cells for 24 hours, the content of protein SH-groups in cells showed a statistically significant reduction on exposure to H_2O_2 over the whole range of studied concentrations, relative to the control values.

0.1 μM — by 46.6% ($p = 0.0003$),
0.5 μM — by 53.0% ($p = 0.0002$),
1 μM — by 62.6% ($p = 0.0002$),
5 μM — by 51.3% ($p = 0.0002$),
10 μM — by 41.4% ($p = 0.0002$),
50 μM — by 27.2% ($p = 0.0036$),
100 μM — by 36.4% ($p = 0.0009$, Figure 1a).

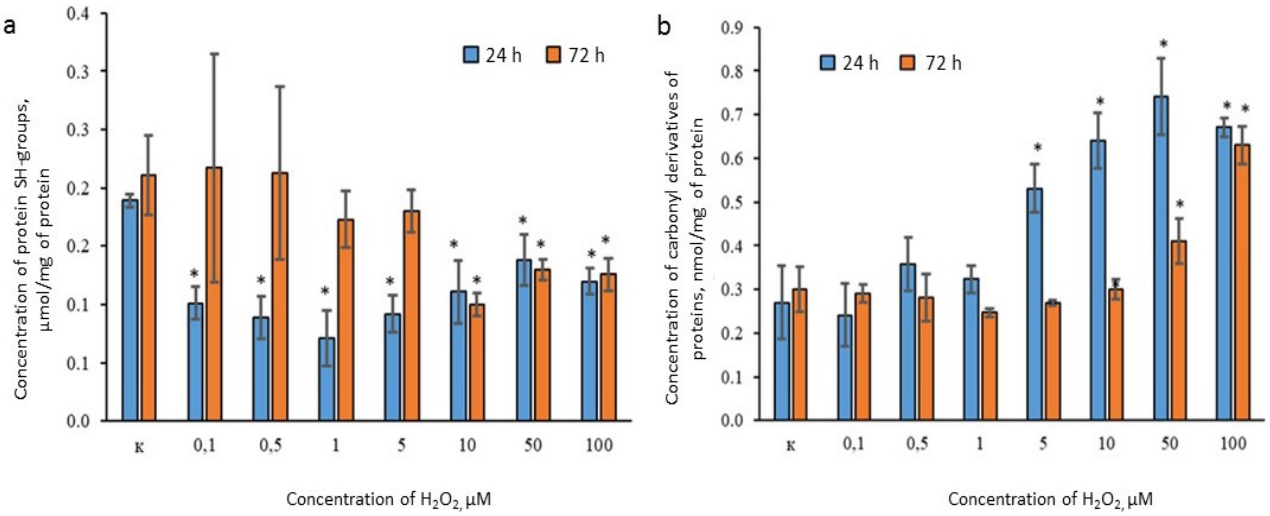

Fig. 1. Evaluation of development of oxidative stress in Caco-2 line cells on exposure to hydrogen peroxide at concentrations of 0.1–100 μM for 24 and 72 hours: concentration of SH-groups (a), concentration of carbonyl derivatives of proteins (b).
Note: * statistically significant differences from control, $p < 0.05$ (Newman–Keuls test).

Increase in the time of incubation to 72 hours caused statistically significant reduction of the content of protein SH-groups in cells on exposure to H_2O_2 at concentrations of 10, 50 and 100 μM by 52.4% ($p = 0.02$), 38.1% ($p = 0.02$) and 40.4% ($p = 0.02$), respectively, in comparison with the data of control groups (Figure 1a).

Incubation of cells of Caco-2 line for 24 hours resulted in significant increase in the level of carbonyl derivatives of proteins on exposure to H_2O_2 at concentrations of 5, 10, 50 and 100 μM in comparison with the norm by 96.3% ($p = 0.03$), 137.1% ($p = 0.003$), 174.1% ($p = 0.001$) and 148.1% ($p = 0.003$), respectively (Figure 1b).

On exposure to H_2O_2 at a concentration of 50 μM for 72 hours, the level of carbonyl derivatives of proteins considerably increased by 36.7% ($p = 0.001$), and at a concentration of 100 μM — by 110% ($p = 0.001$) as compared to control. At the rest of the concentrations, the pro-oxidant did not produce any statistically significant influence on the studied parameter (Figure 1b).

On exposure to H_2O_2 at concentrations of 0.1; 0.5; 1; 5 and 10 μM for 24 hours, the activity of SOD increased by 216.1% ($p = 0.0002$), 187.4% ($p = 0.0002$), 206.7% ($p = 0.0002$), 86.9% ($p = 0.03$) and 78.9% ($p = 0.03$), respectively, relative to the values of the control group (Figure 2).

Exposure to H_2O_2 in the range of concentrations of 0.1–5 μM for 72 hours did not produce any effect on the activity of SOD, and at a concentration of 10 μM led to increase in the activity of SOD by 111.8% ($p = 0.006$) as compared to the control group (Figure 2). At concentrations of H_2O_2 of 50 and 100 μM and the incubation time 24 and 72 hours, a tendency to reduction of the activity of SOD was noted.

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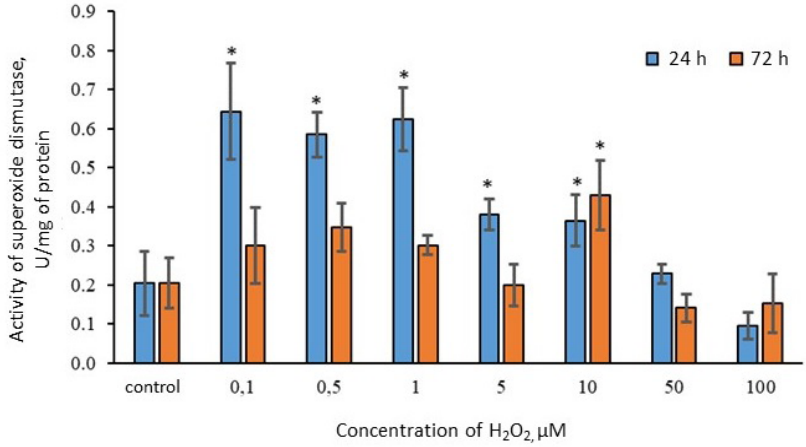

Fig. 2. Activity of superoxide dismutase in cells of Caco-2 line on exposure to hydrogen peroxide at concentrations of 0.1–100 μM within 24 and 72 hours.
Note: * statistically significant differences from control, $p < 0.05$ (Newman-Keuls test).

Quantitative evaluation of the nuclear factor of erythroid 2-related factor (Nrf2) in cells of Caco-2 line in vitro on exposure to H_2O_2 at concentrations of 0.1–100 μM. On exposure to H_2O_2 at concentrations of 0.1; 0.5 and 1 μM on cells of Caco-2 line for 24 hours, the amount of Nrf2 in the cytoplasm decreased by 56.5% ($p = 0.03$), 48.6% ($p = 0.02$) and 51.7% ($p = 0.03$) and increased in the nucleus by 55.2% ($p = 0.003$), 38.1% ($p = 0.001$) and 37.0% ($p = 0.001$), respectively, relative to the control group (Figure 3a). At a concentration of H_2O_2 of 100 μM, the amount of Nrf2 in the cytoplasm did not change and in the nucleus decreased by 80.6% ($p = 0.001$).

In incubation of cells with H_2O_2 at concentrations of 10 and 50 μM for 72 hours, the amount of Nrf2 decreased by 58.8% ($p = 0.009$) and 64.7% ($p = 0.009$), respectively, in the cytoplasm and increased by 47.7% ($p = 0.003$) and 25.8% ($p = 0.05$), respectively, in the nucleus. Exposure to H_2O_2 at a concentration of 100 μM for 72 hours caused decrease in the level of Nrf2 in the cytoplasm by 55.6% ($p = 0.005$) and by 85.1% ($p = 0.0002$) in comparison with the control group (Figure 3b).

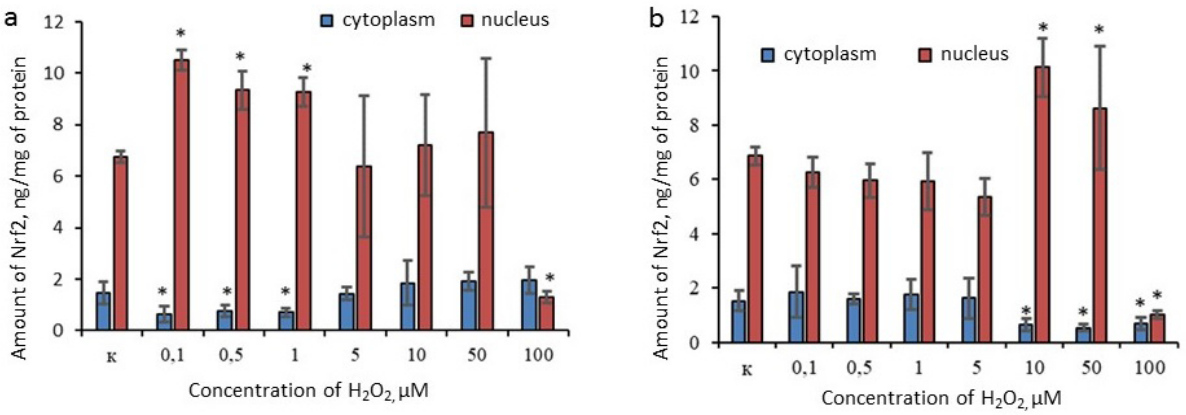

Fig. 3. The amount of nuclear factor erythroid E2-related factor 2 (Nrf2) in the cytoplasm and nucleus of cells of Caco-2 line *in vitro* on exposure to hydrogen peroxide at concentrations of 0.1–100 μM within 24 (a) and 72 hours (b).
Note: c — control; * — statistically significant differences from control, $p < 0.05$ (Newman-Keuls test).

The correlation analysis revealed a direct relationship between the concentration of protein SH-groups and the amount of Nrf2 in the cytoplasm of cells in their incubation with H_2O_2 for 24 hours ($r = 0.44$; $p = 0.03$), 72 hours ($r = 0.34$; $p = 0.05$). In turn, the amount of Nrf2 in the nucleus positively correlated with the activity of SOD in cells on exposure to H_2O_2 for 24 hours ($r = 0.77$; $p = 0.0001$) and 72 hours ($r = 0.36$; $p = 0.06$).

Protective function of Nrf2 was evaluated by the viability of cells of Caco-2 line on exposure to H_2O_2 and to H_2O_2 in the presence of Nrf2 inhibitor — AEM1.

Incubation of cells with H_2O_2 at concentrations of 50 μM and 100 μM for 24 and 72 hours led to statistically significant reduction of cell viability relative to the control by 45.9% (p = 0.001) and 41.2% (p = 0.02), by 73.2% (p = 0.0003) and by 65.7% (p = 0.002), respectively (Figure 4).

In introduction of Nrf2 inhibitor (AEM1, 5 μM) in the nutrient medium 30 minutes before incubation with pro-oxidant, the viability of cells showed statistically significant reduction at H_2O_2 concentrations of 0.1–10 μM (incubation for 24 hours) and 0.5–10 μM (incubation for 72 hours) relative to the viability of cells incubated with only H_2O_2, which indicates the protective role of Nrf2 in the conditions of OS (Figure 4).

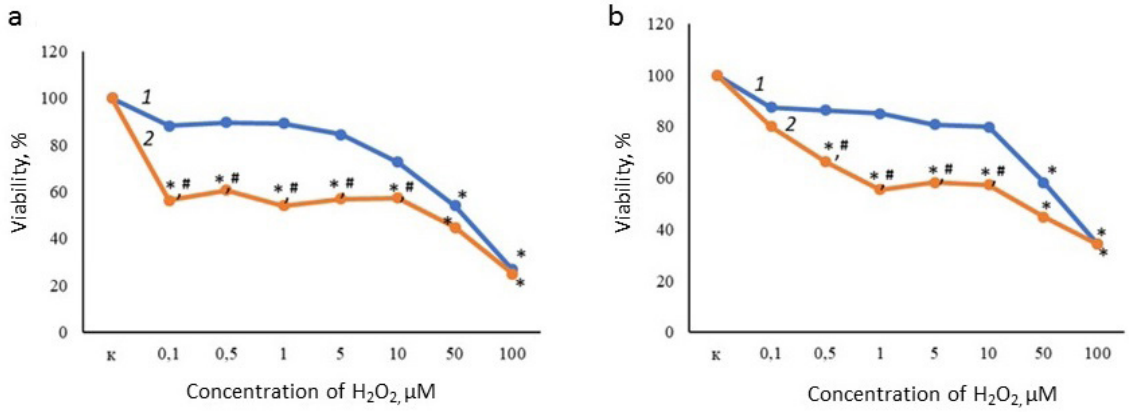

Fig. 4. Viability of cells of Caco-2 line on exposure to hydrogen peroxide at concentrations of 0.1–100 μM for 24 (a) and 72 hours (b) independently and with inhibition of Nrf2 synthesis (2).
Note: c — control; * — statistically significant differences from control, p < 0.05 (Newman–Keuls test); # — cstatistically significant differences from H2O2 group, p < 0.05 (Newman–Keuls test).

DISCUSSION

Overproduction of ROS with reduced capacitance of antioxidant cell defense leads to damage to macromolecules (proteins, lipids, nucleic acids) [16, 17]. Most sensitive to action of pro-oxidants are cysteine residues in protein molecules. Reactive sulfhydryl (SH) groups are regulatory centers that are molecular switches of the activity of proteins [18].

Nrf2-redox-sensitive transcription factor reacts to the change in the ratio of reduced and oxidized SH-groups in proteins. Its expression increases with the development of OS and is aimed at protecting the cell from the effects of free radicals [19]. Under normal conditions, this transcription factor exists in complex with keap1 repressor protein, their binding is regulated by a number of protein kinases. On the one hand, keap1 promotes ubiquitination and proteosomal degradation of Nrf2 (a necessary condition for this process is the presence of two cysteine residues in keap1 molecule), and on the other hand, prevents its penetration into the nucleus from the cytoplasm (Figure 5) [20].

After activation, keap1-Nrf2 complex dissociates, and Nrf2 translocates into the nucleus where it binds with elements of the antioxidant response element (ARE) and activates transcription of protective enzymes [20].

The obtained results demonstrate that in development of OS induced by H_2O_2, keap1-Nrf2 dissociates due to oxidation of SH-group, and Nrf2 translocates into the nucleus (Figure 5), which is confirmed by correlation relationship of the studied parameters.

Antioxidant enzymes play an important role in maintaining redox homeostasis and protecting cells against damage and apoptosis, especially on exposure to pro-oxidants. Superoxide dismutase (EC 1.15.1.1) is a key enzyme of the antioxidant systems of all aerobic organisms that catalyzes the conversion of oxygen anion radical (O_2^-) into hydrogen peroxide and molecular oxygen.

Increase in the amount of Nrf2 in the nucleus leads to increase in the activity of SOD, which is confirmed by a positive correlation relationship under action of H_2O_2 (Figure 5).

On exposure to pro-oxidant for 72 hours, no statistically significant correlation relationship between the activity of SOD and the amount of Nrf2 in the nucleus was found. Probably, on prolonged exposure to H_2O_2 (72 hours), the protective mechanism of Nrf2 is realized not only through activation of SOD, but also through other antioxidant enzymes in which induction Nrf2 participates.

The described cascade of biochemical processes is of importance in maintenance of the viability of cells in

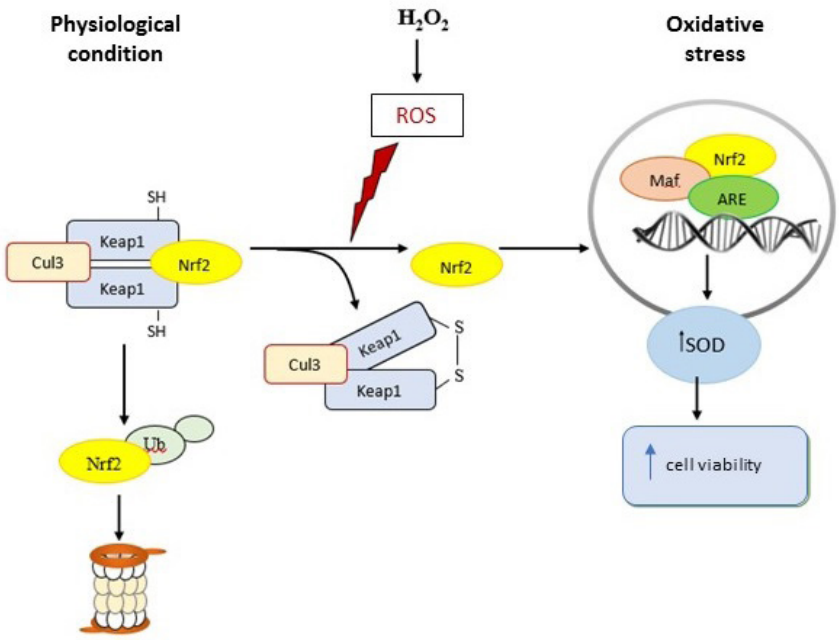

Fig. 5. Mechanism of protective effect of Nrf2 erythroid transcription factor in oxidative stress induced by hydrogen peroxide at concentrations of 0.1–100 μM in vitro.
Notes: ROS — reactive oxygen species; ARE — antioxidant response element ; Cul3 — cullin 3 protein; keap1 — kelch-like ECH associated protein 1; Maf — musculoaponeurotic fibrosarcoma; Nrf2 — nuclear factor E2-related factor 2; SOD — superoxide dismutase; Ub — ubiquitin protein.

OS conditions. On exposure of Caco-2 line cells to H_2O_2 at concentrations of 0.1–10 μM for 24 hours and of 0.5–10 μM for 72 hours, the cell viability was maintained due to transcription factor Nrf2, which is confirmed by a significant reduction of percent of cells that survived in conditions of Nrf2 inhibition by AEM1.

CONCLUSION

In a certain range of concentrations and duration of exposure, hydrogen peroxide induces nuclear translocation of Nrf2, which promotes activation of antioxidant SOD enzyme and preserves viability of Caco-2 line cells in OS *in vitro.*

ADDITIONAL INFORMATION

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Conflict of interests. The authors declare no conflicts of interests.
Contribution of the authors: *Yu. V. Abalenikhina* — conducting the main stages of the experiment, analyzing and interpreting data, writing an article; *P. D. Erokhina* — cell cultivation and conducting the main stages of the experiment; *A. A. Seidkuliyeva* — cell cultivation and biochemical analyses; *O. A. Zav'yalova* — conducting biochemical analyses; *A. V. Shchul'kin* — concept and design development, verification of critical intellectual content, final approval for publication of the manuscript; *E. N. Yakusheva* — data analysis and interpretation, verification of critical intellectual content. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.
Вклад авторов: *Абаленихина Ю. В.* — проведение основных этапов эксперимента, анализ и интерпретация данных, написание статьи; *Ерохина П. Д.* — культивирование клеток и проведение основных этапов эксперимента; *Сеидкулиева А. А.* — культивирование клеток и проведение биохимических анализов; *Завьялова О. А.* — проведение биохимических анализов; *Щулькин А. В.* — разработка концепции и дизайна, проверка критически важного интеллектуального содержания, окончательное утверждение для публикации рукописи; *Якушева Е. Н.* — анализ и интерпретация данных, проверка критически важного интеллектуального содержания. Авторы подтверждают соответствие своего авторства международным критериям ICMJE (все авторы внесли существенный вклад в разработку концепции и подготовку статьи, прочли и одобрили финальную версию перед публикацией).

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