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Comparing the impact of an acute exercise bout on plasma amino acid composition, intraerythrocytic Ca^{2+} handling, and red cell function in athletes and untrained subjects

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ABSTRACT

The N-methyl D-aspartate receptors (NMDARs) mediating Ca^{2+} uptake upon stimulation with glutamate and glycine were recently discovered in red blood cells (RBC) of healthy humans. Activation of these receptors with agonists triggered transient Ca^{2+}-dependent decrease in hemoglobin oxygen affinity in RBC suspension. The aim of this study was to assess the potential physiological relevance of this phenomenon. Two groups formed by either healthy untrained volunteers or endurance athletes were subjected to a stepwise incremental cycling test to exhaustion. Plasma glutamate levels, activity of the NMDARs, and hemoglobin O_2 affinity were measured in blood samples obtained before and after the exercise in both groups. Increase in plasma glutamate levels following exercise was observed in both groups. Transient Ca^{2+} accumulation in response to the NMDAR stimulation with NMDA and glycine was followed by facilitated Ca^{2+} extrusion from the RBC and compensatory decrease in cytosolic Ca^{2+} levels. Short-term activation of the receptors triggered a transient decrease in O_2 affinity of hemoglobin in both groups. These exercise-induced responses were more pronounced in athletes compared to the untrained subjects. Athletes were initially presented with lower basal intracellular Ca^{2+} levels and hemoglobin oxygen affinity compared to non-trained controls. High basal plasma glutamate levels were associated with induction of hemolysis and formation of echinocytes upon stimulation with the receptor agonists. These findings suggest that glutamate release occurring during exhaustive exercise bouts may acutely facilitate O_2 liberation from hemoglobin and improve oxygen delivery to the exercising muscle.

1. Introduction

Acute endurance training bouts induce transient alterations in hematocrit and properties of red blood cells (RBCs). Multiple studies report acute dynamic changes of plasma volume, pH and osmolarity caused by an exhaustive exercise episode. Increase in total hemoglobin concentration associated with training to exhaustion was caused by dehydration (e.g. [1–4]). Acute transient changes in plasma composition associated with water loss impact RBC volume. Reports on the direction of these changes are contradictory ranging from post-exercise RBC swelling presumably associated by acidosis [3] to a decrease in mean cell volume [4]. Furthermore, single episodes of endurance training are known to cause premature loss of RBCs as mild hemolysis was observed during endurance exercise in some but not all sport disciplines [5]. It was suggested that hemolysis is caused by the mechanical contact of feet with the ground (foot strike). However, hemolytic events were also observed in swimmers [6], as well as in runners, but not in cyclists [5]. Regular training also affects RBC properties. In particular, facilitated RBC loss along with the stimulation of *de novo*

Abbreviations: RBC, red blood cells; NMDARs, N-methyl-D-aspartate receptors; MCV, mean cell volume; Gly, glycine; Glu, glutamate; GSH, reduced glutathione; GSSG, oxidized glutathione.

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production of RBC is triggered already within two days after the onset of training giving rise to a younger population of RBC in trained amateur sportsmen and professional athletes [7]. A single training bout may cause alterations in RBC deformability. Notably, these responses are discipline-dependent with both reduction in deformability and increase in RBC rigidity reported for different types of exercise (e.g. [8]).

In an attempt to unravel the mechanisms of mean cell volume regulation during exercise, RBC collected from subjects at rest were exposed to plasma obtained from identical persons during the exercise. This manipulation resulted in the stimulation of both passive K^+ loss and active K^+ re-uptake by the Na,K-ATPase [9] that was not related to adreno-stimulation or acidosis. The factors secreted into the circulation during the exercise in control of these ion movements have never been identified.

Apart from the compound(s) released into the circulation during the training bout, exercise is associated with exposure of the circulating RBC to facilitated shear and oxidative stresses. Oxidative burst is always associated with exercises [10]. Free radicals are generated by the exercising muscles [11] and by partially deoxygenated RBCs themselves [12].

What are the factors involved in exercise-induced responses of RBC? Are any of those changes adaptive? May these exercise-induced responses of RBC be a manifestation of cell damage? And what are the triggers and master regulators of these responses? Oxidative and advanced shear stresses are the two factors that cause alterations in RBC membrane structure and properties. Shear stress was reported to trigger Ca^{2+} uptake [13] and induce NO production in human RBCs [14]. Recent progress in characterization of the conductive Ca^{2+} pathways in the RBC membrane suggests that Ca^{2+} plays a role of a second messenger responding to both stimuli. Mechano-sensitivity of PIEZO1 ion channels mediating Ca^{2+} uptake into human RBC [15–17] is widely discussed. There are some indications for mechano-sensitivity of yet one more ligand-gated Ca^{2+} channel in RBCs, erythroid N-methyl D-aspartate receptors (NMDARs) [18]. These receptors are also sensitive to the alterations in redox state [19]. Acute transient increase in the intracellular free Ca^{2+} occurring upon supplementation of the NMDAR agonists was reported to facilitate oxygen release from hemoglobin in shear stress-free RBC suspension [20]. Whether these findings are of physiological relevance and whether erythroid NMDAR activation actually occurs in RBCs passing through the muscle capillaries, remains unclear. Intensive shear conditions created within the intramuscular capillary circuit during exhaustive exercise bouts would contribute to the activation of one or both types of Ca^{2+} channels. Furthermore, exercising muscles are a potential source of plasma glutamate. Concentration of this amino acid in the muscle exceeds the one in plasma by more than 200-fold [21] and micro-rupture would result in glutamate leakage into the circulation.

When occurring during exercise, transient Ca^{2+} uptake pulses will hit multiple downstream targets in RBC. Those include erythroid NADPH oxidases [22] and endothelial NO synthase [18,20,23]. Both enzymes respond to an increase in Ca^{2+} with activation and a burst of $O_2^{\bullet -}$ and NO production. Long-term effects of repeated cycles of Ca^{2+} accumulation/extrusion in RBC of athletes in the course of regular training sessions will then include repeated oxidative bursts and progressive proteolytic cleavage of multiple targets by the Ca^{2+}-dependent protease μ-calpain [24]. Both oxidation and proteolysis are increased during exercise [25]. However, the impact of Ca^{2+}-homeostasis in RBCs into these processes has never been evaluated.

The present study was designed to monitor the effect of an acute training bout performed by endurance athletes and control subjects with moderate basal level of physical activity on the plasma amino acids' composition, special attention paid to glutamate levels. Activity of the erythroid NMDARs (Ca^{2+} uptake and the number of receptors active in contact with autologous plasma) was assessed before and after the training in both groups and related to the basal intracellular Ca^{2+} levels in RBCs and total number of NMDARs per cell available for activation. We furthermore detected basal P_{50} of hemoglobin in RBCs collected before and after the training as well as the changes in O_2 affinity of hemoglobin upon maximal NMDAR stimulation with agonists of the receptors. The obtained results support our hypothesis on the impact of endurance exercises on the intraerythrocytic Ca^{2+} levels. Alterations of plasma glutamate levels along with facilitated Ca^{2+} uptake by the NMDARs in cells of athletes caused by the exercise bout point to the potential participation of glutamate and the erythroid NMDARs in exercise-induced alterations of RBC properties in humans.

Table 1
Study participants indices.

	Athletes	Controls	p-value
Sex (m/w)	6/6	6/6	n.a.
Age (years)	24 ± 5	25 ± 3	0.73
Heigth (cm)	176 ± 10	173 ± 9	0.52
Body mass (kg)	65 ± 10	69 ± 12	0.41
BMI (kg/m^2)	21 ± 1	23 ± 3*	**0.04**
Pmax (W/kg)	5,2 ± 0,5	3,5 ± 0,7**	**<0.01**

BMI = body mass index; Pmax = maximal anaerobic power.

2. Materials and methods

2.1. Participants

12 elite endurance athletes (6 males, 6 females; 10 triathletes, 2 cyclists) as well as 12 age- and sex-matched, moderately active controls were recruited. All athletes compete on an international level and were mostly recruited at the Olympic training center Saarbrücken. Two of the study subjects participated in the 2012 Olympic games. Controls were matched for sex and age (±2 years) and are engaged in recreational physical activities only. Subject characteristics are summarized in Table 1.

All subjects gave written informed consent prior to participation. The study was approved by the local ethics committees (Ärztekammer des Saarlandes; ID 115/12).

2.2. Blood sampling

Participants reported to the laboratory between 8 and 10 a.m. after abstaining from physical exercise for at least 36 h. A fasting period was not required to enable the recruitment of elite athletes. After a supine rest period of 10 min venous blood samples were collected from the antecubital vein by standard techniques. Post-exercise blood samples were collected in the same way 30 min after cessation of exercise. All blood samples were processed immediately.

2.3. Exercise testing protocol

An exhaustive, stepwise exercise test was conducted on a calibrated cycle ergometer (Excalibur Sport, Lode B.V., Groningen, Netherlands). Initial load was 50 W for women and 100 W for men. Step duration and increment were 3 min and 50 W, respectively. Verbal encouragement was given to all subjects during the final stages of the exercise test. Capillary blood samples for the determination of blood lactate concentration were taken from the hyperemizied earlobe at rest, during the last 15 s of each step as well as 30 min after cessation of exercise. Samples were immediately hemolyzed and analysis carried out using an enzymatic-amperometric system (Super GL, Greiner, Flacht, Germany).

Objective criteria of exhaustion (maximal blood lactate concentration of >8 mmol/l and maximal heart rate of >200-age (years)) were met by all subjects.

2.4. Timing of detection of various blood parameters

Conventional blood cytology and biochemistry analyses were performed immediately after blood collection. Plasma was collected immediately upon blood sampling and stored for amino acid analysis. Ca^{2+} uptake by RBC and blood rheology were assessed on the day of blood collection. Determination of the number of receptors ([^{3}H]-MK-801 binding assay), reduced and oxidized glutathione, annexinV binding, the number of CD71-positive cells, and the effect of glutamate/NMDA on the hemoglobin oxygen affinity was performed 12–24 h after withdrawal, time required for a courier delivery of samples from Saarland University to the Zurich University (shipment temperature within 10–20 °C, duration 12–24 h). Effects of transportation on the parameters we were measuring were studied prior to this study and taken into consideration when interpreting the obtained results.

2.5. Plasma amino acid measurements

Plasma amino acids were measured by AccQ-Tag and MassTrak running on an ACQUITY UPLC® System (Waters) at the Functional Genomics Centre Zurich.

2.6. Intracellular Ca^{2+} monitoring using microfluorescence assay

Intracellular Ca^{2+} measurements were performed within the first six hours after blood withdrawal by imaging intracellular Ca^{2+} using the fluorescent Ca^{2+} indicator Fluo-4 [26]. RBC were loaded with 5 μM Fluo-4 (Life Technologies, Darmstadt, Germany) for 1 h at 37 °C. Fluo-4-loaded RBCs were plated onto coverslips and 15 min were allowed for cell sedimentation and dye de-esterification. Fluorescence was measured as previously described [27] with the following adaptations: Cells were placed on an inverted microscope (TE2000, Nikon, Tokyo, Japan) equipped with a 60x Plan Apo 1.4 objective. A video-imaging device (TILL Photonics, Munich, Germany) was attached to the microscope and contained a monochromator (Polychrome IV), a camera (Imago), the imaging control unit and acquisition software (TILL-LA). Fluorescence intensity in RBC was recorded for 1 min in Tyrode solution containing (in mM): 135 NaCl, 5.4 KCl, 10 glucose, 1 $MgCl_2$, 1.8 $CaCl_2$ and 10 HEPES. The pH was adjusted to 7.35 using NaOH to get the baseline values. Then 300 μM NMDA and 100 μM Glycine (NMDA/Gly) was added by the perfusion system and recorded for another 14 min. Images were analysed on a cell based fitting algorithm as recently reported [28] starting with the definition of regions of interest in ImageJ (Wayne Rasband, National Institute of Mental Health), and the resulting traces were further processed by IGORpro software (WaveMetrics Inc., USA) and custom-developed macros. Intracellular Ca^{2+} was shown to be stable over 24 h of shipment at room temperature.

2.7. Rheometrical measurement to test for alteration in RBC aggregability

Rheological measurements of whole blood were performed as previously described for RBC suspensions in dextran solutions [29]. Experiments were carried out using a commercial constant shear rheometer Mars II, Thermo Scientific, Karlsruhe, Germany equipped with a cone-plate geometry (diameter: 60 mm, angle: 1 u) at a temperature of 23 °C. Samples were pre-sheared at 100 s^{-1} for one minute to homogenise the samples, limit sedimentation and obtain comparable initial conditions. Treatment with NMDA/Gly was performed directly prior to the measurements. Paired Student's *t*-test following the normality test was used to assess the significance in the training-induced alterations in the groups of non-trained subjects and athletes.

2.8. NMDAR activity in RBC fractions

Whole blood samples in duplicates (one control blood sample and one more supplemented with 300 μM NMDA and 300 μM Gly) were incubated with [^{3}H]MK-801 (20 Ci/mmol; Perkin Elmer, Schwerzenbach, Switzerland) at a concentration of 5×10^{-7} mmol/ml (1 h, room temperature). Thereafter, both blood samples were mixed with isotonic 90% Percoll solution and RBCs fractionated according to their density by centrifugation in Percoll density gradient at 45′000*g* for 30 min. Light (L), medium (M) and dense (D) fractions were collected as shown elsewhere [20]. Cells forming these fractions were washed with calcium-free buffer containing (mM): 145 NaCl, 5 KCl, 10 glucose, 0.1 EDTA, 10 Tris·HCl (pH 7.4 room temperature) supplemented with 0.1% bovine serum albumin. One part of the cell pellet was then mixed with 30 parts of the lysis buffer containing 25 mM NaH_2PO_4 and 1 mM EDTA (pH 7.0 at 0 °C) and incubated on ice for 30 min to achieve complete hemolysis. Thereafter, RBC membranes were sedimented by centrifugation (45′000*g*, 30 min at 4 °C), membranes lysed in 10 ml of Quicksafe A scintillation fluid (Zinsser Analytic, UK) and radioactivity of the RBC membrane fractions assessed using Packard 1600TR liquid scintillation analyzer. The number of [^{3}H]MK-801 molecules per RBC was calculated according to the following equation:

$$N = \frac{A_{cells}}{A_{sp} \times N_A \times N_{cells}}$$

where N is a number of [^{3}H]MK-801 binding sites per cell, A_{cells} is radioactivity of membrane fraction in Bq corrected for the non-specific binding (see below), A_{sp} is specific activity of [^{3}H]MK-801 used for labelling Bq/mol, N_A is the Avogadro constant (6.022×10^{23} mol^{-1}), and N_{cells} is a number of RBCs from which the membranes were prepared.

Specificity of binding of [^{3}H]MK-801 was confirmed by performing the non-specific binding test in which the radiolabelled antagonist was allowed to bind to the cells in the presence of 1000-fold excess of non-labelled MK-801 (or detailed information see [23]). No changes in the number of NMDARs detected in RBCs with the [^{3}H]MK-801 binding assay were shown within 24 h of transportation.

2.9. Morphological responses to the NMDAR stimulation

RBC were washed with the medium containing (mM) 145 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.1% bovine serum albumin (BSA), and allowed to sediment at the glass bottom of the imaging chamber. Images of RBCs were taken before and within 12 min after supplementation of stock solution of buffered mixture of glutamate and glycine (30 mM each) to reach a final concentration of 300 μM of each amino acid. Images of cells were taken by Axiovert 200 M fluorescence microscope (Zeiss) equipped with Axiocam camera and oil emersion phase contrast ×100 objective.

2.10. Oxygen dissociation curve and P_{50} measurements

Hemox blood analyzer (TCS Scientific, New Hope, PA) was used for monitoring of P_{50} in blood samples. Five microliters of packed RBCs resuspended in buffer containing 145 mM NaCl, 5 mM KCl, 10 mM Imidazole-HCl (pH 7.40 at 37 °C), 10 mM glucose, 0.1 mM EDTA, pH 7.4, 37 °C supplemented with 2 mM $CaCl_2$ were added to

5 ml of buffer containing or free from the saturating concentrations of NMDAR agonists (300 μM NMDA and 100 μM glycine), conditions identical to the ones we have applied to assess the effects of Ca^{2+} on O_2 affinity of hemoglobin in our earlier studies [23]. Oxygen dissociation curves were recorded at 37 °C and P_{50} calculated from them using Hemox Analytical Software (HAS).

2.11. Intracellular glutathione content

Intracellular glutathione levels were measured in RBCs using Ellmann's reagent. Briefly, 200 μl of blood were added to 800 μl of 5% trichloracetic acid solution and stored in liquid nitrogen. Blood hematocrit was measured in triplicates using capillaries and used for normalization. Protein sediment was removed by centrifugation and the amount of reduced non-protein thiols was assessed by mixing aliquots of supernatant with Ellmann's reagent dissolved in phosphate buffer and measuring extinction at 412 nm by Lambda spectrophotometer (Perkin Elmer). In parallel samples were prepared in which oxidized glutathione (GSSG) was reduced by a mixture of glutathione reductase and NADPH. After the reduction step was completed the levels of non-protein thiols were measured in these samples and GSSG levels calculated as a difference between the GSH levels in sets of samples with and without glutathione reductase supplementation. Earlier on we have shown that 24 h of shipment cause a decrease in the intracellular GSH content by ~35% (Makhro and Bogdanova, unpublished). This consideration was taken into account when interpreting the obtained date.

2.12. Detection of the number of reticulocytes, white blood cells and annexinV-positive cells

RBCs were fixed in isotonic phosphate buffer containing 1% glutaraldehyde and stained with the following antibodies and fluorescent markers. Mouse monoclonal antibody against transferrin receptor (CD71) conjugated to APC (eBiosciences/affymetrix, #17-0719-42) was used to assess the number of reticulocytes, mouse monoclonal antibody against CD45 conjugated to PC7 (Beckman/Coulter, #IM35480) was used as a marker for white blood cells. Phosphatidylserine exposure to the outer leaflet of plasma membrane was detected by treating the cells with annexin V conjugated to eFluor 450 (eBioschence/affymetrix, #88-8006-74). All compounds were used in dilutions suggested by the companies. Flow cytometry was used to assess the number of RBCs positive for the markers.

3. Results

3.1. Effects of acute exercise on plasma amino acid composition

In both control group and athletes cycling exercise induced glutamate release (Fig. 1), that was more pronounced in control subjects (Table 2). Increase in plasma glutamate did not differ between male and female study participants. Exercise was associated with a 3.5% hemoconcentration (hemoglobin levels changing from 13.5 ± 1.1 g/dL before to 14.0 ± 1.3 g/dL after the exercise bout, mean ± SD p = 0.000552). But, even when corrected for it (see the post-exercise values for amino acids' concentrations in brackets in Table 2), the significance of alterations caused by the exercise was preserved for all amino acids measured. Along with glutamate, cycling to exhaustion caused an increase in plasma alanine levels as well as in taurine and histidine, but only in plasma of athletes. In plasma of control subjects glycine levels were insignificantly lower after the exercise whereas a decline in plasma of athletes was sounder and statistically significant. In all subjects the levels of glycine remained above the saturating threshold for the NMDARs at baseline and after the exercise. Similar pattern

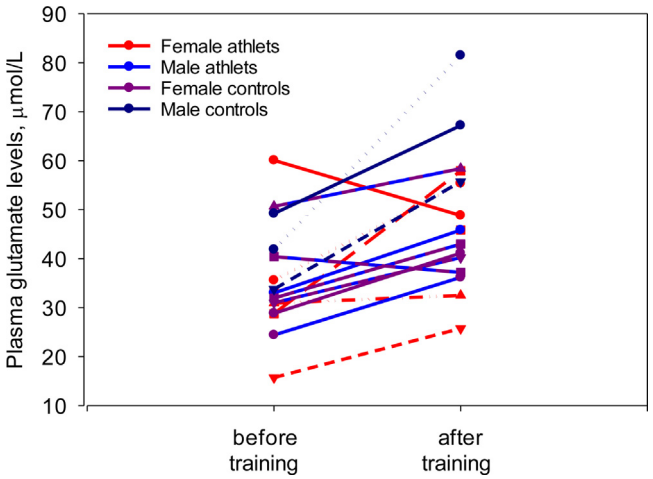

Fig. 1. Plasma glutamate levels in blood of study participants (N = 19) before and after the short exhaustive cycling bout. Each pair of symbols connected with the line represent single individual. Presented in red are data for female athletes, in light blue are the values for male athletes, violet and dark blue stand for female and male untrained control subjects respectively. P value for the paired *t*-test (at rest vs post-training) is shown.

was monitored for lysine and serine. In post-exercise plasma of athletes post-exercise plasma histidine accumulation was more pronounced and statistically significant. Decrease in plasma levels of some essential amino acids including valine, isoleucine, leucine, phenylalanine, and tryptophan was recorded after the training in control group. In athletes, exhaustive cycling bout was followed by depletion of plasma ornithine, and threonine pools as well as reduction in leucine and isoleucine levels whereas the changes of phenylalanine and tryptophan were not statistically significant.

3.2. NMDAR activity in RBCs before and after the exercises

Erythroid NMDARs are the targets for plasma-borne glutamate, aspartate and glycine [20]. The changes in activity of erythroid NMDARs following the exercise bout were assessed using microfluorescence live imaging and [^{3}H]-MK-801 binding assay.

Basal Ca^{2+} levels in RBCs of untrained controls and athletes loaded with Ca^{2+}-sensitive dye Fluo 4-AM were detected at rest (baseline) and after the exercise bout in the agonists-free Ca^{2+}-containing Tyrode solution. In addition Ca^{2+} uptake triggered by the stimulation of NMDARs with saturating doses of specific agonists NMDA (300 μM) and glycine (100 μM, NMDA/Gly) was detected at rest and post-exercise. RBCs of athletes contained significantly less free Ca^{2+} at rest as well as after the exercise than the cells of untrained control subjects (Fig. 2A). Following the cycling bout intracellular Ca^{2+} levels were decreased in both groups compared to the values measured at rest (Fig. 2A). Maximal activation of the NMDARs in RBC with NMDA/Gly triggered Ca^{2+} uptake manifested as an increase in fluorescence intensity (F) over the baseline levels before the NMDAR stimulation (F0) in both groups at any occasion (Fig. 2B). In agreement with our previous observations a great spread of the amplitudes of the NMDA/Gly-inducible upstroke of Ca^{2+} uptake (F/F0) in individual cells was observed [20]. The median amplitude of NMDAR-mediated Ca^{2+} uptake at rest was lower in athletes than in untrained controls, but increasing after the exercise. In contrast to that, initially high Ca^{2+} uptake triggered by the agonists of NMDARs in control group remained unaltered by the exhaustive exercise (Fig. 2B).

The number of NMDARs in mature erythrocytes detected forming the medium density fraction in Percoll density gradient was similar in athletes and untrained controls making 9.1 ± 2.7 (n = 10) and 8.5 ± 4.2 (n = 8) receptors per cell respectively. Plasma gluta-

Table 2
Amino acid levels in plasma of control subjects and athletes before (basal levels) and 30 min after the exercise.

	Controls, N=7			Athletes, N=11		
	basal	training	*p*	basal	training	*p*
Ala	351.4±41.9	467.7±69.1 (453.6)	5.00E-04	317.7±79.3	439.4±84.7 (418.7)	5.00E-06
Glu	36.7±7.9	51.2±17.8 (49.7)	0.019	35.2±11.8	44.2±9.3 (42.0)	0.02
Ser	87.2±17.0	84.3±10.7	ns	87.6±22.1	80.3±13.6 (76.4)	0.025
Gly	205.6±43.1	200.5±54.2	ns	241.5±39.2	222.5±39.2 (212.9)	0.017
Tau	42.6±6.3	53.2±14.6	ns	36.5±8.1	47.2±12.0 (45.1)	0.012
Orn	40.2±8.7	36.3±10.1	ns	46.2±13.2	39.9±11.7 (38.1)	4.00E-03
Lys	150.6±21.7	151.1±18.9	ns	138.3±34.6	119.3±17.6 (113.4)	0.011
His	81.6±7.3	85.7±9.5	ns	80.8±17.2	88.0±15.2 (83.6)	0.017
Val	218.6±45.8	196.3±38.2 (190.3)	0.036	207.7±42.6	194.8±29.3 (185.0)	ns
Ile	63.5±16.6	51.8±12.1 (50.2)	0.02	58.6±16.9	50.1±8.4 (47.5)	0.02
Leu	123.6±31.0	105.9±21.5 (102.7)	0.038	120.3±30.0	106.9±13.4 (101.5)	0.03
Phe	51.5±9.9	46.6±8.7 (45.3)	0.028	51.1±8.8	49.4±6.5 (47.1)	ns
Trp	47.2±9.5	37.1±7.4 (36.0)	1.00E-03	42.5±8.8	38.6±4.8 (36.7)	ns
Thr	112.9±32.8	117.4±26.4	ns	98.0±29.3	89.6±21.8 85.1	0.017

Essential amino acids are highlighted in bold. Highlighted in orange are the amino acids released into the circulation during the exercise whereas the amino acids depleted during the exercise are highlighted in green.
Presented are the mean values in μmol/L ±SD and the p values for the Student's paired *t*-test comparing the values before and after the exercise for each participant. Presented in brackets are the mean values for the amino acid concentrations corrected for hemoconcentration (see text).

mate levels also did not differ between the groups (Table 2). Based on all these findings one can suggest that the lower Ca^{2+} uptake by the NMDARs of athletes and its increase upon exercise is a result of signaling processes controlling the receptor function.

The impact of the NMDARs on the regulation of intracellular Ca^{2+} was assessed as free Ca^{2+} levels in RBC in both groups were plotted against the number of NMDARs remaining active in plasma measured using [^{3}H]MK-801 binding (Fig. 2C). A weak but significant negative correlation was obtained between these parameters. We also evaluated how many of NMDARs remain active RBCs suspended in autologous plasma. Out of 4–10 receptors per cell that were detected in the M fraction of RBCs of various subjects (see above) maximum 1–6 remain active in autologous plasma (Fig. 2D). It was never the case that all receptors were found in active state when cells were plasma-borne.

3.3. Oxygen affinity of hemoglobin: effect of the NMDAR stimulation in athletes and untrained control subjects

We have recently shown that stimulation of the erythrocytic NMDARs was associated with acute temporary decrease in hemoglobin oxygen affinity [20]. Due to the technical difficulties, we could not assess oxygen affinity directly in RBC passing through the muscle vascular beds. We hence explored the changes in oxygen dissociation curves in RBCs of untrained control subjects and athletes before and after the exercises in the absence or in the presence of saturating concentrations of the NMDAR agonists. When plotted against the basal pseudo-steady state intracellular Ca^{2+} levels at rest, the P_{50} values obtained for the same conditions and same subjects followed a bell-shaped dependence (Fig. 3A). Non-linear dependence of P_{50} from free intracellular Ca^{2+} was earlier on confirmed for hemoglobin solutions [20]. The "outlier" point for RBCs with maximal intracellular Ca^{2+} levels associated with low P_{50} levels was obtained for a male athlete participant presented with the highest levels of post-exercise plasma glutamate levels (41.8 vs 81.5 μmol/L basal vs post-exercise values). Further analysis revealed the lack of correlation between plasma glutamate levels (both basal and post-exercise values) and P_{50} measured in RBCs collected from the study participants at rest (supplementary Fig. 1).

In agreement with earlier reports of other groups, basal P_{50} levels in cells of athletes at rest were significantly higher than P_{50} of untrained control subjects. Acute stimulation of the NMDARs in RBC of non-trained subjects and athletes resulted in further up-regulation of P_{50} in both groups. The difference in absolute P_{50} values between the cells of athletes and those of untrained controls was maintained after the stimulation, giving rise to maximal O_2 release capacity in stimulated RBC of athletes (Fig. 3B).

3.4. Intracellular glutathione levels

Reduced (GSH) and oxidized (GSSG) glutathione levels were measured in RBCs before and after the exercise (supplementary Table 1). GSH levels slightly, but insignificantly decreased after the exhaustive cycling bout in both groups (95.7 ± 2.9% of basal values observed in non-trained controls and 97.6 ± 4.6% in athletes). Intracellular GSSG showed a modest inverse increase to 104 ± 21% in control, and to 101 ± 16% in athletes. The limitation of this finding is the delayed (12–24 h after collection) assessment of redox state as blood samples. The measurements were performed in RBC12–24 h after the collection. We have estimated a decrease in intracellular GSH to reach 36% of baseline levels within 24 h of

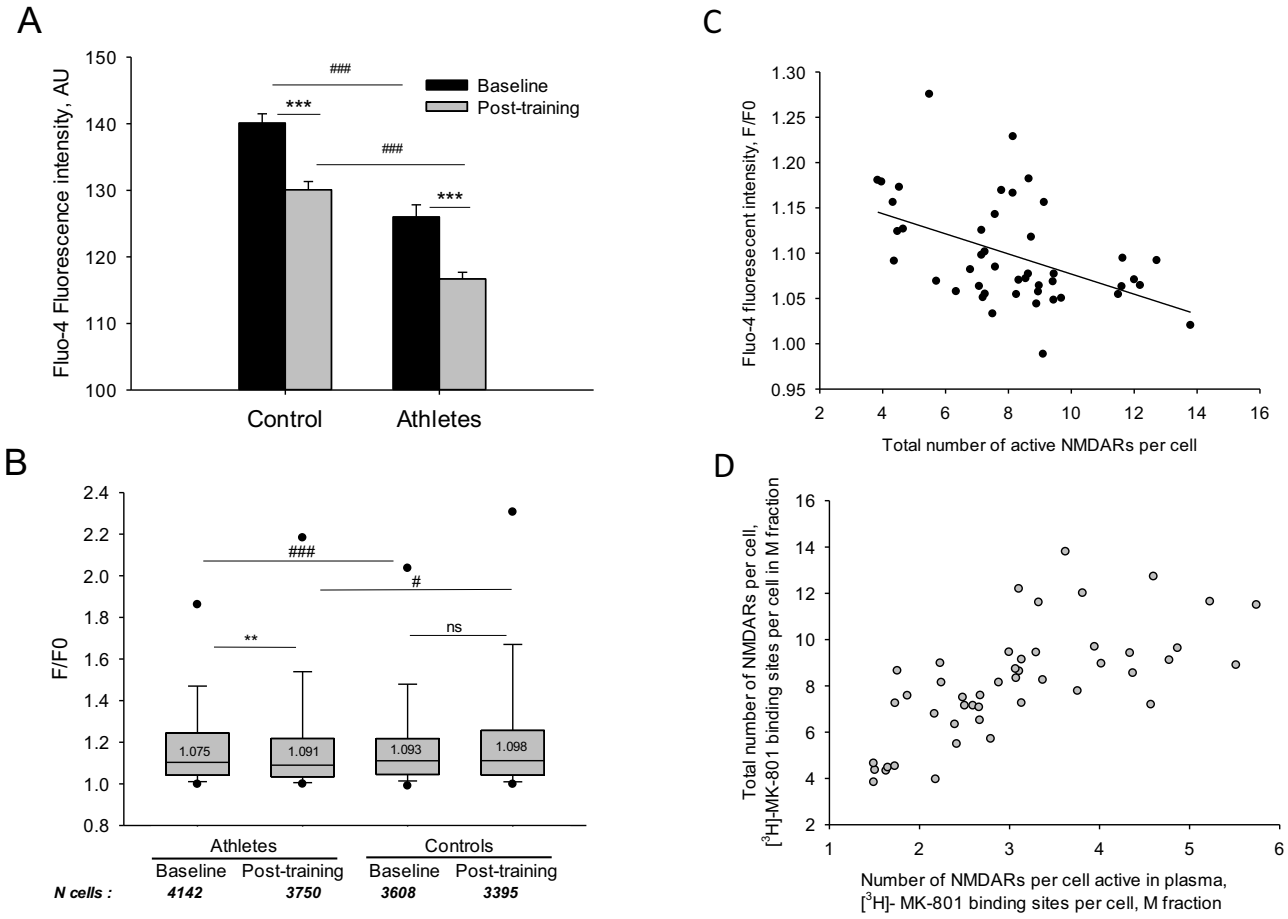

Fig. 2. Cross-talk between the training bout, intracellular Ca^{2+} levels and activity of the NMDA receptor in RBCs of 12 non-trained controls and 13 athletes.
A: basal intracellular Ca^{2+} levels in cells suspended in own plasma assessed by means of microfluoscence imaging (fluo-4 AM) in control subjects and athletes at baseline (black bars) and after (grey bars) the training. Values are mean fluorescence intensity in arbitrary units. B: box plots show medians of maximal increase in Fluo-4 fluorescence intensity (F) triggered by the stimulation of RBC with saturating NMDA/Gly concentrations normalized to that in non-stimulated RBCs (F0) in athletes and non-trained controls before (baseline) and after the exercise (post-training). Presented are medians (numbers within the boxes) of all cells (N) tested. Mann Whitney test was used to determine significance of differences between the means or median values as the normality of distribution of the values was not confirmed. ** denotes $p < 0.01$ *** stands for $p > 0.001$ when values before and after the training were compared. # and ### stand for $p < 0.05$ and 0.001 when comparing the corresponding values between the athletes and non-trained controls. NS stands for "not significant". C: intracellular Ca^{2+} in RBCs obtained before and after the exercise re-suspended in plasma supplemented with saturating NMDA and glycine concentrations (panel B) as a function of the total number of NMDARs per cell mature RBC fraction ((M) fraction) in all study participants. Each point represents a single measurement (N = 45) Linear regression correlation: R = 0.4675, p = 0.00028. D: the inter-individual variability of NMDAR activity in plasma as a function of total NMDAR availability in RBCs (N = 47).

simulated postage at room temperature (Makhro and Bodganova, unpublished observations). However, the relative differences in GSH levels are expected to be preserved. Furthermore, no differences between the groups as well as within the groups between the conditions was observed in methemoglobin levels (supplementary Table 2).

3.5. RBC lysis and shape changes in response to acute exercise

Exercise was associated with hemolysis and echinocytosys in 8 out of 22 tested persons forming the "training-sensitive" group. This was a mixed group consisting of 4 athletes and 4 untrained controls. The examples of ghost formation and echinocytes' appearance after the exercise are exemplified in Fig. 4A and B. Of note, the A2 subject showing particularly prominent post-exercise hemolysis is the "outlier" with maximal intracellular Ca^{2+} levels from Fig. 3A (see Section 3.3). Sensitivity of RBCs to training-related stress was associated with higher basal plasma glutamate levels (Fig. 4C) although no correlation was found between specific shape changes (echinocytes or stomatocytes) and plasma glutamate levels. Plasma glutamate levels measured after the training were not significantly different between the "sensitive" and "insensitive" individuals.

A single training bout caused no increase in the amount of cells in which phosphatidylserine was exposed to the outer membrane leaflet (all numbers within 0.44–0.58% of total cell population as measured by the annexinV binding test). The amount of CD71+ cells was also unchanged by the training. Athletes, but no the untrained controls showed a slight, but significant increase in CD45+ cells (0.37 ± 0.07% before vs 0.51 ± 0.08% of total cell population after the training, $p = 0.0061$ in paired *t*-test). This was not the case in untrained controls in which number of CD45+ cells equaled to 0.45 ± 0.11% of total population before and 0.50 ± 0.12% after the training bout.

3.6. RBC rheology as a function of cycling bout

Rheology of RBC in whole blood of athletes and untrained controls was monitored before and after the training bout. As shown in Table 3, a single training episode was not altering cellular deformability in any of the groups. A Student's paired *t*-test did not reveal

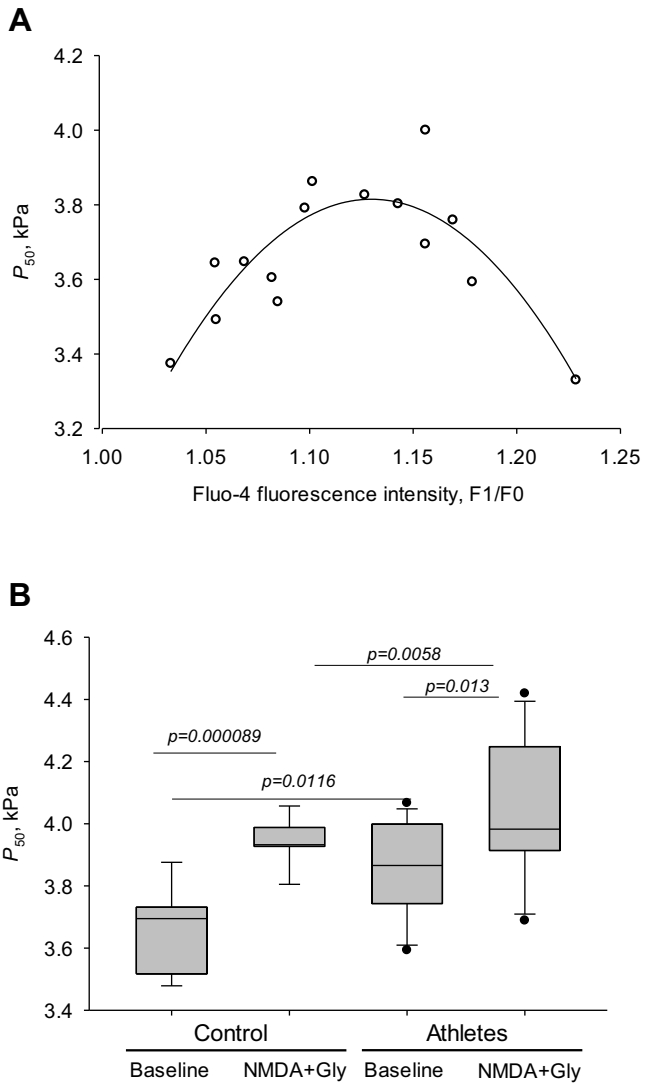

Fig. 3. Oxygen affinity of hemoglobin as a function of intracellular Ca^{2+} and the NMDAR stimulation with the agonists. A: Oxygen partial pressure required for half-saturation of hemoglobin (P_{50}) as a function of intracellular Ca^{2+} in all subjects tested. Each point represent one study participant (n = 15) B: basal P_{50} levels and the ones immediately after acute stimulation with the receptor agonists (Control group: n = 7, Athletes: n = 12). The data were analyzed using paired or unpaired Student's *t*-test following the normality test. The obtained P values are shown in the graph.

Table 3
Shear rate viscosity of RBC of untrained control subjects and athletes suspended in dextran solution before and after the exercise bout at baseline and after the stimulation with NMDA/Gly.

	Before training	After training
Control Baseline	0.0186 ± 0.0102	0.0205 ± 0.016
NMDA/Gly	0.0230 ± 0.0236	0.0284 ± 0.0228
Athletes Baseline	0.0169 ± 0.0072	0.0190 ± 0.0084
NMDA/Gly	0.0171 ± 0.0082	0.0289 ± 0.0186

Values are expressed in Pa. Data are means ± SD for 12 subjects in each group.

any statistically significant differences in viscosity before to after the training and before vs after the NMDA/Gly stimulation.

4. Discussion

This is the first study emphasizing the potential impact of single exercise and long-term training on Ca^{2+} movements across the RBC membrane and the pseudo-steady state free Ca^{2+} levels in the cells. The alterations in plasma glutamate occurring during a single training bout were revealed. These changes were assessed in venous blood. Although we could not directly confirm the muscular origin of glutamate release, the local levels of plasma glutamate could be even more prominent than those we have recorded within this study. Increase in plasma glutamate could trigger stimulation of NMDARs in RBC of exercising subjects causing transient Ca^{2+} uptake episodes. Increase in the intracellular Ca^{2+} in turn appears to have an immediate impact on the hemoglobin oxygen affinity, but not on blood rheology or redox state. This study is not powered to draw any definitive conclusions on the association of high plasma glutamate levels with shortening of the RBC life span in athletes. However, the data obtained for the A2 subject as well as the findings summarized in Fig. 4C suggest that it may be the case.

4.1. Plasma glutamate alterations during the exercise

Alterations in plasma amino acids in our experimental settings were not related to the food ingestion, as the study participants were abstaining from the meal before and during the training session were, thus, clearly exercise-induced. Increase in plasma glutamate levels reflects the amino acid release or reduction in its reabsorption by the peripheral organs, of which skeletal muscle is the most abundant. Exercising muscle is commonly viewed as a secretory organ capable to support optimal blood supply. Secreted factors called "myokines" include proton equivalents, metabolites, NO, and hundreds of peptides of which interleukin-6 and 10 are most studied [30]. Further studies and novel fast in vivo monitoring techniqus are necessary to get direct evidence that amino acids, in particular glutamate, may be added to the myokines' list. Earlier on Rennie et al. [31] shed light to the multifaceted function of amino acid transporters in exercising striated muscles. It remains unclear whether glutamate release we have observed was mediated by a specific amino acids- or osmolyte transporter or whether it occurred passively following the gradient from ~4000 to 30 μM between the muscle and the plasma compartments [32]. Glutamate transporters expressed in striated muscle cells include excitatory amino acid transporter 1 EAAT1A3 also known as GLAST, [33] and EAAT1A5 [34]. However these anionic amino acid transporters are usually involved in $Na^+/K^+/H^+$-coupled glutamate and aspartate uptake into the myocytes rather than their release into the circulation. Lactoacidosis, however, may inverse the direction of glutamate transport making it driven by the proton gradients.

4.2. Calcium levels and exercise

Several mechano-sensitive conductive pathways, one of which is also responding to the changes in plasma glutamate levels, mediate Ca^{2+} uptake by human RBC [17,18]. Both glutamate release and shear stress are thus expected to add up in an acute increase in the intracellular Ca^{2+} and transient dehydration [20]. Limitation of the approach we used for testing of the impact of glutamate on P_{50} is the lack of shear stress that would contribute to the maintenance of Ca^{2+} at higher levels increasing the opening probability of mechano-sensitive Ca^{2+} channels. Transient increase in the intracellular Ca^{2+} following the NMDAR stimulation stimulates K^+ loss through the Gardos channel [18,20,35].

RBCs of athletes contain less free Ca^{2+} at rest than the cells of untrained controls (Fig. 3A) and the activation of NMDARs in them results in a lower amplitude of transient Ca^{2+} uptake than in untrained subjects (Fig. 3B). However, exercises trigger facilitation of the Ca^{2+} uptake via the NMDARs upon their stimulation with agonists in RBCs of athletes (Fig. 2B). This suppression of the Ca^{2+} uptake capacity by the NMDARs (NMDA-glycine-inducible pathway) contributes to the lower intracellular Ca^{2+} levels in RBCs observed before training but does not explain even further decrease

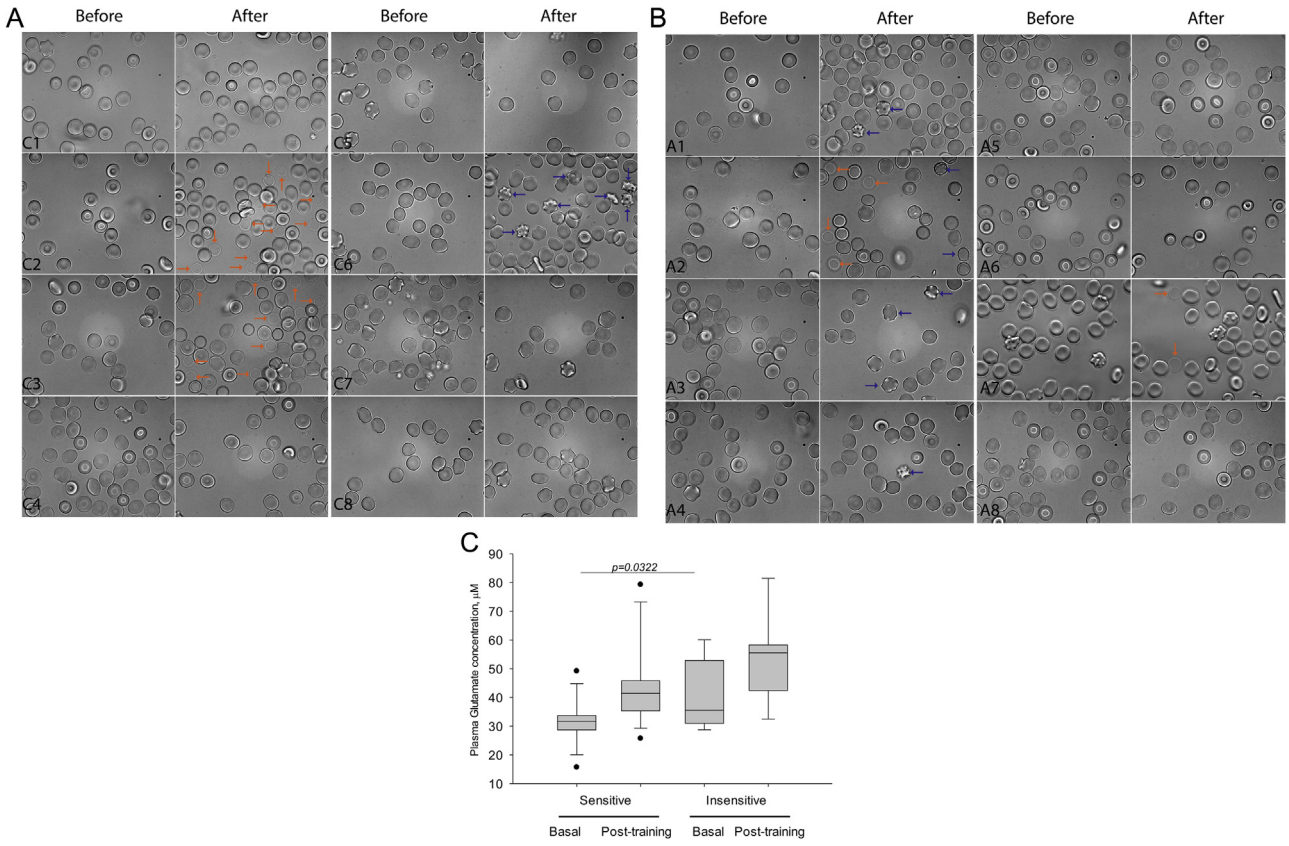

Fig. 4. Representative bright field images of RBC of 8 control non-trained subjects (panel A) and 8 athletes before and after the exercise (panel B). Ghosts and echinocytes formed after the exercise are highlighted with arrows. C: plasma glutamate levels in blood of participants found sensitive (showing hemolysis and morphological abnormalities, n = 8) and insensitive (n = 14) to the exercise-induced stress before and after the exercise. Mann Whitney test showed significant difference between the plasma glutamate levels in plasma of "sensitive" and "insensitive" groups at rest (basal).

in Ca^{2+} levels observed after the exercise was completed. Extrusion of Ca^{2+} by the Ca^{2+} pump should be equally efficient to quickly bring the intracellular Ca^{2+} down after the training. Increase in activity of the plasma membrane Ca^{2+} pump was reported earlier on as a result of limited proteolysis by Ca^{2+}-sensitive protease μ-calpain [36]. Periodic activation of μ-calpain is most likely caused by Ca^{2+} oscillations in RBCs during the regular exhaustive training sessions. Effects of long-term regular training on the activity of Ca^{2+} pump await further investigation and are out of the scope of the present study.

4.3. Hemolysis and calcium

Ca^{2+} ions are known to be involved in regulation of the spectrin-actin interaction. Interaction of Ca-Calmodulin with the band 4.1R protein decreases affinity of the latter to the docking proteins band 3, glycophorin C and p55 destabilizing thereby RBC cytoskeletal structure [37–39]. Long-term regular practicing of endurance exercise may in addition cause progressive cleavage of α- and β-spectrins, ankyrin, and band 4.1 by calpain [40–42]. We have not observed any morphological abnormalities in RBC of all participants at rest. However, 8 subjects out of 22 belonging to both athletes and untrained control group responded to a single exercise bout with formation of ghosts (hemoglobin-free RBC membranes) and echinocytosis. Cycling-induced hemolysis was not reported before according to our knowledge (e.g. [5]). Hemolytic events are known to follow intensive physical exercises including skiing [43], swimming [6], and marathon running [44] and several other sport disciplines reviewed elsewhere [25]. Imitation of conditions to which RBCs are exposed during the exercises (deoxygenation and shear stress) ex vivo also resulted in induction of hemolysis [45].

In our experimental settings enhanced susceptibility to hemolysis and morphological alterations associated with exhaustive exercises strongly correlated with the up-regulation in plasma glutamate levels. This observation suggests the importance of the erythroid NMDARs activity in sportive blood trauma. Cyclic oscillations of intracellular Ca^{2+} in RBC in the course of regular training bouts may be involved in progression of cell damage associated with exhaustive exercises. In our experimental settings this damage, however, did not correlate with an increase in phosphatidylserine exposure, as no difference was observed in both basal and post-exercise levels of annexinV binding. We also could not detect any changes in reticulocyte counts as the percentage of CD71+ cells did not differ between the athletes and non-trained individuals at any occasion. Thus, the observed hemolysis induced by a single cycling bout had no immediate effect on the RBC turnover and could easily be overseen in the automated blood analysis settings.

4.4. Changes in hemoglobin oxygen affinity

A rightward shift of the oxygen dissociation curve of people regularly practicing exhaustive exercises correlates with the higher Bohr effect. This phenomenon is supposed to be related to the higher ATP and 2,3-diphosphoglycerate in their RBC [46–49]. In part, higher P_{50} values reported in athletes may also be attributed to the facilitated clearance of senescent cells and *de novo* RBC production in these individuals rendering the RBC population "younger" [7,50]. Young cells were shown to higher metabolic rates and higher

2,3-diphosphoglycerate and ATP levels and lower affinity [51,52]. In addition to that reticulocytes and young RBCs are also characterized by the higher abundance of NMDARs and a higher capacity to increase Ca^{2+} levels upon their activation [20]. According to our observations Ca^{2+} uptake may contribute to maintenance of low O_2 affinity of hemoglobin.

As follows from Fig. 2A, prolonged training is associated with a decrease in the intracellular Ca^{2+} levels, although P_{50} remains high (Fig. 3B). Thus, the lower affinity of hemoglobin to O_2 in RBCs athletes at rest is maintained by the factor(s) dominating over Ca^{2+}-driven regulation. Among these factors are intracellular pH, ATP, 2,3-diphosphoglycerate, and Cl^- concentrations are the most well characterized [53]. Training affects most of these parameters [46–48]. At present we may view Ca^{2+} as a short-term messenger that is capable to mediate fast reversible changes in P_{50} (Fig. 3B). Unfortunately, detection of P_{50} using Hemox analyzer cannot be used to accurately monitor the rate of changes in P_{50} induced by Ca^{2+} uptake as the measurement takes several minutes for completion. Indirect measurements of the shifts in the intracellular pH caused by Ca^{2+} supplementation of hemoglobin solution show that response may be instantaneous in they are driven by the pH alterations [20]. Of importance is the fact that responsiveness of P_{50} to the activation of NMDARs occurs readily in both athletes and non-trained control (Fig. 3B). As O_2 affinity of Hb in RBCs of athletes is lower than that of untrained subject, the trained group profits from decreasing it even further upon activation of NMDARs.

Contribution of amino acids to the regulation of O_2 binding to hemoglobin has never been considered before. The attempts to mimic the shifts in oxygen dissociation curve observed *in vivo* in RBC samples collected after the exercise failed [46]. Back in 1975 the authors suggested that decrease in O_2 affinity was caused by the presence of "rapidly decaying unknown substance" in plasma of the exercising humans [46]. Inactivation of the NMDARs after the stimulation with glutamate and/or the lack of glutamate and glycine in the incubation medium could at least in part contribute to this mysterious lack of reproducibility.

4.5. The lack of effect of a single cycling bout on RBC rheology

Ca^{2+} uptake and the following activation of Gardos channels are involved in transient dehydration which could be reversed within minutes in RBCs o healthy human subjects [20]. In addition, shear stress applied ex vivo was reported to induce RBC shrinkage, which is Ca^{2+}-independent and makes cells less deformable [54]. Being transient, these processes did not appear to cause a measurable impact into the gross RBC deformability in our experimental settings.

4.6. Study limitations and concluding remarks

This study was attempting to assess the possible effect of exercise on the activity of erythroid NMDARs. Based on our ex vivo studies we knew that activation of NMDARs causes acute transient increase in the intracellular Ca^{2+} associated with a transient shift in P_{50}. The current study design did not allow to monitor these transitory events in vivo. We were bound to follow the receptor activity in RBC and intracellular Ca^{2+} levels minutes to hours after blood collection. Blood plasma, on the contrary, was separated immediately upon blood withdrawal and was accurately representing amino acid levels before and right after the exercise. Acute effects (Ca^{2+} uptake by the NMDARs, GSH levels and P_{50}) had to be imitated by acute supplementation of NMDA and glycine to plasma or plasma-like extracellular medium. Evaluation of the acute effects of the NMDAR stimulation on the Ca^{2+} pump function in intact RBCs of the study participants also remained out of the scope of this study. The obtained data emphasize the differences in Ca^{2+} handling by RBC of athletes and untrained people. They favor our hypothesis and suggest that exercise-induced modulation of Ca^{2+} plays an important role in regulation of oxygen carrying capacity and, in the long term, in defining the life span of the circulating RBCs.

Authors' contribution

This study was designed by AB, AM, LK and AH. AM, TH, JW, NB, PS, CW, TM, LK and AB performed the experiments, analysed the data and prepared the figures. AB, AM, LK, AH, CW and MG contributed to the interpretation of the data and to the manuscript preparation.

Conflict of interest

Co-authors have no conflict of interests to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ceca.2016.05.005.

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