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Ammonium Salts Promote Functional Adaptation of Rat Erythrocytes on the Model of Forced Swimming

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Ammonium, an end-product of catabolism, in low doses can promote adaptation of metabolic pathways in erythrocytes under conditions of extreme physical exercise. We compared the effects of two ammonium salts, ammonium chloride and ammonium carbonate, in two doses on biochemical parameters of rat erythrocytes 1 day after extreme physical exercise in a 4-week cycle of forced swimming. Of 16 analyzed parameters, the maximum number of significant shifts from the control was revealed in the groups of rats receiving ammonium chloride in doses of 20 and 10 mg/kg, and the minimal number of differences was found in groups treated with ammonium carbonate in the same doses. The comparison of the levels of reduced glutathione and 2.3-bisphosphoglicerate and activities of 5'-nucleotidase and Ca^{2+}- and Na/K-ATPases attested to more rigorous control of the mechanism of oxygen delivery to tissues by erythrocytes after administration of ammonium chloride in a dose of 20 mg/kg.

Key Words: *extreme load; adaptation; ammonium; erythrocytes*

Ammonium is constantly produced in all organs and tissues of the body. The main producers of ammonium are the organs with high metabolism of amino acids and biogenic amines, such as the nervous tissue, liver, intestine, and muscles [4]. The main biochemical sources of ammonium are non-oxidative deamination (hydrolytic for serine, threonine, and cysteine, and intramolecular for histidine), oxidative deamination (direct aerobic deamination mediated by amino acid oxidases and direct anaerobic deamination mediated by glutamate dehydrogenase in all tissues except for muscle tissue; indirect oxidative deamination or transdeamination), deamidation of glutamate and asparagine, catabolism of biogenic amines, degradation of purine and pyrimidine bases catalyzed by adenylate cyclase and AMP-deaminase. Ammonium is toxic, thus the mechanisms of ammonium binding (intoxication) resulting in the production of glutamate, glutamine, asparagine, and carbamoyl phosphate exist in tissues. Treatment with arginine and citrulline, compounds of urine cycle, for neutralizing of ammonium generated during the utilization of branched-chain amino acids allows to improve the endurance of long-distance runners [3]. However, little is known about the role of erythrocytes as a transporter of lactate and ammonium under extreme conditions, even though the clinical parameters, transport proteins, and certain regularities of transport of these metabolites in and out erythrocytes have been widely discussed last 50 years [7,11,13]. Our recent study was focused on the effects of ammonium chloride as a stimulator of physical performance under conditions of its separate and combined administration with green tea extract [2]. We hypothesized that ammonium as an end product of catabolism affecting ion balance of cells and acid-base balance of the body and a substance freely entering erythrocytes and other cells, in low doses can promote adaptation of metabolic pathways to extreme physical loads, optimize the main functions of these pathways, and thus improving organism endurance. The stimulating effect of ammonium chloride on physical performance of rats in the forced swimming test was previously dem-

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onstrated. Multidirectional adaptive changes in biochemical parameters of erythrocytes were also found in the group of rats treated with ammonium chloride and green tea extract. On the basis of these data, a mechanism of optimization of oxygen transporting and shuttle functions of erythrocytes by ammonium chloride during intense physical load was proposed.

Here we compared the effects of ammonium chloride and carbonate in two doses on the biochemical parameters of rat erythrocytes 1 day after extreme load in 4-weeks cycle of forced swimming. Comparative analysis of these ammonium salts was inspired by the fact that carbonate-anion along with ammonium ion is an essential end-product and regulator of acid-base metabolism, but its bioavailability in mineral drinks is low due to rapid disintegration by saliva and GIT carbonic anhydrases; at the same time, the increase in carbonate anion bioavailability in the ammonium carbonate can provide additive effect of ammonium-bicarbonate preconditioning.

MATERIALS AND METHODS

The experiments were performed in accordance to the Regulation for Animal Experiments approved by the Ethical Committee of I. M. Sechenov Institute of Evolutionary Physiology and Biochemistry. The previously described forced swimming test [2] was slightly modified, in particular, preliminary testing was performed at the end of week 2 of swimming and load weight was corrected relative to body weight of rats more often. After adaptation of rats to water, 6 groups were formed: group 1, intact animals (negative control, n=6); group 2 (positive control; n=7), swimming+NaCl (10 mg/kg; oral administration of physiological saline); groups 3 and 4, swimming+ammonium chloride (ACh) in doses of 10 mg/kg (n=9) and 20 mg/kg (n=8), respectively, 5 min before swimming; groups 5 and 6, swimming+ammonium carbonate (AC) in doses of 10 mg/kg (n=11) and 20 mg/kg (n=7), respectively; 5 min before swimming. The methods of measuring of metabolite concentration and activity of erythrocyte enzymes were described previously [2].

Statistical analysis of the data included the calculation of the mean and median. Significance of differences between the groups was evaluated using Mann—Whitney test. The differences in dependent samples were evaluated using Wilcoxon's T test. Correlations were analyzed using Spearman's test (Rho coefficient, two-tailed). The differences were considered significant at the level of significance of 95% (p<0.05). The differences at the level of significance of 90% were considered not as significant, but as a tendency to change (p<0.1). The calculations were made in two compatible software Microsoft Excel 2016 and Past 3.24.

RESULTS

The level of reduced glutathione in erythrocytes of rats receiving NaCl solution was slightly reduced in comparison with that in intact animals (by 10.6%, p<0.1; Table 1). In rats receiving 10 mg/kg ACh and AC in doses of 10 and 20 mg/kg, the concentration of reduced glutathione did not differ from that in intact control, but in rats receiving 20 mg/kg ACh, this parameter was significantly higher than in other groups (except the group receiving 20 mg/kg AC): by 11.2% in comparison with intact control (p=0.024), by 24% in comparison with positive control (p=0.01), by 21.2% in comparison with rats treated with 10 mg/kg ACh (p=0.003), and by 18.1% in comparison with rats treated with 10 mg/kg AC (p=0.008).

Activity of type 1 glutathione peroxidase in erythrocytes of rats receiving 20 mg/kg ACh was significantly lower than in the positive control group and groups treated with AC in both doses; the decrease from the level of intact control was insignificant (Table 1). The decrease in glutathione reductase activity in erythrocytes of rats receiving 10 mg/kg ACh was significant only in comparison with the positive control group (by 29%, p=0.048). No significant differences in activity of glutathione-S-transferase (GST) and glucose-6-phosphate dehydrogenase (G6PDD) were observed in erythrocytes of animals of all groups in comparison with intact control and between the treatment groups. Thus, the increase in the level of reduced glutathione in erythrocytes of rats receiving 20 mg/kg ACh can be explained by its lower utilization in the reaction catalyzed by glutathione peroxidase 1. Hydrogen peroxide was primarily detoxified by catalase and its activity was significantly increased in this group in comparison with intact control (by 14.5%) and rats receiving 10 mg/kg AC (by 17%).

Activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in erythrocytes of rats receiving 20 mg/kg ACh slightly decreased in comparison with the positive and negative controls. However, GAPDH activity in erythrocytes of rats receiving 10 mg/kg ACh was significantly lower (by 20%) than in positive and negative controls and animals receiving 20 mg/kg AC (Table 1).

At the end of the cycle of forced swimming, activity of Ca^{2+}-ATPase decreased by 19-20% in erythrocytes of rats receiving 10 mg/kg ACh in comparison with positive control (p=0.031) and intact control (p<0.1). However, administration of 20 mg/kg ACh was followed by a slight increase in enzyme activity in comparison with the control groups. This parameters in animals treated with 20 mg/kg ACh was significant-

ly higher than in rats treated with 10 mg/kg ACh (by 39%, p=0.03) and 10 mg/kg AC (by 18%, p=0.034). Swimming was followed by a decrease in 5'-nucleotidase activity in erythrocytes in positive control group (by 24%, p=0.020). Administration of ACh in doses of 10 and 20 mg/kg and AC in a dose of 10 mg/kg was associated with a significant decrease in this parameter in comparison with the intact control (by 12.7%, p=0.04; by 20.3%, p=0.03; by 19.0%, p=0.047, respectively). After administration of 20 mg/kg AC, enzyme activity did not differ from the level of intact control and was significantly higher than after administration of 10 mg/kg AC (by 23.4%, p=0.04). A slight decrease in activity of Na/K-ATPase was found in erythrocytes of rats receiving 20 mg/kg ACh in comparison with the control groups. However, this parameter tended to increase after administration of 10 mg/kg AC. The differences in these groups were significant (33%). We found no significant between-group differences in the level of 2,3-biphosphoglycerate in erythrocytes except for a tendency to a decrease in this parameter (by 23%) after administration of 20 mg/kg ACh in comparison with 10 mg/kg AC. The concentration of methemoglobin in the positive control group was significantly higher in comparison with the intact control group (by 17%, p=0.02). Methemoglobin level also significantly increased after administration of 20 mg/kg ACh (by 13%, p=0.03) and 20 mg/kg AC (by 22.6%, p=0.03) in comparison with the intact control. This parameter tended to increase after the treatment with AC and ACh in doses of 10 mg/kg in comparison with intact control (Table 1).

Forced swimming was followed by a slight increase (by 60%, p<0.1) in the rate of transmembrane electron transfer (TMET) in erythrocytes of positive control rats. However, TMET in the groups of rats treated with ammonium salts was either similar to this level in the intact control (ACh, 10 mg/kg), or tended to decrease (ACh, 20 mg/kg; AC, 10 and 20 mg/kg). ATP level in erythrocytes of positive control rats and all treatment group rats tended to increase in comparison with the intact control (by 18-28%). The least pronounced changes in this parameter were observed in rats receiving 20 mg/kg ACh, and the most pronounced changes were found in the groups treated with 20 mg/kg AC and NaCl. Lactate dehydrogenase (LDH) activity in erythrocytes of rats receiving 10 mg/kg ACh also tended to increase in comparison with the intact control (by 31%; Table 1).

Among the 16 studied biochemical parameters of functional state of erythrocytes, the maximal number of significant changes (5 parameters) was observed after administration of 20 mg/kg ACh, then 10 mg/kg ACh (4 parameters). The minimum number of changes

TABLE 1. Biochemical Parameters of Rat Erythrocytes One Day after the Cycle of Forced Swimming (median±MAD)

Parameter	Intact control (n=6)	Positive control (NaCl, n=7)	ACh, 10 mg/kg (n=9)	ACh, 20 mg/kg (n=8)	AC, 10 mg/kg (n=11)	AC, 20 mg/kg (n=7)
Reduced glutathione, μmol/g Hb	6.3±0.2	5.7±0.5	5.8±0.2	7.0±0.1**$^{+}$	6.0±0.3	6.2±0.5
Glutathione peroxidase-1, μmol GSH/min/g Hb	691±29	787±54	806±87	678±40^{++}	746±41	756±48
Glutathione reductase, nmol GSH/min/g Hb	632±149	703±136	498±73^{+}	571±176	623±137	607±85
Glutathione-S-transferase, μmol GSH/min/g Hb	2.20±0.23	2.28±0.35	2.23±0.14	2.26±0.41	2.01±0.21	1.97±0.41
G6PDD, $NADP^{+}$/min/g Hb	14.7±0.2	15.0±0.3	14.3±0.5	15.3±0.4	14.5±0.8	15.0±0.5
GAPHD, μmol NAD^{+}/min/g Hb	131±5	129±2	102±17*$^{++}$	116±13	123±6	126±8
Catalase, mmol H_2O_2/min/g Hb	106±13	108±13	108±13	124±8*	103±9	129±20
TMET, nmol/min/g Hb	84±19	134±10	85±12	51±26	66±32	76±16
5'-Nucleotidase, μmol Pi/h/g Hb	79±2	60±13*	69±11*	63±7*	64±4*	79±5
Ca^{2+}-ATPase, μmol Pi/h/g Hb	79±7	77±2	62±2^{+}	86±6	73±6	77±2
Na/K-ATPase, μmol Pi/h/g Hb	32±6	33±6	30±2	27±2	36±3	30±5
ATP, μmol/g Hb	6.8±1.1	8.5±1.8	8.2±0.3	8.0±1.3	8.2±0.7	8.7±1.3
BPG, μmol/g Hb	35.2±3.9	35.9±3.8	33.8±2.6	31.8±3.4	39.2±6.4	35.4±5.1
Methemoglobin, %/g Hb	0.53±0.03	0.62±0.06*	0.58±0.05	0.60±0.06*	0.63±0.07	0.65±0.10*
LDH, μmol NADH/min/g Hb	52±1	55±2	68±11	56±11	52±10	56±4
MDA, μmol/g Hb	21.8±1.6	21.8±2.6	16.7±2.7	21.8±0.4	25.0±1.9	20.5±3.8

Note. G6PDD, glucose-6-phosphate dehydrogenase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; TMET, transmembrane electron transfer; BPG, 2,3-biphosphoglycerate. *p<0.05, **p<0.01 in comparison with intact control; ^{+}p<0.05, ^{++}p<0.01 in comparison with positive control.

was revealed after treatment with AC in both doses (one parameter each). Thus, erythrocytes of rats receiving ACh before physical load were most liable to adaptive changes or were faster renewed. In rats treated with 20 mg/kg ACh, the antioxidant system of erythrocytes was characterized increased level of reduced glutathione and higher activity of catalase and lower activity of glutathione peroxidase-1. More than 2-fold difference in TMET between the positive control group (NaCl) and groups treated with ACh (20 mg/kg) and AC (10 mg/kg) attests to adequate functioning of intracellular systems of antioxidant defense and high adaptation capacity of erythrocytes in rats receiving ACh and AC in these doses. Moreover, 33% increase in Na/K-ATPase activity in the group treated with 10 mg/kg AC in comparison with the group receiving 20 mg/kg ACh can reflect more potent activation of P_2X-receptors of erythrocytes by exogenous ATP leading to Na^+ entry, which is considered as the primary mechanism of erythrocyte swelling for dosed release of ATP [8]. Increased activity of Ca^{2+}-ATPase associated with reduced activity of 5'-nucleotidase in the group treated with ACh (20 mg/kg) is a sign of positive adaptation [12]. Reduced activity of 5'-nucleotidase is an adaptive systemic response aimed at stimulation of ATP release due to erythrocyte swelling, activation of endothelial P_2Y-receptors, and generation of nitrogen oxide. At the same time, regulated swelling of erythrocytes is more preferable than erythrocyte shrinkage associated with high risk of apoptosis induction. The required functional effect in erythrocytes of ACh group (20 mg/kg) was mediated by the combination of changes in antioxidant defense system, ATP generation, and ATP utilization by ATPases. Comparison of 2,3-biphosphoglicerate level and activities of 5'-nucleotidase and Ca^{2+}- and Na/K-ATPases in erythrocytes of rats receiving ACh (20 mg/kg) and AC (10 mg/kg) attested to more rigorous regulation of oxygen transfer to tissues in the ACh group (20 mg/kg). Probably, ATP release in these animals is mediated primarily by pannexin-1 phosphorylated by protein kinase A (PKA). The second pathway of ATP release via potential-dependent anion channels (VDAC) activated by Ca^{2+} ions is not activated due to timely removal of Ca^{2+} ions [10]. The latter determines one more important aspect, in particular, prevention of activation of calcium-dependent proteases leading to cytoskeleton degradation and erythrocytic death via eryptosis. On another hand, accurate functioning of a more precise mechanism of regulation of methemoglobin level via its timely elimination in microvesicles allows extending erythrocyte lifetime and functional activity [9]. This suggestion is confirmed by the dynamics of changes in methemoglobin level.

The use of nutraceutical substances, such mineral salts and endogenous metabolites, plays an important role in the sport of records and is actively studied [5]. Bicarbonate is one of the most studied among the used metabolites. It is administered to control the acid-base metabolism of sportsmen to enhance performance during extreme loads [6]. The observed improvement of physical performance is far from significant. At the same time, we showed that treatment with ammonium salts in low doses can significantly affect performance and enhance ultrastructural parameters of muscle fibers in rats during forced swimming [1,2]. Here we asked which of these ions, ammonium or bicarbonate, plays a pivotal role in registered adaptive changes in erythrocytes, and whether bicarbonate ions are worth to be added. We presume additive effect of ammonium and carbonate ions of the compared salts, but additional studies are required to confirm this hypothesis. Considering changes in the glutathione system and ATPase activity, the increase in the dose of ammonium ion can provide more potent adaptive effect due to more rigorous regulation of physiological activity of erythrocytes.

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