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THE EFFECT OF EXHAUSTIVE EXERCISE ON THE CONCENTRATION OF PURINE NUCLEOTIDES AND THEIR METABOLITES IN ERYTHROCYTES

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Abstract. In this study we tried to obtain a complete overview of purine nucleotide metabolism in erythrocytes before and during an incremental, intermittent exhaustive exercise bout protocol for sportsmen (high-performance rowers) and untrained, healthy, active volunteers. Erythrocyte levels of the main nucleotides (ATP, ADP, AMP, GTP, GDP, GMP, IMP, NAD^+ and $NADP^+$), nucleosides (Ado, Guo, Ino) and the base Hyp were measured using the HPLC method. The parameters that can be deducted from their concentrations: TAN, TGN and AEC, GEC expressed by the ratio of high/low energy nucleoside phosphates were calculated. The effects of graded rowing exercise on the concentration and metabolism of erythrocyte purine and pyridine nucleotides and the activity Na^+, K^+-ATPase in both trained and untrained individuals. In the group of sportsmen, ATP, ADP and AMP concentrations were decreasing during the effort (for ATP r_s=-0.65). However, ADP and AMP concentrations were significantly lower only during recovery ($p<0.0001$). We observed a negative correlation (r_s=-0.72) between the ATP concentration and the Ado concentration during maximal effort. In the sportsmen group, NAD^+ concentration was decreasing during the effort (r_s=-0.68). Lower activity of Na^+, K^+-ATPase in erythrocytes was significantly correlated with the decrease in ATP concentration after maximal effort in sportsmen. This study confirms that erythrocyte energy metabolism depends on the body's adaptation to effort and the physical efficiency of the participants. ***(Biol.Sport 25:35-55, 2008)***

Key words: Graded exhaustive exercise - Purine and pyridine nucleotides - ATPase - HPLC

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Abbreviations
ADA-adenosine deaminase (EC 3.5.4.4)
Ade- adenine
Ado-adenosine
ADP-adenosine-5'-diphosphate
AEC- adenylate energy charge
AK-adenosine kinase (EC 2.7.1.20)
AMP- adenosine-5'-monophosphate
AMP-D-AMP deaminase (EC 3.5.4.6)
APRT-adenine phphosphribosyltranferase (EC 2.4.2.7)
ATP- adenosine-5'-triphosphate
1,3-BPG- 1,3-Biphosphoglycerate
2,3-BPG- 2,3-biphosphoglycerate
GDP-guanosine-5'- diphosphate
GEC-guanylate energy charge
GMP- guanosine-5'- monophosphate
GTP- guanosine-5'- triphosphate
Gua-guanine
Guo-guanosine
HGPRT-hypoxanthine-guanine phosporybosyltransferase (EC 2.4.2.8)
HRmax-maximal heart rate
Hyp-hypoxanthine
IMP-Inosine-5'- monophosphate
Ino-inosine
NAD^+-nicotinamide adenine dinucleotide
$NADP^+$-nicotinamide dinucleotide adenine phosphate
5'-NT-5'-nucleotidase (EC 3.1.3.5)
PNP-purine nucleotidase phosphorylase (EC 2.4.2.1)
PRPP-5'-phosphoribosyl 1-pyrophosphate
TAN-total adenine nucleotides
TGN-total guanine nucleotides

Introduction

Prolonged physical effort yields many functional and metabolic changes in the system, and training leads to an increased physical efficiency (function) and tolerance to effort [2,7]. The training-influenced changes result from adaptation processes within systems, organs and tissues; the human body increases activities

supplying oxygen and energy substrates for muscles, and increases the excretion of metabolic products and excess heat.

The study of purine nucleotide metabolism is very important for understanding of disruptions in energy metabolism as the purine nucleotides participate in most energy-requiring metabolic reactions and act as coenzymes. Purine nucleotide metabolism in erythrocytes is based on two fundamental pathways: the salvage pathway and the catabolic pathway. The salvage pathway can readily form mononucleotides from purine bases their nucleosides. HGPRT catalyzes the formation of IMP from hypoxantine (Hyp) and GMP from guanine (Gua). APRT catalyzes the conversion of adenine (Ade) to AMP. HGPRT returns the major products of purine nucleotide catabolism to nucleotide forms. Since the erythrocytes do not exibit xantine oxidase (EC 3.5.4.3) activity, Hyp is the end product of ATP degradation in the cells. Thus, adenine nucleotide catabolism can be measured as Hyp formation in erythrocytes [10,13,40,42]. In erythrocytes adenosine (Ado), guanosine (Guo) and inosine (Ino) can be phosphorylated to AMP, GMP, IMP, respectively [3,4,28] Catabolic pathway of erythrocytes is involved in the following activities: AMP-D, 5'-NT, ADA, PNP [1,6,10,11,15,37,42]. ATP plays a significant role in transferring energy within the cell, diffusing from where it is produced to where it is utilized. ATP in erythrocytes is utilized in ion transport processes that account for almost 30% of total ATP consumption. Na^+ and K^+ transport utilizes much more ATP, while Ca^{2+} transport requires markedly less energy. There is no consensus in literature on the dependence of Na^+, K^+-ATPase (and Ca^+-ATPase)on ATP. That is why we also attempted to find out the relations between the ATP concentration and the activity of Na^+, K^+-ATPase (EC 3.6.1.37).

The previous studies on the influence of physical effort on the cell energy status in blood are not consistent [2,18,20,36]. It may be the result of the fact that bioenergetic processes are strongly affected by the nature of the effort, its intensity and duration, and the fitness of the individuals involved in the study [9,17,23]. The nucleotide concentration influences the efficiency and endurance of the system; it also plays a crucial role in adaptational processes responding to an increased oxygen demand during effort [22,43,47]. The red blood cells and ATP (also ADP and AMP) might be involved in the regulation of local blood flow and O_2 delivery by signaling O_2 availability in the erythrocyte. The red blood cells release ATP in response to a fall in hemoglobin O_2 saturation [16,22].

In this study we tried to obtain a complete overview of purine nucleotide metabolism in erythrocytes before and during an incremental, intermittent exhaustive exercise bout protocol for sportsmen (high-performance rowers) and

untrained, healthy, active volunteers. First we measured erythrocyte levels of the main nucleotides (ATP, ADP, AMP, GTP, GDP, GMP, IMP, NAD^+ and $NADP^+$), nucleosides (Ado, Guo, Ino) and the base Hyp were measured using the HPLC method. The parameters that can be deducted from their concentrations: TAN, TGN and AEC, GEC expressed by the ratio of high/low energy nucleoside phosphates were calculated. The effects of graded rowing exercise on the concentration and metabolism of erythrocyte purine and pyridine nucleotides and the activity Na^+, K^+-ATPase in both trained and untrained individuals.

Materials and Methods

The study involved 15 high-performance rowers and 10 healthy, active, but not trained volunteers. Subjects were informed of the experimental procedures and possible risks before giving their consent to participate. All studies were approved by the Local Ethics Committee.

All the participants were 21-23 years old, 175±4 cm tall, 75±4 kg. The test took place two weeks before the rowers' camp, during a general preparation period. It was carried out on a Concept II rowing ergometer with an increasing load, which allowed a computerized determination of peak power, rate, mean power and total work. The test involved 4 attempts; each subsequent attempt having a higher load. Both groups, rowers and untrained individuals, exercised with a load inducing 50%, 70%, 85% and 100% maximal heart rate (HRmax) calculated according to Karvonen's method [26]. The particular attempts took 5 min (except the 100% one that took only a min) and the breaks between the exercises were 5 min each.

Measurements during recovery were carried out 5 min after the maximal effort. Sample blood was taken from the dorsal vein in the hand six times: before the effort, after each exercise cycle and during recovery. During the experiment we controlled heart rate, work, speed, mean power and time.

HPLC separation of purine:

1. Chemicals: Purines used as chromatographic standards were obtained from Sigma- Aldrich. HPLC- grade acetonitrile was obtained from Merk. HPLC-grade potassium dihydrogen orthophosphate, potassium chloride and tripotassium orthophosphate were obtained from Fluka Chemie GmbH. HPLC-grade water was prepared using a Milli-Q (Millipore purification system). All HPLC- solvents were filtered through 0.22 µm nylon filters (Supelco) prior to use.

2. Instrumentation: For HPLC analysis, a Hewlett Packard series 1100 chromatographic system was used. The system consisted of a quaternary pump system with degasser and continuous seal wash option (G1311 A), variable-

wavelength detector (G1314) and thermostatted column compartment (G1316A). The analytical column (100x4.6 mm LC) was packed with 18.3 μm of Hypersil BDS-C (Hewlett Packard). Samples were introduced using a Rheodyne 7725 injection valve equipped with a 20 μl loop. Sample peaks were integrated and quantified using an HPLC chromatography data system operating on Chemstation Software for Windows 98 (Hewlett Packard).

3. HPLC separation of purine: The samples (500 μl) were deproteinized with 500 μl of 1.3 M perchloric acid in 1.5 ml Eppendorf tubes. Extract mixtures were centrifuged (16000 G for 10 min, at 4 ^{0} C). The supernatant (600 μl) was neutralized with approximately 60- 90 μl of 3 M potassium orthophosphate solution (pH range of the sample to 6.0-7.0). The neutralized extract was again centrifuged and filtered through a 0.22 μm nylon filter. The obtained clear filtrate was used directly for HPLC assay or stored at -80 ^{0}C.

4. Chromatographic conditions: Purine and pyridine nucleotides: ATP, ADP, AMP, GTP, GDP, GMP, IMP, NAD^+, $NADP^+$; nucleosides: Ado, Guo, Ino and bases Hyp were measured using the Smolenski *et al.* HPLC method [41]. Sample aliquots (100 μl) were injected into the chromatograph column and nucleotides were separated using a linear phosphate buffer gradient system (buffer A: 150 mM KH_2PO_4, 150 mM KCl adjusted to pH 6.0 with K_2HPO_4; buffer B: 15% v/v solution of acetonitrile in buffer A) at a flow rate of 0.666 ml/min. Peaks were detected by absorption measurements at 254 nm. The composition of the mobile phase was controlled by a low-pressure gradient mixing device. The cycle time was 12.8 min between injections. The analytical column was maintained at constant temperature 20.5 ^{0} C.

Measurements of ATPase activities: The Na^+, K^+ -ATPase activities were determined on erythrocyte plasma membranes by Choi's method [12]. For the determination of membrane ATPase, aliquots of a 25 μL membrane suspension, containing 25-50 μg of protein, were preincubated at 37^{0}C for 5 min in the following media: 20 mM KCl; 100 mM NaCl; 0,5 mM EGTA; 30 mM histidine-imidasole buffer (pH 7,0); 0,5 mM $MgCl_2$ .The enzymatic reaction was started by adding 2 mM ATP. After 30 min incubation at 37^{0}C, the reaction was stopped by adding 1 mL of ice cold 20% trichloric acid. Then the mixture was centrifuged at 20 000 G for 2 min and the inorganic phosphate released into the supernatant was measured, according to the Goldberg and Fernanden method [21]. Enzyme activity is expressed as of nmol of P_i per mg membrane proteins per 60 min determined according to Lowry *et al.* [29].

Hematocrit index value, RBC, WBC, hemoglobin concentration: The study was carried out with a Technicon RS-500 biochemical analyzer and a bioMerieux test battery.

Glucose concentration: The study was carried out using an enzymatic method with glucose oxidase and glucose peroxidase, using a Technicon RS-500 biochemical analyzer and a bioMerieux test battery.

Ionized calcium, potassium and sodium concentrations: The study was carried out with ionselective electrodes with an AVL-988/3 apparatus and a Hoffman-La Roche test battery.

Statistical analysis of data: Statistical analysis was conducted using v.5.1 of Statistica software. Non-parametric ANOVA Kruskal-Walis rank and U-Mann Whitney tests were used to check the significance of difference ($p<0.05$). A test for Spearman rank correlation coefficient was used to check the statistical significance of observed correlations.

Results

The concentration of ATP in erythrocytes was significantly higher in sportsmen than in untrained men (rest and 50%, 70% and 85% HRmax, $p<0.01$) (Fig. 1). Erythrocyte ADP and AMP concentrations were significantly higher in sportsmen than in untrained men (rest, 50%, 70% and 85% HRmax, $p<0.0001$). In the group of sportsmen, ATP, ADP and AMP concentrations decreased during the effort (for ATP r_s=-0.65). ATP concentration in that group was significantly lower during the maximal effort and recovery than in the other measurement points ($p<0.01$). ADP and AMP concentrations were significantly lower during the maximal effort ($p<0.001$) and recovery ($p<0.0001$) than in the other measurement points.

The concentration of GTP in erythrocytes in both groups remained at a similar level (Fig. 2). The concentration of GDP in erythrocytes was significantly higher in sportsmen than in the untrained group (in rest, 50%, 70% and 85% HRmax) $p<0.0001$. In the sportsmen we observed a significant increase in GDP concentration during recovery compared with the measurements before and during the effort ($p<0.0001$). GMP concentration in the sportsmen group was significantly higher than in the untrained group.before the effort, at 50% and 85% HRmax ($p<0.01$, $p<0.05$ $p<0.01$, respectively). GMP concentration during the maximal effort and recovery was significantly lower compared with other measurements ($p<0.05$ and $p<0.0001$, respectively).

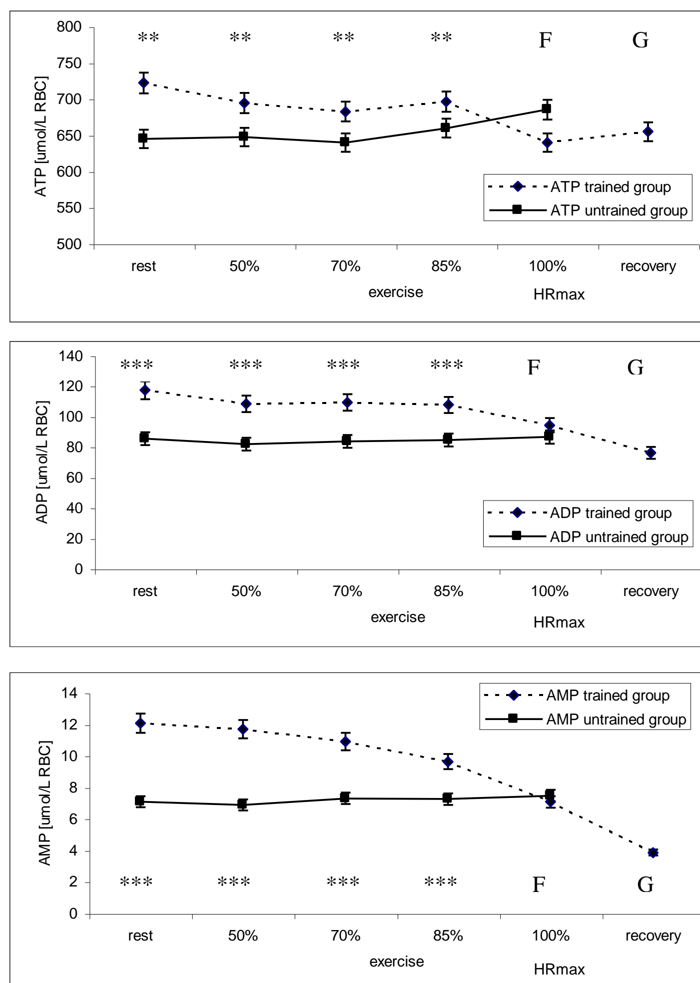

Fig. 1
The concentration of ATP, ADP, AMP (µmol/L RBC) in erythrocytes in trained and untrained individuals
Values are means ±SD, *p<0.05, **p<0.01, ***p<0.001 different from the untrained group. ATP - significance of differences between the mean values of ATP concentration in sportsmen (F) and (G) p<0.01 vs. rest, 50%, 70%, 85%; ADP, AMP - significance of differences between the mean values of ADP, AMP concentration in sportsmen (F) p<0001 vs. rest, 50%, 70%, 85%, recovery; (G) p<0.0001 vs. rest, 50%, 70%, 85%, 100%.

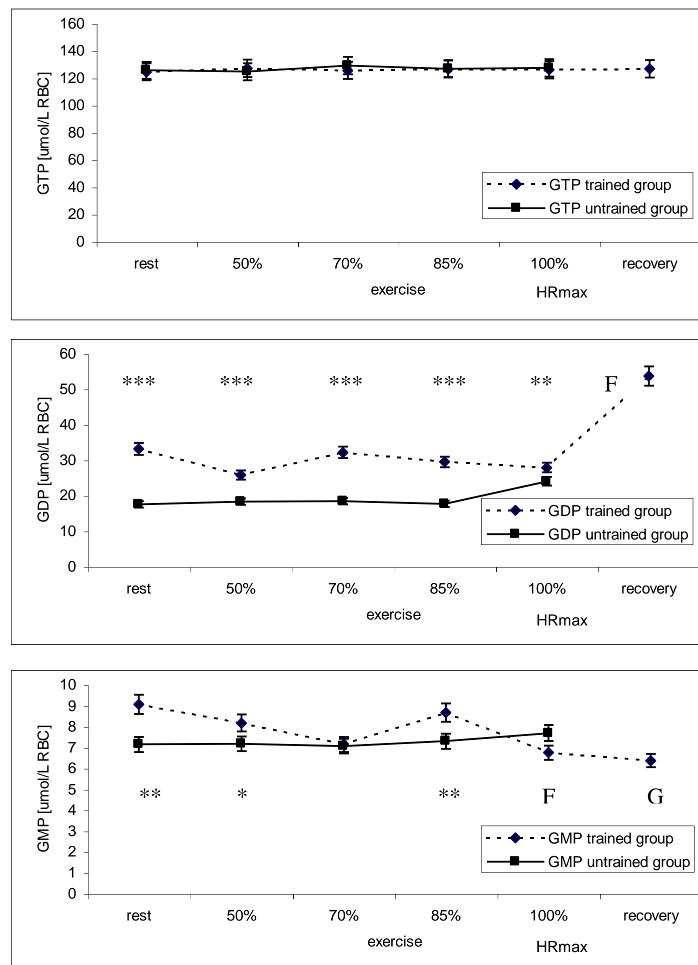

Fig. 2
The concentration of GTP, GDP, GMP (μmol/L RBC) in erythrocytes in trained and untrained individuals
Values are means ±SD, *p<0.05, **p<0.01, ***p<0.001 different from the untrained group. GDP - significance of differences between the mean values of GDP concentration in sportsmen (F) p<0.0001 vs. rest, 50%, 70%, 85%, 100%; GMP - significance of differences between the mean values of GMP concentration in sportsmen (F) p<0.05 vs. rest, 50%, 70%, 85%, recovery, (G) p<0.0001 vs. rest, 50%, 70%, 85%, 100%.

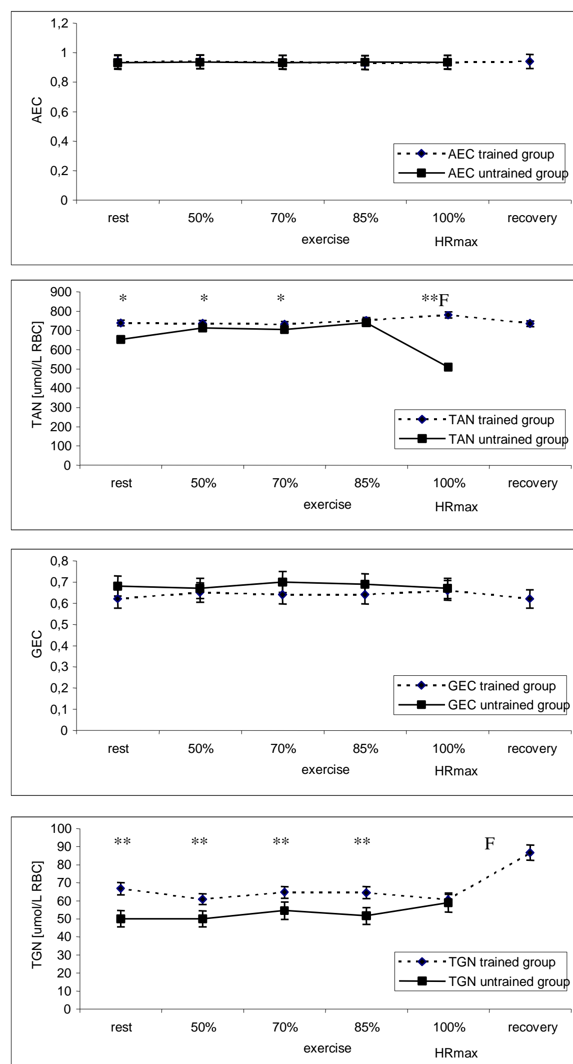

Fig. 3
AEC, GEC and TAN, TGN in trained and untrained group
Values are means ±SD, *p<0.05, **p<0.01, ***p<0.001 different from the untrained group. TAN - significance of differences between the mean values of TAN in the untrained group (F) p<0.0001 vs. rest, 50%, 70%, 85%, recovery. TGN - significance of differences between the mean values of TGN in sportsmen (F) p<0.0001 vs. rest, 50%, 70%, 85%, 100%

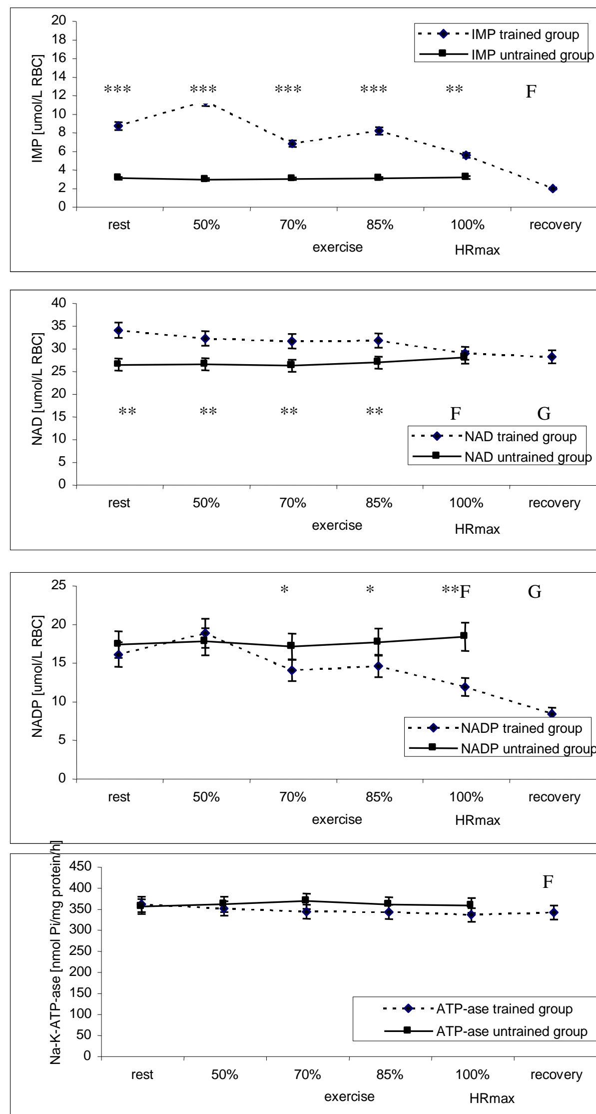

Fig. 4
The concentration of IMP, NAD^+, $NADP^+$ (µmol/L RBC) and Na^+, K^+ ATP-ase activity (nmol Pi/mg protein/h) in trained and untrained group
Values are means ± SD, *p<0.05, **p<0.01, ***p<0.001 different from the untrained group. IMP - significance of differences between the mean values of IMP concentration in sportsmen (F) p<0.01 vs. rest, 50%, 70%, 85%, 100%; NAD^+ -

significance of differences between the mean values of NAD^+ concentration in sportsmen (F) p<0.01 vs. rest, 50%, 70%, 85%, (G) p<0.01 vs. rest, 50%, 70%, 85%, 100%; $NADP^+$ - significance of differences between the mean values of $NADP^+$ concentration in sportsmen (F) p<0.01 vs. rest, 50%, 70%, 85%, (G) p<0.001 vs. rest, 50%, 70%, 85%, 100%, Na^+, K^+ ATP-ase activity - significance of differences between the mean values of Na^+, K^+ ATP-ase activity in sportsmen (F) p< 0.01 vs. rest, 50%, 70%, 85%, 100%.

The ATP, ADP, AMP stoichiometrically bind all of the metabolic sequences of a living cell. The amount of metabolically available energy that is momentarily stored in the adenylate system is linearly related to the mole fraction of ATP plus half the mole fraction of ADP; this parameter has been termed the AEC of the adenylate pool (evaluated according to Atkinson's formula [5]): AEC=([ATP]+0.5[ADP]) / ([ATP]+[ADP]+[AMP]). The absolute intracellular ATP concentration depends on the TAN and the AEC, that is, on the fraction of ATP in the TAN. TAN was calculated from the median concentrations [ATP], [ADP] and [AMP]: TAN=[ATP]+[ADP]+[AMP]. In both groups, AEC value was at a similar level during effort (Fig. 3). TAN was significantly higher in the sportsmen group compared with the untrained group (p<0.05). In the untrained group TAN decreased significantly during effort at 100% of maximal heart rate (p<0.0001). GEC value ([GTP]+0.5[GDP]) / ([GTP]+[GDP]+[GMP]) was similar in both groups and did not change during effort (Fig. 3). TGN calculated from the median concentrations [GTP]+[GDP]+[GMP] in erythrocyte in the sportsmen group was significantly higher compared with the untrained group (p<0.01). In the sportsmen group TGN increased significantly during recovery (p<0.0001).

The erythrocyte concentration of IMP in the sportsmen group before the effort, at 50%, 70%, 85% and 100% HRmax, was higher compared with the untrained group(p<0.001, p<0.0001, p<0.001, p<0.0001, p<0.01, respectively) (Fig. 4). IMP concentration in the sportsmen group during recovery was significantly lower compared with other measurement points (p<0.01).

A significant increase in Ado and Guo concentration in blood was seen in training group. Ado concentration for rest, 50%, 70% and 85% HRmax and during the maximal effort was significantly higher in the sportsmen group compared with the untrained group (p<0.001) (Fig. 5). In the sportsmen group, during the effort with 70% HRmax and during recovery, we observed a significant increase in Ado concentration (p<0.01 and p<0.0001 respectively). In the untrained group Ado concentration was constant. We observed a negative correlation (r_s=-0.72) between the ATP concentration and the Ado concentration during the maximal effort. Guo

concentration in the sportsmen group was significantly higher than in the untrained group at the rest and during the effort at 50%, 70% and 85% HRmax ($p<0.05$, $p<0.02$, $p<0.01$ and $p<0.01$, respectively). In the sportsmen group during the maximal effort and recovery we observed a significant decrease in Guo concentration compared with the measurement before the effort ($p<0.01$) and during the effort at 50%, 70% and 85% HRmax ($p<0.001$). Guo concentration in the untrained group was constant. Ino concentration before the effort, at 50%, 70%, 85% HRmax and during the maximal effort, was significantly higher in the sportsmen group compared with the untrained group ($p<0.01$) (Fig. 5). In the sportsmen group, at the 70% HRmax and during the recovery, we observed a significant increase in Ino concentration ($p<0.01$ and $p<0.0001$). In the untrained group Ino concentration was constant. Adenine nucleotide catabolism can be measured as Hyp formation (the product of adenine nucleotide degradation) and Hyp concentration in both groups was at a similar level before the effort, at 50%, 70%, and 85% HRmax (Fig. 5). In the sportsmen group we observed a 3-fold increase in Hyp concentration at 100% HRmax ($p<0.001$) and 10-fold during recovery ($p<0.0001$). A significant increase of Hyp (and Ado) in sportsmen group compared with the untrained group, especially during the maximal effort and during recovery, suggest the increased dephoshorylation of adenine nucleotides.

The concentration of NAD^+ in erythrocytes was significantly higher in the sportsmen group compared with the untrained group (before the effort, at 50%, 70% and 85% HRmax, $p<0.001$) (Fig. 4). In the sportsmen group NAD^+ concentration was decreasing during the effort (r_s= -0.68). NAD^+ concentration in that group , during the maximal effort and recovery, was significantly lower than in other measurement points ($p<0.01$). The concentration in erythrocytes of $NADP^+$ in both groups, during the rest and at 50% of the maximal heart rate, remained at a similar level (Fig. 4). In the sportsmen group $NADP^+$ concentration decreased during the effort (r_s= -0.57). $NADP^+$ concentration in that group during the maximal effort and during recovery was significantly lower compared with other measurement points ($p<0.001$, $p<0.0001$). We did not observe any significant changes in Na^+, K^+ ATP-ase activity between both groups (Fig. 4). In the sportsmen group Na^+, K^+ ATP-ase activity decreased during effort and was significantly lower during recovery compared with earlier measurements. ($p<0.01$). RBC, WBC, hematocrit index values, hemoglobin concentration, glucose, sodium, potassium and ionized calcium concentration is shown in Table 1. We did not observed any significant changes in these parameters both in the sportsmen group and the untrained group. The heart rate, work, speed, mean power and time are presented in Table 2.

Table 1

Clinical and hematological characteristic of trained (n=15) and untrained (n=10) individuals: RBC, WBC, hemoglobin concentration, hematocrit index, concentration of glucose, sodium potassium and ionized calcium

parameters	glucose (mmol/L)		Na (mmol/L)		K (mmol/L)		Ca (mmol/L)	
group	trained	untrained	trained	untrained	trained	untrained	trained	untrained
Rest	4.84±0.51	4.92±0.41	142±1	144±2	4.51±0.31	4.2±0.42	1.013±0.04	1.02±0.08
50% HRmax	4.71±0.56	4.88±0.57	141±2	143±2	4.64±0.33	3.8±0.35	0.950±0.08	1.01±0.08
75% HRmax	4.75±0.63	5.35±0.53	138±3	141±3	4.23±0.42	3.8±0.33	0.915±0.07	0.98±0.03
80% HRmax	5.24±0.82	5.5±0.77	143±2	141±3	4.18±0.51	3.9±0.41	1.035±0.08	0.95±0.05
100%HRmax	5.98±0.74	6.14±0.84	143±3	143±4	4.08±0.50	3.9±0.39	1.083±0.05	0.98±0.02
Recovery	6.37±0.71		144±3		4.1±0.37		1.051±0.07	

parameters	RBC (10^6/uL)		WBC (10^6/uL)		Ht (%)		hemoglobin (g%)	
group	trained	untrained	trained	untrained	trained	untrained	trained	untrained
Rest	4.96±0.18	4.52±0.19	6.43±1.08	5.81±0.77	46±3	43±2	22.2±3.08	20.1±2.21
50% HRmax	5.10±0.29	4.71±0.22	7.31±0.98	6.82±0.82	47±4	43±2	20.4±2.18	21.2±1.55
75% HRmax	5.27±0.22	4.70±0.33	8.47±0.87	7.31±0.99	48±3	42±3	20.3±2.32	21.3±1.12
80% Rmax	5.17±0.31	4.82±0.47	8.63±1.12	7.33±1.26	47±2	46±2	21.3±3.12	20.4±2.74
100%HRmax	5.21±0.29	4.89±0.37	9.07±0.80	7.52±0.88	49±3	48±3	21.7±3.31	20.3±2.22
Recovery	5.22±0.35		8.81±1.25		50±4		21.1±2.65	

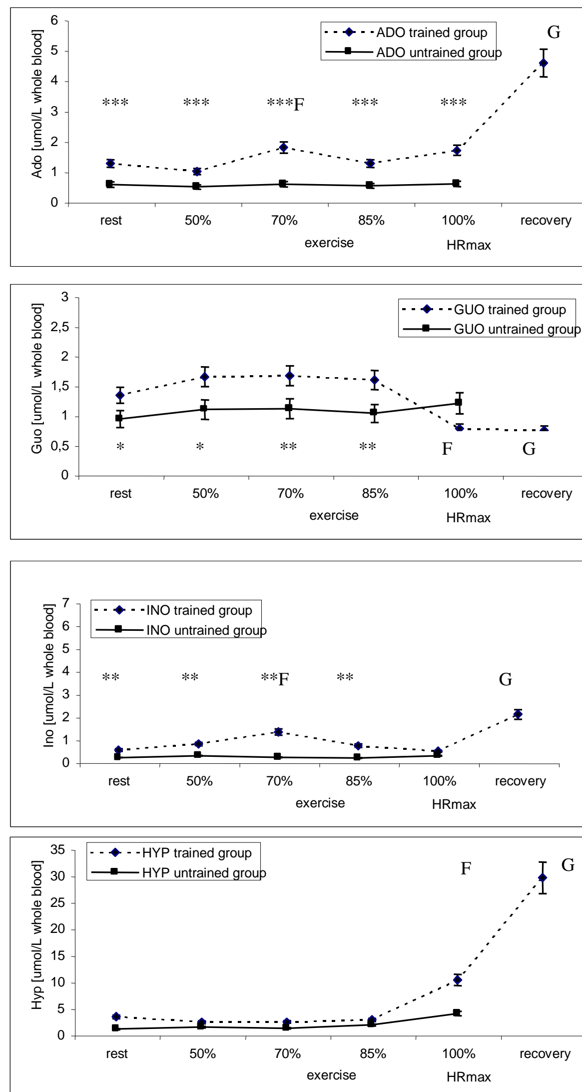

Fig. 5
Purine and pyridine nucleosides content in trained and untrained group. The concentration of Ado, Guo, Ino, Hyp are expressed in µmol/L whole blood
Values are means ±SD, *p<0.05, **p<0.01, ***p<0.001 different from the untrained group. Ado - significance of differences between the mean values of Ado concentration in sportsmen (F) p<0.01 vs. rest, 50%, 85%, 100%; (G) p<0.0001 vs.

rest, 50%, 70%, 85%, 100%. Guo - significance of differences between the mean values of Guo concentration in sportsmen (F) $p<0.01$ and (G) $p<0.001$ vs. rest, 50%, 70%, 85%; Ino - significance of differences between the mean values of Ino concentration in sportsmen (F) $p<0.01$ vs. rest, 50%, 85%; 100%, recovery; (G) $p<0.0001$ vs. rest, 50%, 70%, 85%, 100%; Hyp - significance of differences between the mean values of Hyp concentration in sportsmen (F) $p<0.001$ vs. rest, 50%, 70%, 85%; (G) $p<0.0001$ vs. rest, 50%, 70%, 85%, 100%.

Table 2
Parameters of effort monitoring before and during an incremental, intermittent exhaustive exercise: heart rate values (heart beats/min.), total work (cal), power (W), rate (beats/min)

parameters	Heart rate (beats /min)		Total work (kJ)		Power (W)		Exercise rate (beats/min)	
Group	trained	untrained	trained	untrained	trained	untrained	trained	untrained
Rest	58±2	66±3						
50% HRmax	100±4	90±3	51.8±0.8	28.1±2.8	94.8±5.3	12.7±3.2	18±2	12±4
75% HRmax	139±3	141±5	80.7±6.8	34.2±3.5	196.1±4.2	34.2±5.6	21±2	18±4
80% HRmax	169±9	155±10	111.3±5.7	41.2±9.6	302.3±11.5	58.4±7.9	25±3	21±3
100%HRmax	189±11	188±17	32.5±6.08	19.3±8.5	551.7±27.3	271.3±34.2	43±3	35±5
Recovery	101±7							

Discussion

Our studies on the purine metabolism of erythrocytes during an incremental, intermittent exhaustive exercise resulted in significant changes in purine nucleotide concentration and their derivatives in the studied sportsmen.

During effort we could observe a gradual decrease of ATP concentration in the sportsmen group. In the untrained group the values were significantly lower than in the sportsmen group and were at the constant level, with a small and insignificant increase during the final stage of the effort (85% and 100% of the maximal heart rate).

ATP concentration in erythrocytes in the sportsmen observed after maximal effort, decreased by 13% compared to the value before the effort; ADP and AMP decreased by 20% and 42% respectively. This decrease in adenine nucleotide

concentration may be caused by temporary hypoxia and a decrease in blood pH, both of which can inhibit glycolysis in erythrocytes. Interestingly, our untrained group did not show any significant changes in adenine nucleotide concentration; similar results were observed by Harkness *et al.* [23] in erythrocytes of non-sporting men after a short and intense effort. Markiewicz *et al.* [32], in their study on 20 year old non-sporting students, observed an insignificant increase in ATP, but a significant decrease in ADP and an increase in AMP concentrations; in only one case they observed a significant decrease in ATP concentration. Similar results were presented by Jaroszewicz *et al.* [25]. The authors of the aforementioned articles suggested that the direct reason for the decrease in ADP and the increase in ATP and AMP may be ADP phosphorylation by adenylate kinase (EC 2.7.4.3), the activity of which is stimulated by the increase in partial blood oxygen pressure during effort [25,32]. The erythrocyte functions as an O_2 sensor, contributing to the regulation of skeletal muscle blood flow and O_2 delivery, by releasing ATP depending on the number of unoccupied O_2 binding sites in the hemoglobin molecule [22]. ATP in turn can induce vasodilatation by binding to P_{2y} – purinergic receptors located on the vascular endothelial cells to release NO – and /or endothelium-derived hyperpolarization factors, which diffuse to the vascular smooth muscle and result in vasodilatation [16, 22]. Gonzales-Alonso [22] reported an increase in ATP concentration in plasma during incremental knee-extensor exercise and after recovery.

It seems that human body reactions to effort may vary in particular individuals. This notion has been confirmed by Błaszczyk *et al.* [8] in their studies on high performance cyclists, where some individuals displayed increased and some decreased ATP concentration. According to the authors, that could be explained by an individual's erythrocyte metabolic response, depending on the initial metabolic state. If ATP concentration at rest was higher, then after effort it decreased; when the ATP concentration was lower, then after effort it increased.

Many authors have reported that submaximal effort results in a decrease in ATP concentration [19,25,32]. It is possible that the changes are caused by the growing metabolic hypoxia both inside and outside erythrocytes, which affects regulatory enzyme activity and hence lowers the rate of glycolysis [14,27,45]. An increase in glucose utilization during initial muscle work and the speeding up of phosphopenthose pathway conversions should not be overlooked here.

It is important to remember that maximal effort may bring out radically different responses in the system than in the case of submaximal effort. The direction of changes in red blood cells after maximal effort may depend on fitness and the system’s efficiency, and also on the initial lactic acid concentration [8].

Lower activity of Na^+, K^+-ATPase in erythrocytes was significantly correlated with a decrease in ATP concentration after maximal effort in sportsmen. One may suppose that the decrease in the enzyme's activity was also influenced by the hyperoxemia of the intra- and extra-cellular environment. ATPases are the enzymes bonding certain ionic forms of ATP, connected to Mg^{2+} or not. The enzymes utilizing energy use Mg^{2+}-ATP. Free ATP may bond with the enzyme but then it inhibits the ATPase reaction [35]. Kinetic studies showed that Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase have high ATP-affinity sites on the cytoplasmic side of the membrane. ATP, as a substrate, may be substituted by other nucleotides, but with lower activity. Quantitative and qualitative distribution of Na^+ and K^+ on both sides of the membrane is carried out by the active transport system (P-type ATPases) [30,34]. Probably about 30% erythrocyte ATP is utilized by the sodium-potassium pump (Na^+, K^+-ATP-ase), which actively transports Na^+ and K^+ [39]. There is a close relationship between Na^+, K^+-ATP-ase activity, the Na^+ and K^+ concentrations, and ATP concentration.

The coenzymes NAD^+/NADH and $NADP^+$/NADPH are redox pair of pyridine nucleotides: their biological effects are determined by the redox state. NAD^+ and its derivatives NADH, $NADP^+$ and NADPH have regulatory functions in the generation of triose phosphates and pyruvate from glucose. NAD^+/NADH catalyzes reactions of glycolysis and sorbitol pathway in the cytosol [33]. The redox pair $NADP^+$/NADPH regulates steps of the sorbitol and pentose phosphate pathway. In this study, we observed a NAD^+ concentration decrease after maximal effort. The decrease may be an additional factor slowing the rate of glycolysis, because NAD^+ deficiency makes it impossible to convert 3-phosphoglycerate into 1,3 - BPG, and in consequence to resynthesize ATP. However, Mairbaurl *et al.* [31] reported a decrease in NAD^+/NADH ratio and 2.3-BPG parallel to acidosis. According to those authors this can be explained by an interruption of red cell glycolysis on the PK and GAP-DH step caused by a lactate and pyruvate influx into the erythrocyte, as well as an intraerythrocytic acidosis and a drop in the NAD^+/NADH ratio. Mairbaurl *et al.* [31] reported a decrease in NAD/NADH ratio and 2.3-BPG parallel to acidosis. According to them, this can be explained by an interruption of red cell glycolysis on the PK and GAP-DH step caused by a lactate and pyruvate influx into the erythrocyte, as well as an intraerythrocytic acidosis and a drop in the NAD^+/NADH ratio. As a result AEC value decreases [5]. A lack of changes in AEC and GEC at the stable level of TAN and TGN in sportsmen erythrocytes may indicate that the sportsmen had very high adaptation abilities.

In this study, we observed sportsmen to have a lower erythrocyte IMP concentration during maximal effort and recovery, and a simultaneous Ino and Hyp

concentration increase. Harkness *et al.* [23], in their study on non-sporting men, also observed a significant (2/4-fold) Hyp concentration increase both in erythrocytes and plasma; IMP concentration did not change significantly. Sahlin *et al.* [38] observed an eightfold increase in Hyp concentration in plasma during prolonged exercise in humans.

Purine bases Ade, Hyp and Gua, and nucleosides Ado, Ino and Guo, present in blood, come from conversions in erythrocytes and other tissues. They may be easily uptaken by erythrocytes, the liver, kidneys and muscles, and then utilized on the purine conversion pathways [44,47]. In erythrocytes IMP does not undergo deamination into AMP, and production of IMP from Hyp is based on salvage reactions. Maybe that was the reason we observed lower IMP concentration and higher Hyp content in the blood. Most likely reason for the increase in RBC Hyp observed is an increased uptake because of an increased release into the circulation form the intensely contracting muscle.

In this study, the IMP decrease in sportsmen parallel to Ino and Hyp increase in blood may have been the consequence of lower erythrocyte AMP deaminase activity. It is confirmed by Hellsten-Westing *et al.* [24] in their studies on muscle bioptate of sprinters after exhaustive training.

In this study, the decrease in phosphorylated nucleotides (ATP, ADP, AMP, IMP) in sportsmen after exhaustive effort, parallel to a degradation products (Ino and Hyp) increase, suggests an intensified nucleotide dephosphorylation [38,44,47]. However, Yamamoto *et al.* [46] reported a significant increase in IMP and Hyp in erythrocytes. A recovery duration of <3 min is too short to allow significant recovery of ATP [43], then, an increased number of exercise bouts per training session may elevate muscle purine loss, with a simultaneous increase in the concentration of degradation products in the full blood. This may occur because the enzymes associated with the degradation pathway of IMP have a longer period in which they are exposed to an elevation of substrate concentrations.

Zhao *et al.* [47] and Sahlin *et al.* [38] confirmed the changes in Hyp and IMP concentration as the markers of the extracellular catabolism of purine nucleotides in muscles subjected to prolonged maximal effort.

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