

Stress, Aging and Reliability of Antioxidant Enzyme Defense

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Abstract: Clinical and experimental data point to existence of disturbances of adaptive ability of aged organism to extreme impacts. However mechanisms of these disturbances are not clear yet.

The purpose of the investigation was to study age-related changes in reaction of erythrocyte antioxidant enzyme system in response to acute psycho-emotional stress and a possible role of these changes in age-related alterations of oxygen blood transport in nonhuman primates.

Ten young (6-8 years) and ten old (20-26 years) healthy female rhesus monkeys were subjected to acute moderate psycho-emotional stress (two hours squeeze cage restraint) at 1500h. Plasma cortisol, lipid peroxidation products (TBARS) and activities of superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase (GR), and glutathione-S-transferase in erythrocytes were measured before stress and at 30, 60, 120, 240 min and 24 hours after beginning of the stress.

We have found for the first time that SOD activity decreased in response to the stress in young monkeys while it increased in the half of old monkeys. Young animals also demonstrated essentially higher increase in GR activity and plasma cortisol level in response to the restraint in comparison with old monkeys. Level of TBARS did not practically change in response to the stress in young animals and significantly increased in old monkeys.

The study demonstrated that the age-related alterations in SOD and GR stress responsiveness lead to activation of peroxide oxidation of lipids that may be considered as an important factor of aging damage of erythrocyte functioning and reliability of oxygen transport to tissues under stress conditions.

Keywords: Psycho-emotional stress, erythrocyte antioxidant enzymes, cortisol, aging, monkeys.

Some clinical and experimental data point to existence of disturbances of adaptive ability of aged organism to extreme impacts. So, level of morbidity and death rate among the persons of the old age who have undergone surgical interventions are considerably higher in comparison with young subjects [1, 2]. Among old subjects neuropsychic diseases, such as depression and anxiety with the etiology significantly conditioned by stress are much more often widespread [3, 4]. However the mechanisms of these disturbances are not clear yet. The leading role in this phenomenon is attributed to age-related alterations in functioning of the hypothalamic-pituitary-adrenal (HPA) axis [4, 5, 6] and immune system [7, 8]. Actually there are no data on character of functioning of antioxidant enzyme system of erythrocytes in response to stress and features of its reaction in old age. At the same time age-related changes in reliability of an antioxidant system defense with the subsequent changes in reactive oxygen species (ROS) level and intensity of peroxide oxidation of lipids in erythrocytes could have important pathophysiological consequences in the form of age-related disturbances in mobility of erythrocytes and transport of oxygen under the conditions of extreme impacts.

The purpose of this work was to study age-related changes in reaction of erythrocyte antioxidant enzyme system in response to acute psycho-emotional stress and

possible role of these changes in age-related alterations of oxygen blood transport in monkey model (female rhesus monkeys). We have found for the first time that SOD activity decreased in response to the stress in young monkeys while it increased in the half of old monkeys. Young animals also demonstrated essentially higher increase in GR activity in response to the restraint in comparison with old monkeys. We have also found that the age-related alterations in stress responsiveness of SOD and GR lead to increase of products of peroxide oxidation of lipids (TBARS) in erythrocytes of old animals that may be an important factor of aging damage of erythrocyte functioning and reliability of oxygen transport to tissues, which are experiencing increased oxygen demand in the face of a psycho-emotional stress.

Ten young adult (6-8 years) and ten old (22-26 years) healthy female rhesus monkeys were used in the experiments. The animals were kept in open enclosures or cages designed for group housing in the monkey colony of the Institute of Medical Primatology, Sochi-Adler, Russia. During the observation period they were kept in individual cages under conditions of controlled temperature (20-25°C) and controlled illumination (from 0600h to 1800h). Body weight of the animals ranged from 4.4 to 6.8 kg. The mean body weight of the young group was 5.10 ± 0.28 kg and of the old group 5.30 ± 0.30 kg. All experiments were carried out in the period June-September when ovarian cycles are not typical for female rhesus monkeys. The animals were fed pellets prepared by the Institute according to the technique of the firm "Altromin" (Lage, Germany). The pellet diet was complemented with bread, boiled eggs, and fresh vegetables and

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fruit. Water was available *ad libitum*. Before the experiments, the animals were adapted to living in metabolic cages and to the procedure of bleeding for at least four weeks.

Animals of both age groups underwent an acute stress procedure: moderate restraint in a metabolic cage for two hours. Restraint was achieved by using a conventional

squeeze board to press the animal to the front wall of the metabolic cage. The body and extremities of the animal were not tightly immobilized. Animals were subjected to the stressor at 1500h. The animals were fasted overnight. Blood samples were taken before immobilization (0), at 30, 60, and 120 min during application of the stressor, and at 240 min, i.e., two hours after termination of the stressor. Besides

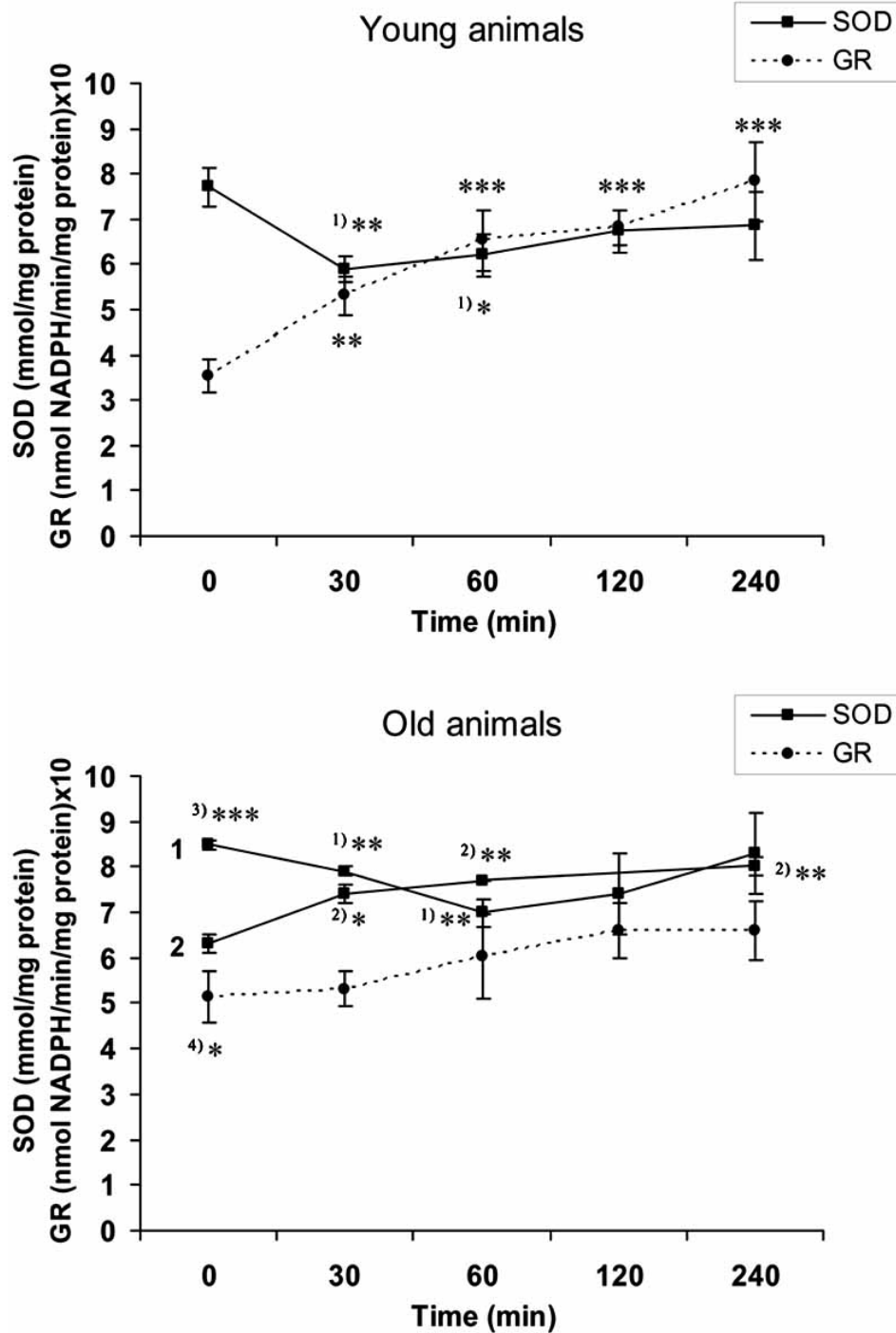


Fig. (1). Dynamics of erythrocyte GR and SOD activities in young (n = 10) and old (n = 10) rhesus monkey females in response to the stress imposed at 1500h (mean ± SEM).

P < 0.01, *P < 0.001 – vs. the values for GR before stress (0 min); ¹*P < 0.05, ¹**P < 0.01 – vs. the values for SOD before stress (0 min); ²*P < 0.05, ²**P < 0.01 – vs. the values for SOD in 2nd subgroup before stress; ³***P < 0.001 – vs. the relative values for SOD in 2nd subgroup; ⁴* P < 0.05 – age-related differences for GR.

blood samples were taken at 24 hours after onset of immobilization. At each time point 2.5-3.0 ml of blood was taken from the cubital or femoral vein with heparin as anticoagulant and immediately centrifuged at 2000G at +4°C. Plasma and erythrocytes were separated and stored at -70°C. The erythrocytes were hemolyzed and the hemolysates were immediately used for measurement of activities of superoxide dismutase (EC 1.15.1.1, SOD, Cu, Zn-SOD), glutathione peroxidase (EC 1.11.1.9, GSH-Px), glutathione reductase (EC 1.6.4.2, GR), and glutathione transferase (EC 2.5.1.18, GST). The SOD activity was measured using the modified polarographic method of catalytic currents [9]. The recording of polarographic oxygen waves was carried out at room temperature in 0.1 M sodium borate buffer, pH 9.8, using the polarographer PU-1 (Russia). It has 3 electrodes. The working electrode was the dropping mercury electrode and the mercury layer at the bottom of the device has served as an auxiliary electrode. A chlor-silver electrode was the third one. The calibration was performed with use of superoxide dismutase solution (erythrocyte, Serva). The activity of SOD was expressed in mmol enzyme per mg of total erythrocyte protein.

The activity of GSH-Px was measured by kinetic spectrophotometry on the basis of rate of oxidation of reduced glutathione by tertiary-hydroxybutyl during 10 min incubation at 37°C. The rest of reduced glutathione was measured with Ellmann' reagent. The activity of GSH-Px was expressed as mmol of reduced glutathione (GSH) per min per mg of total erythrocyte protein [10]. The activity of GR was measured by kinetic spectrophotometry on the basis of rate of oxidation of NADPH during 5 min incubation at 37°C and expressed as nmol NADPH per min per mg of total erythrocyte protein [10]. The activity of GST was measured by kinetic spectrophotometry on the basis of rate of formation of glutathione conjugate with c 1-chlor-2, 4-dinitrobenzol (GSDNB) during 3 min incubation at 37°C and expressed as μmol glutathione conjugate with GSDNB per min per mg of total erythrocyte protein [10].

Erythrocytes were assayed for lipid peroxidation products as thiobarbituric acid-reacting substances (TBARS) by spectrophotometry [11].

The plasma samples were used for measurements of cortisol (F) by immunoenzyme assay using standard hormone

kits (Alkor Biotechnologies, Inc., St. Petersburg, Russia). The intra- and interassay variation coefficients for cortisol did not exceed 10%.

The experimental values are presented in the tables and figures as means ± SEM. Statistical tests for circadian and age effects were performed using one and two-way analysis of variance (ANOVA) followed by post hoc significant difference (HSD) test for paired comparisons [12].

RESULTS

Activity of Antioxidant Enzymes and of the Adrenal Cortex and Dynamics of Lipid Peroxidation Products in Young Rhesus Monkey Females Under Conditions of Acute Moderate Psycho-Emotional Stress

In our studies the greatest changes in response to stress was detected with GR and SOD. In young animals, GR activity was significantly increased 30, 60, 120 and 240 min after stress exposure (Fig. 1). Unlike GR, activity SOD in response to stress influence decreased. The most considerable decrease in activity SOD was marked 30 min after the stress beginning, and then activity SOD gradually increased, coming nearer to initial values 240 min after stress exposure (Fig. 1). Note that in a number of young animals activity SOD and GR was measured in addition 5 and 15 min after the onset of stress. It has appeared that opposite changes in activity SOD and GR were observed in all experimental animals already 5 and 15 min after the beginning of stress (Table 1).

Activity of GR and SOD returned to initial baseline values 24 hours after stress (0.41 ± 0.04 versus 0.35 ± 0.04 nmol NADPH/min/mg protein before the stress for GR and 7.25 ± 0.20 versus 7.20 ± 0.60 mmol/mg protein before the stress for SOD).

The correlation between GR activity and corticosteroid concentrations was observed throughout the four hours of the studies (Fig. 2). The correlation coefficient was 0.78 ± 0.09 . In the first two hours the correlation coefficient was close to 1.00 ($r = 0.98 \pm 0.04$).

Activities of GSH-Px and GST did not change significantly in response to the stress (Table 2).

Table 1. Dynamics of Activities of SOD and GR in Erythrocytes and F Level in Peripheral Blood Plasma of Young (n = 3) Rhesus Monkey Females in Response to the Stress Imposed at 1500h (mean ± SEM)

Time, min	SOD, mmol/mg protein	GR, nmol NADPH/min/mg protein	F, nmol/l
0	8.37 ± 0.68	0.36 ± 0.09	760 ± 50
5	8.10 ± 0.60	0.32 ± 0.02	870 ± 110
15	7.70 ± 0.50	0.58 ± 0.15	950 ± 80
30	5.40 ± 0.27*	0.56 ± 0.06	1020 ± 40*
60	6.10 ± 0.03*	0.56 ± 0.02	1220 ± 90*
120	7.30 ± 0.10	0.74 ± 0.13	1380 ± 110**
240	7.90 ± 0.70	0.73 ± 0.11	890 ± 70

* $P < 0.05$, ** $P < 0.01$ – vs. the relative values before stress (0 min).

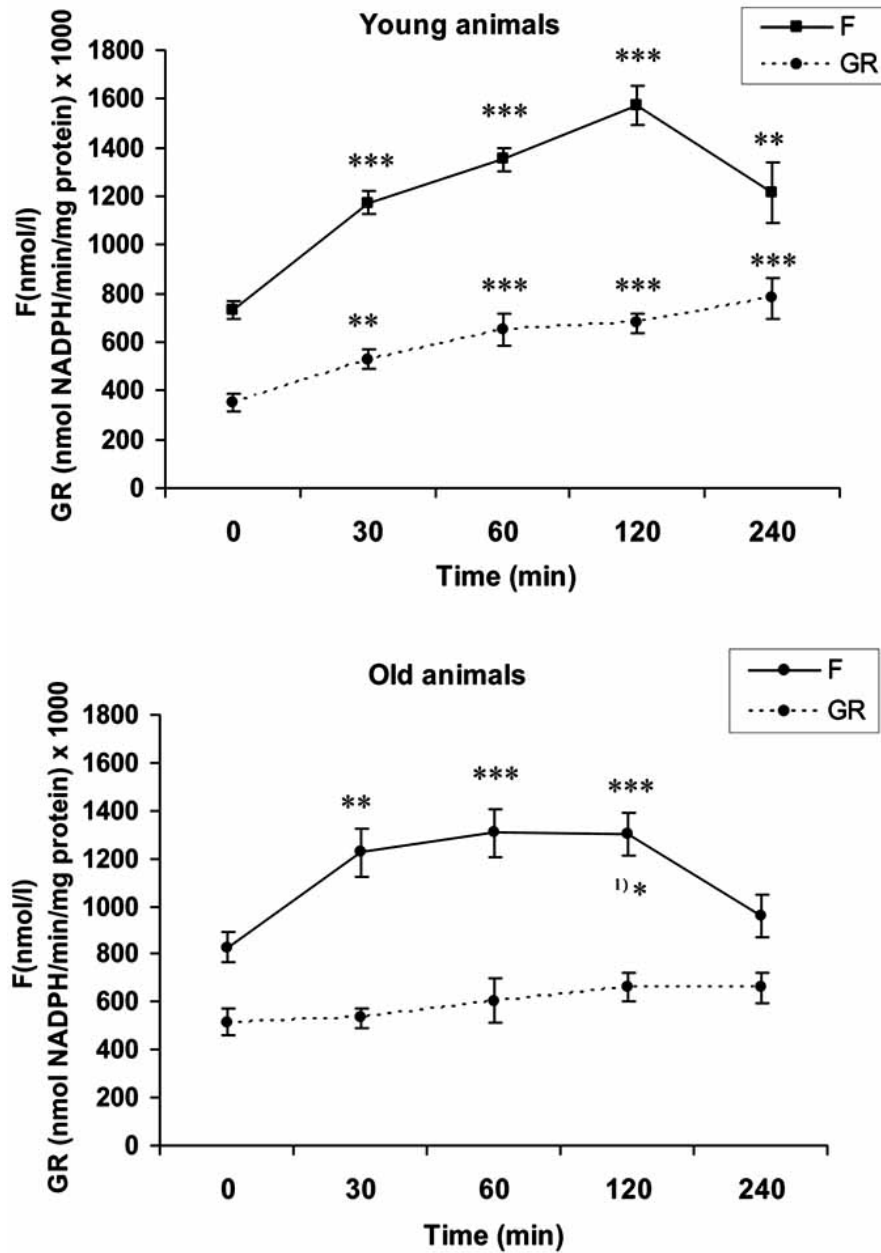


Fig. (2). Dynamics of erythrocyte GR activity and plasma F level in young (n = 10) and old (n = 10) rhesus monkey females in response to the stress imposed 1500h (mean ± SEM).

P < 0.01, *P < 0.001 – vs. the relative values before stress (0 min); ¹*P < 0.05 – age-related.

In young animals a wide variation was found between the initial levels of TBARS (0.141 – 0.348 nmol/mg protein) with no changes in response to acute stress effect. Dynamics of the average of TBARS values in erythrocytes of young rhesus monkeys in response to the stress is shown in Table 3.

Activity of Antioxidant Enzymes and of the Adrenal Cortex and Dynamics of Lipid Peroxidation Products in Old Rhesus Monkey Females Under Conditions of Acute Moderate Psycho-Emotional Stress

In old animals basal activity of GR was significantly higher in comparison with young animals (0.514 ± 0.056 versus 0.353 ± 0.036 nmol NADPH/min/mg protein in

young animals, $P < 0.05$) (Fig. 1). In response to acute stress GR activity in old animals, unlike young animals, showed only the tendency toward increase 30, 60, 120 and 240 min after beginning of the immobilization (Fig. 1). Therefore increase of GR activity expressed in percentage of initial baseline values in old animals was essentially lower 60, 120 and 240 min after the onset of restraint in comparison with young animals (Table 4).

By basal activity and character of SOD changes under stress conditions old animals could be grouped in 2 sub-groups. The first subgroup consisted of 5 animals in which basal SOD activity and its dynamics in response to stress were similar to those parameters in young animals (Fig. 1).

Table 2. Dynamics of Activities of Erythrocyte GSH-Px and GST in Young (n = 10) and Old (n = 10) Rhesus Monkey Females in Response to the Stress Imposed at 1500h (mean ± SEM)

Time, min	GSH-Px, mmol GSH/min/mg protein	GST, μmol GSDNB/min/mg protein
<i>Young animals</i>		
0	0.639 ± 0.069	4.447 ± 0.474
30	0.704 ± 0.081	4.523 ± 0.222
60	0.686 ± 0.080	3.989 ± 0.277
120	0.637 ± 0.079	3.934 ± 0.299
240	0.697 ± 0.086	4.170 ± 0.250
<i>Old animals</i>		
0	0.667 ± 0.050	3.858 ± 0.679
30	0.598 ± 0.048	3.437 ± 0.722
60	0.738 ± 0.059	3.222 ± 0.516
120	0.747 ± 0.059	3.455 ± 0.340
240	0.728 ± 0.076	3.614 ± 0.435

Table 3. Dynamics of Lipid Peroxidation Products (TBARS) in Erythrocytes of Young (n = 5) and Old (n = 5) Rhesus Monkey Females in Response to the Stress Imposed at 1500h (mean ± SEM)

Time, h					
0	0.5	1.0	2.0	4.0	24.0
TBARS, nmol/mg protein					
<i>Young animals</i>					
0.262 ± 0.039	0.269 ± 0.029	0.285 ± 0.045	0.268 ± 0.026	0.277 ± 0.036	0.291 ± 0.068
<i>Old animals</i>					
0.259 ± 0.005	0.273 ± 0.007	0.298 ± 0.024	0.286 ± 0.007*	0.310 ± 0.010*	0.304 ± 0.048

P* < 0.05 – vs. the relative values before stress (0 min).Table 4. Dynamics of GR Activity in Erythrocytes of Young (n = 10) and Old (n = 10) Rhesus Monkey Females in Response to the Stress Imposed at 1500h (mean ± SEM)**

Time, min				
0	30	60	120	240
GR activity (% initial level)				
<i>Young animals</i>				
100	150 ± 20	185 ± 20	193 ± 20	220 ± 26
<i>Old animals</i>				
100	104 ± 20	118 ± 10 ^{1)*}	129 ± 10 ^{1)*}	129 ± 16 ^{1)*}

^{1)*}*P* < 0.05 – age – related differences.

The second subgroup consisted of 5 old animals in which basal SOD activity was significantly lower than in animals of the first subgroup (6.3 ± 0.2 versus 8.5 ± 0.1 mmol/mg protein, respectively, $P < 0.001$) and raised in response to stress unlike animals of the first subgroup and young animals (Fig. 1). Note that despite distinctions in initial SOD level and in dynamics of its activity in response to acute stress values of SOD in both subgroups reached similar level 30 min after beginning of the immobilization and further, that is 60, 120 and 240 min after stress exposure, practically did not differ (Fig. 1). Thus SOD in animals of 1st subgroup 240 min after stress exposure practically reached initial level (Fig. 1); it is similar to SOD dynamics in response to stress in young animals. At the same time in animals of 2nd subgroup SOD activity 240 min after the onset of restraint was statistically significantly higher in comparison with initial level (8.0 ± 0.2 and 6.3 ± 0.2 mmol/mg protein, respectively, $P < 0.001$) (Fig. 1).

Activity of GR and SOD returned to initial baseline values 24 hours after the stress (0.545 ± 0.050 versus 0.514 ± 0.056 nmol NADPH/min/mg protein before the stress for GR and 8.45 ± 0.15 versus 8.50 ± 0.10 mmol/mg protein before the stress for SOD for animals of 1st subgroup and 6.70 ± 0.2 mmol/mg protein before the stress for SOD for animals of 2nd subgroup).

We found a correlation between changes in GR activity and changes in F in response to stress. However this link was less strong in comparison with young animals. The correlation coefficient between the GR activity and the F response in the range of 0 – 240 min was 0.52 ± 0.15 and in the range of 0 – 120 min was 0.67 ± 0.13 . It is worth noting that in old animals the increase of F level 120 min after the beginning of stress was essentially lower than in young animals (Fig. 2).

Activities of GSH-Px and GST did not change significantly in response to the stress (Table 2).

In old monkeys, unlike young animals, the initial levels of TBARS varied insignificantly (within 0.250 – 0.269 nmol/mg protein) and were increasing in all animals in response to stress exposure. As it is shown in Table 3, the average values in the levels of TBARS unlike those in young ones were significantly increased 120 and 240 min after stress exposure.

DISCUSSION

We have previously reported increases in erythrocyte GR activity in female rhesus monkeys in response to moderate acute stress [13, 14]. There are other reports of increased GR activity in nerve cells in response to mild hypoxia [15], and in fibroblasts and muscle cells after heat stress [16-18]. Together with such increased GR activity, an increase in levels of GSH has also been found [16-21].

The increase in GR activity and GSH concentration in cells in response to stress, apparently, is of important adaptive significance – creates stock GSH – the major low-molecular antioxidant and a substratum for GP. The increase in reduced glutathione levels makes cells by less vulnerable to a more severe stress influence. In favor of this conclusion,

there are reports [15-18] that moderate stress can also lead to resistance to a subsequent more severe stress that would otherwise kill the cells.

The possible mechanism of increase GR activity in erythrocytes is stimulating influence from high cortisol concentration in plasma of peripheral blood, probably, mediated through activation of the pentose-phosphate cycle and stimulation of NADPH production [14].

Unlike dynamics of GR activity, activity of erythrocyte SOD under the conditions of stress in young animals decreased already 5 min after the beginning of stress and reached minimum values 30 min after the stress exposure (see Table 1, Fig. 1). Then SOD activity slowly raised, however did not reach the initial values neither 120 min after the stress beginning, nor 240 min, i.e., two hours after termination of the stressor. Differently, in young animals dissociation was observed in character of change of two major antioxidant enzymes – SOD and GR in the conditions of acute psycho-emotional stress.

Why SOD activity decreases? It is known that in response to various kinds of stressors short-term increase of intracellular concentration ROS and development of oxidative stress comes about which helps to switch on cellular adaptation of an organism [22-24]. In particular rise of ROS causes induction of heat shock proteins (Hsps), which are considered to be important factors, in the intracellular adaptation [25, 26]. So, it has been revealed that over expression of Hsps, including human Hsp27 and its mouse homologue Hsp25, in fibroblasts [16, 17] and in muscle cells [18] increased the levels of GSH, GR activity, glucose-6-phosphate dehydrogenase [16] and cell resistance to different types of stress.

Probably, in erythrocytes in response to stress-mediated adrenergic stimulation, increase of superoxide-free radical occurs mainly due to easy fall of SOD activity because mitochondria – the basic source of superoxide radical in cells – in erythrocytes are absent. In support of this assumption the data obtained by us that the lipid peroxidation products only slightly increased in young healthy animals under conditions of acute psycho-emotional stress can testify (see Table 3).

Only in half of the old animals SOD activity, both in basal conditions and under the stress conditions, had similar character with young animals (1st subgroup, see Fig. 1). In other half of the old animals (2nd subgroup, Fig. 1) basal SOD activity was essentially lower in comparison with young animals and animals of 1st subgroup. In response to the stress SOD activity in 2nd subgroup of old animals did not decrease and, on the contrary, in young animals, it rose.

Decrease of basal activity SOD in erythrocytes in old rhesus monkey females is consistent with the literature data marking fall of activity of this enzyme (Cu/Zn SOD) with aging in erythrocytes in laboratory rodents [27, 28] and in humans [29-32]. Decrease of basal SOD activity in old monkeys (2nd subgroup), apparently, leads to parity change between prooxidant and antioxidant processes in erythrocytes towards maintenance of higher values of ROS in comparison with young animal and old animals with invariable basal SOD levels (1st subgroup). Apparently, only certain ROS concentration possesses signaling function in cells that

switch on processes of intracellular adaptation, therefore in response to stress SOD activity in erythrocytes in animals of 2nd subgroup raises. Perhaps, signaling concentration of ROS is reached 30 min after the stress beginning. As it is possible to see from a Fig. (1), in old animals of both subgroups, activity of SOD reached practically identical values 30 min after the stress beginning and further, that is 60, 120 and 240 min, did not come to light essential distinctions in SOD activity in animals of discussed 2 subgroups.

In the literature there are a few reports on reaction of erythrocyte Cu, Zn-SOD in response to stress [27, 33, 34], including restraint stress [33, 34]. Nevertheless, available data are quite in concordance with our data. So, in young rats SOD activity in erythrocytes decreased in response to restraint stress [33, 34]. The heat stress led to decrease in activity Cu, Zn-SOD in young rats, but to its increase in old animals [27]. The increase in SOD activity in response to the exercise stresses has been revealed in a muscular tissue in aged men [35].

Basal GR activity in old animals was higher, than in young (see Fig. 1). Similar data were marked by us earlier for females of rhesus monkeys for this time of days that is at 1500h while in the morning age-related changes in activity GR were absent [14]. We also reported the higher GR activity in old rhesus monkey females in comparison with young animals in the night (2200h) [36]. The increase of basal GR activity in erythrocytes has been noted and in aged people, mainly at centenarians [32].

In response to acute stress GR activity in old animals rose, however in essentially smaller degree than in young animals (Figs. 1, 2). Mainly it occurred owing to higher basal GR level in old animals. The maximum values of increase of GR activity in response to the stress did not undergo significant differences in young and old animals.

Revealed disturbances in stress-responsiveness of GR activity in old animals, perhaps, are caused by alteration in functioning of the HPA axis which controls responsiveness of GR to acute stress. As it was earlier marked [13, 14], reaction of the HPA axis and GR activity on stress in young rhesus females depends on time of days: higher during afternoon and evening time in comparison with morning hours. Old animals had a smoothing of this rhythm and even its some distortion to higher stress-responsiveness in the morning. Additionally, in comparison with young monkeys, the old ones demonstrated essentially lower responsiveness of the HPA axis and GR activity in response to the immobilization at 15.00. This study also revealed age-related differences in stress responsiveness of corticosteroids and GR in the afternoon. In old animals the reaction of F and GR was lower than in young animals (Fig. 2, Table 4). Accordingly the correlation coefficient between dynamics of cortisol concentration and dynamics of GR activity under the conditions of stress in old animals was lower than in young animals (0.98 ± 0.04 in young animals and 0.67 ± 0.13 in old animals, $P < 0.05$). Apparently, reliability of hormonal regulation of GR activity under stress decreases with aging.

Age-related alterations in stress responsiveness of SOD and GR, main enzymes of antioxidant enzyme defense, lead to increase of lipid peroxidation products (TBARS) in eryth-

rocytes of old animals (see Table 3). Activation of peroxide oxidation of lipids in turn inevitably leads to damage of a cellular membrane of erythrocytes that can be accompanied with disturbances in mobility of erythrocytes. This is consistent with the data of Krylov and Deryugina who observed obligate changes in electrophoretic mobility of rat erythrocytes in response to various stress procedures [37]. The degree and directionality of changes in electrophoretic mobility depended on the concentration of lipid peroxidation products in erythrocytes [37]. Age-related disturbances in mobility of erythrocytes could lead to diminishing reliability of oxygen transport to tissues that are experiencing increased oxygen demand in the face of a psycho-emotional stress.

In conclusion, it is clear that erythrocyte antioxidant enzyme system (mainly SOD and GR) promptly reacts to moderate acute psycho-emotional stress. SOD activity decreased in response to the stress in young monkeys while it increased in half of old monkeys. Young animals also demonstrated essentially higher increase in GR activity in response to the restraint in comparison with old monkeys. We have also found that the age-related alterations in stress responsiveness of SOD and GR are accompanied by disturbances in lipid peroxidation products in erythrocytes of old animals. We hypothesize that the revealed age-related changes in reaction of erythrocyte antioxidant enzyme system in response to acute psycho-emotional stress may be considered as important factors of aging damage of erythrocyte functioning and reliability of oxygen transport to tissues under stress conditions.

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