

Adhesion of Erythrocytes to Endothelial Cells After Acute Exercise: Differences in Red Blood Cells from Juvenile and Adult Rats

A. TEMIZ ARTMANN¹, M. AKHISAROGLU², Z. SERCAN³, H. RESMI⁴,
B.M. KAYATEKIN², K. YORUKOGLU⁵, G. KIRKALI⁴

¹University of Applied Sciences in Aachen, Department of Cell Biophysics, Jülich, Germany and Dokuz Eylul University School of Medicine, Departments of ²Physiology, ³Medical Biology and Genetics, ⁴Biochemistry, and ⁵Pathology, Izmir, Turkey

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Summary

Erythrocytes (RBC) from untrained male Wistar rats and rat glomerular endothelial cells (EC) were used to investigate the effects of acute exercise (speed: 20 m/min, slope: 0, duration: 1 hour) on RBC membrane protein oxidation and adhesion to cultured EC. Experimental animals were divided into juvenile (age 10 weeks) and adult (age 30 weeks) groups for these studies. Immediately following exercise, juvenile rat RBC membrane protein oxidation was significantly enhanced. Adult rat RBC showed significantly higher basal protein oxidation than juvenile RBC; but the level of adult rat RBC membrane protein oxidation was unaffected by exercise. Prior to exercise, adult rat RBC showed significantly higher adhesion to EC than RBC of juvenile rat. There was no difference in plasma fibronectin or fibrinogen levels following exercise. Only juvenile rat RBC showed a significant decrease in sialic acid residue content following exercise. These experiments show that there are changes in RBC-EC interactions following exercise that are influenced by animal age.

Key words

Erythrocyte • Endothelium • Adhesion • Exercise

Introduction

In recent years, the physiological and pathophysiological changes accompanying exercise have attracted considerable attention. It has been suggested that during strenuous exercise, an inflammatory response is triggered (Camus *et al.* 1994) characterized by leukocyte activation and alterations in the cytokine system (Mertens *et al.* 1996). The mitochondrial electron transport chain as well

as the polymorphonuclear leukocyte xanthine oxidase system have been identified as major sources of intracellular free radical generation during exercise (Buttrum *et al.* 1994). The significance of these changes is of interest because reactive oxygen and nitrogen species oxidatively modify a wide variety of molecules; the consequences of these modifications are unknown (Southern and Powis 1988).

Increased levels of leukocyte activation after

exercise may affect erythrocytes (RBC) and endothelial cells (EC) (Kokot *et al.* 1988, Temiz *et al.* 2000). Furthermore, strenuous exercise may cause transient ischemia and hypoxia, especially in active muscles. Ischemia and hypoxia may also result in local inflammation, cytokine release and leukocyte activation culminating in free radical release (Kokot *et al.* 1988, Temiz *et al.* 2002). Activated leukocytes are known to generate reactive oxygen species capable of damaging cells in physical proximity to leukocytes (Claster *et al.* 1984, Weiss 1980, Saltman 1989).

RBC deformation and passage through the circulation are determined by cellular membranes and cytosolic properties (Shiga *et al.* 1990). Rheological properties of red blood cells are affected by oxidant attacks after exercise. Aerobic exercise causes large quantities of ambient oxygen consumption. Oxidative cellular damages include membrane lipid peroxidation and protein oxidation; these changes modify membrane mechanics (Temiz *et al.* 2000, 2002). RBC adhesion to the vascular endothelium is another factor affecting their circulation and function (Chappey *et al.* 1994, Parise and Telen 2003). Abnormal RBC adhesion to the endothelium has been found in several common disorders associated with vascular occlusion (Chappey *et al.* 1994, Parise and Telen 2003). Furthermore, RBC adherence to the endothelium may initiate and promote intravascular slugging and occlusions leading to tissue ischemia and organ damage in such conditions as retinopathy, dermal ulcer and stroke.

The RBC membrane sialic acid residue content (Dhermy *et al.* 1987) might be one of the cellular components that has an effect on the adhesiveness of RBC to the vascular endothelium besides plasma factors like fibronectin and fibrinogen (Carraro *et al.* 1990). Fibronectin is a multi-domain protein found in the plasma and subendothelial extracellular matrix that induces attachment of a variety of cell types to the endothelium; fibronectin also promotes the migration of many cell types across the endothelium and has been implicated in the microvascular thrombosis of venules (Altankov and Serafimov-Dimitrov 1990, Kumar *et al.* 1996). Oxygen radicals may liberate fibronectin from the extracellular matrix and leukocytes may synthesize and release fibronectin into the plasma (Peters *et al.* 1986, Vincent *et al.* 1988). In some studies fibronectin was shown to be significantly elevated at the end of an exercise session, while fibrinogen was significantly elevated in recovery from exercise (Carraro *et al.* 1990, Wautier *et al.* 1983).

Sialic acid content of red blood cell membranes is considered as a red blood cell life time marker in the literature, although this needs further research. Neuraminidase enzyme that removes the sialic acid from red blood cell membranes decreased the life time of red blood cells (Bocci 1976). Flow cytofluorimetric analysis of young and senescent human erythrocytes probed with lectins gave evidence that sialic acid control their life span (Bratosin *et al.* 1995). The sialic acid residue content of RBC membranes is another factor potentially affecting red blood cell adhesion to EC (Chappey *et al.* 1994).

This study was performed to investigate effects of acute exercise on RBC adhesion to cultured rat glomerular EC. Parallel studies on plasma fibronectin and fibrinogen levels were performed in response to exercise. RBC membrane protein oxidation and sialic acid residue content were measured as well. All studies were performed in juvenile and adult rats to determine if previously reported age related phenomena (Dortant *et al.* 2001, Tamaya-Mori *et al.* 2003) could be replicated. Animal weight was used as a surrogate for assignment to the juvenile and adult groups, as suggested by others (Tamaya-Mori *et al.* 2003, Dortant *et al.* 2001).

Methods

Animals

Experiments were performed on male Wistar rats, source 87.5 % homogeneity. Juvenile rats (10 weeks old) had a mean body mass of 117±12 g, and adult rats (~30 weeks old) had a mean body mass of 298±19 g. Rats were kept for a period of 12 h in the light (07:00-19:00), and 12 h in the dark (19:00-07:00) at a temperature of 25±2 °C. They were given standard pellet diet and drinking water *ad libitum*. Four cohorts of juvenile and four cohorts of adult animals (n=7 each) were studied prior to exercise (controls), immediately after the exercise, 24 h after exercise, and 48 h after exercise. At the end of each of the time intervals, heparinized blood samples were collected from tail veins. All analyses were completed within 3 h after the collection of blood samples. The study was performed in accordance with the Helsinki declaration. Animal studies were approved by the ethics committee.

Exercise protocol

The first 4 days prior to experimental procedures, the animals were kept on the treadmill for

4 min. The speed of treadmill was 4 m/min. On the day of experimental procedures, the animals ran at a speed of 20 m/min for approximately one hour or until exhaustion (Ji *et al.* 1988, Paulin *et al.* 1991).

Preparation of blood samples

Venous blood samples were collected from the rat tail veins into heparinized tubes. RBC were separated from whole blood as described below.

RBC membrane preparation

Heparinized blood was centrifuged at 2000 rpm for 10 minutes. The plasma was discarded, the buffy coat was removed, and RBC membrane preparation was performed according to the method of Dodge *et al.* (1963).

Estimation of protein oxidation

Assay of sulphur-containing amino acid oxidation was based on the method of Schwartz *et al.* (1991). Dichlorodiamine platinum (cDDP) specifically binds to cysteine or to methionine residues in proteins. However, if these residues are already oxidized, then cDDP is not bound. Thus, cDDP binding reflects the extent of residue reduction, with less binding indicating more oxidation. In our study sulphur containing amino acid oxidation in RBC membrane proteins shown as cDDP binding in mol-cDDP/mg protein that the amount of bound cDDP- in the reaction mixture was determined. The binding of cDDP decreases as the oxidation of SH-groups increases.

RBC membrane sialic acid determination

RBC membrane sialic acid (N-acetyl neuraminic acid) residues were measured colorimetrically using the thiobarbituric acid method (Warren 1959).

Plasma fibrinogen and fibronectin determinations

Plasma fibrinogen was determined using an AMAX CS190 instrument (Sigma Diagnostics Procedure No:886) (Claus 1957). The plasma fibronectin level was assessed with an ELISA kit (Biomedical Technologies Inc.(bti)-USA-cat No:BT-700) (Ruoslahti *et al.* 1981).

Endothelial cell culture

EC cultures were derived from *Rattus norvegicus* (covering Wistar, Sprague Dawley, Holtzman, Hooded and Long Evans strains) glomerular endothelial cell line RGE (DSMZ-German collection of

microorganisms and cell cultures No: ACC 262/Germany) and open culture was performed (Laulajainen *et al.* 1993). Cells were cultured to confluence on a glass microscopic slide in RPMI 1640 medium containing 10 % fetal bovine serum.

Flow chamber studies: sample fixation and staining

To assess RBC-EC adhesion, a flow chamber setup was used. It consisted of a microscope, a flow chamber and a computer controlled micropump (Peng *et al.* 1997, Sirois *et al.* 1998). The chamber formed a rectangular parallel-plate flow channel (10 mm x 50 mm x 0.1 mm). EC cultured on top of a glass slide were placed on the bottom part of the flow channel. After assembling the chamber, a 1 ml blood sample diluted in phosphate-buffered saline (PBS) to a final hematocrit of 1 % was passed through the chamber at a constant wall shear stress of 0.5 Pa. After the 1 ml sample passed through the chamber, 4 ml of isotonic sodium chloride was used to wash out all non-adherent material under the same wall shear stress. For counting the adherent RBC, the flow chamber was taken apart. Cells were fixed and stained with a Dade Behring Diff-Quik Stain Set (B4132-1, Switzerland).

Microscopy

The flow chamber was mounted on a microscope Olympus BH-2 (Olympus Corporation, Tokyo, Japan) equipped with a CCD video camera (Model 72, Dage-MTI, Michigan City, IN). Oil-immersion light microscopy was performed after the wash out described above was completed and cells were fixed and stained. In each sample, 1000 endothelial cells were evaluated. The number of adherent red blood cells per endothelial cell was determined; as was the percentage of EC that had at least one adherent RBC. If an RBC was adherent to more than one EC, only one EC was scored. Samples were reviewed by two individuals and the coefficient of variation was below 5 %.

Hematological data

Hemoglobin, hematocrit, RBC count, WBC counts, and RBC mean corpuscular volume (MCV) were obtained with an electronic hematological analyzer (Coulter Electronics, Hialeah, FL, USA).

Statistical analysis

All results are presented as means \pm SD. Comparisons between the groups were made using a

Mann-Whitney U test after Kruskal Wallis Variance Analysis. Statistical analysis was performed with the SPSS software statistical program. Differences between groups at $p < 0.05$ were considered statistically significant.

Results

Differences in hematological indices were found between juvenile and adult groups that had significant differences in body weight. Juvenile animals had higher RBC counts, hemoglobin concentrations, and hematocrit values than adult animals. After exercise, juvenile animals showed a significant reduction in each of these

parameters, while adults showed no change in these parameters following exercise (Table 1). Juvenile rat RBC had lower MCV than adult rat RBC. Muscle activity had no effect on MCV in either group.

Figure 1 shows the percentage of EC that had at least one RBC attached. Before exercise, approximately 18 % of EC had adherent RBC from adult animals, which was nearly double the percentage with adherent RBC from juvenile animals. After exercise, there was an initial rise in adherent RBC from juvenile animals, but this increase disappeared by 48 h after exercise. RBC from adult animals showed elevated adherence to EC at baseline. No change was found following exercise.

Table 1. Hematological data obtained before and after acute exercise.

n=7	RBC number (per fl)	Hb (g/dl)	Hct (%)	MCV (fl)
Juvenile - C	11.08±0.6 †	17.32±0.6 †	55.38±2.1 †	49.96±2.5 †
Juvenile 0 h	9.66±1.3	15.55±1.9	50.07±5.6	51.97±3.1
Juvenile 24 h	7.51±0.5 *	13.80±0.6 *	38.50±2.5 *	51.26±3.0
Juvenile 48 h	9.20±1.0 *	14.68±1.0 *	46.91±3.6 *	51.16±2.6
Adult - C	7.57±0.8	15.97±1.8	43.96±4.6	58.08±1.3
Adult 0 h	7.60±0.3	16.33±0.7	44.96±4.6	57.23±1.8
Adult 24 h	7.27±1.0	14.42±0.4	46.14±1.2	57.88±2.1
Adult 48 h	6.69±1.3	13.54±1.0	33.72±3.5	57.76±2.2

(* significant difference as compared to control, † significant differences between juvenile and adult rats, $p < 0.05$). Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume.

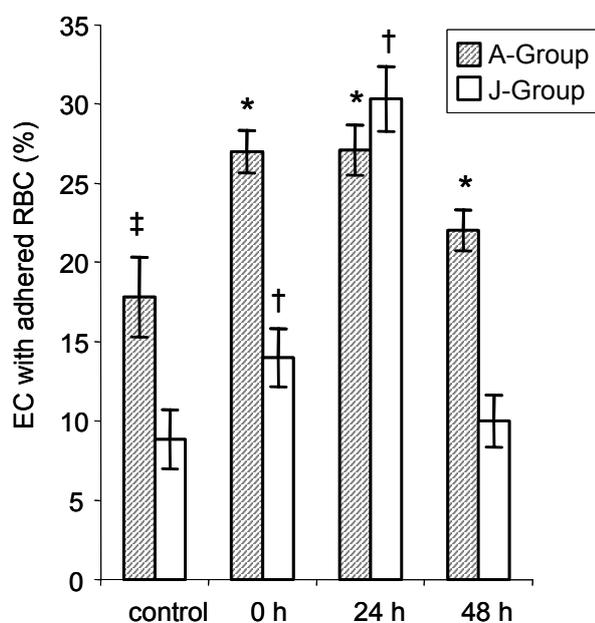


Fig. 1. Percentage of EC with adherent RBC (n=7, data are shown as mean ± S.E.M.; significant differences as compared to control adult *, and control juvenile † groups; † significant differences between the control juvenile and control adult RBC, $p < 0.05$).

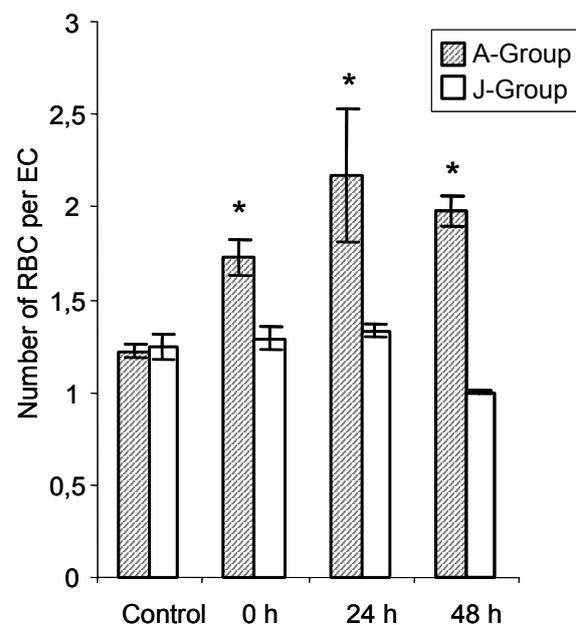


Fig. 2. Average number of attached RBC per EC. The number of EC included all EC, those who showed RBC adhesion plus those who did not. (n=7, data are shown as mean ± S.E.M.; * significant difference as compared to control adult RBC, $p < 0.05$).

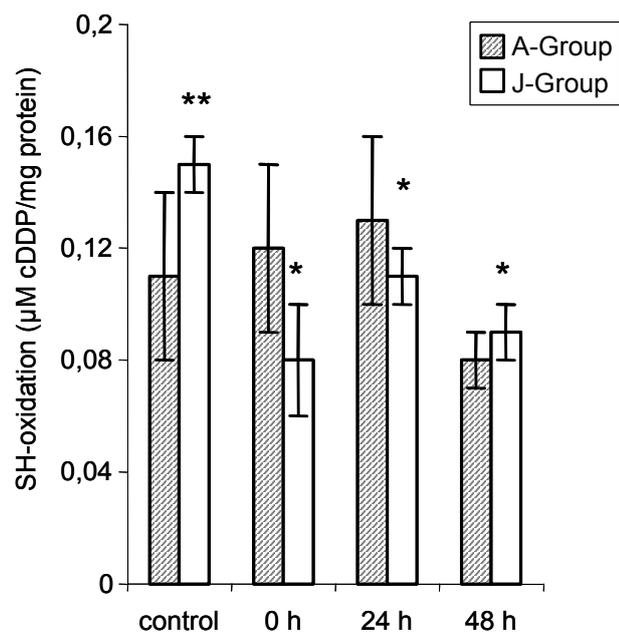


Fig. 3. Sulphur containing amino acid oxidation in RBC membrane proteins shown as cDDP binding in mol-cDDP/mg protein. The binding of cDDP decreases as the oxidation of SH-groups increases (n=7, data are shown as mean \pm S.E.M., * significant difference as compared to controls, $p < 0.05$. † significant difference between control juvenile and control adult RBC, $p < 0.05$).

The number of attached RBC per EC after exercise was markedly increased in the adult group 24 h after exercise, and remained constant thereafter (Fig. 2). There was no change in the number of attached RBC per EC in the juvenile group after exercise.

The fibrinogen level before exercise was 1.76 ± 0.26 $\mu\text{g/ml}$ in the juvenile group and 1.75 ± 0.23 $\mu\text{g/ml}$ in the adult group. Neither group showed any significant change at all time intervals after exercise, except for the adult group immediately after exercise: the fibrinogen level rose to 1.96 ± 0.14 $\mu\text{g/ml}$ ($p < 0.05$) immediately after exercise.

Protein oxidation was assessed by determining the amount of reduced sulphur containing amino acid residues available for oxidation assay *in vitro*. There was a significant difference observed between the two control groups. The adult control group showed a significantly higher level of protein oxidation (Fig. 3) than the juvenile control group. After exercising, the extent of protein oxidation was significantly higher in the juvenile group, but the extent of protein oxidation was unchanged in the adult group.

Similar amounts of RBC membrane sialic acid residues were found in RBC from juvenile and adult control animals. In addition, there was no immediate

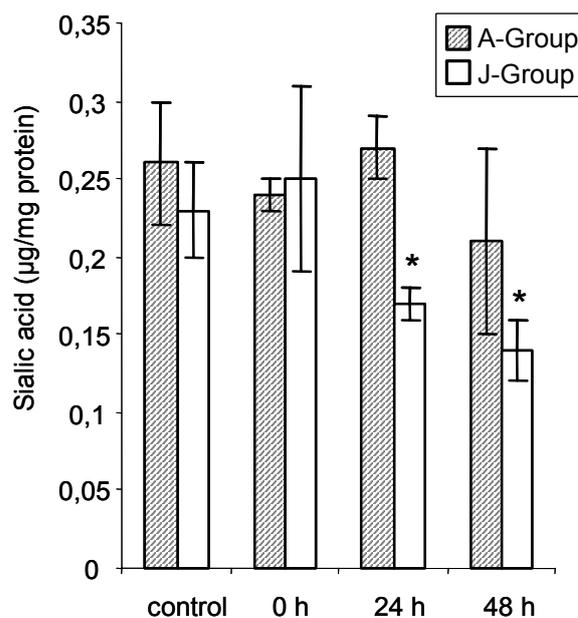


Fig. 4. Sialic acid content of RBC membranes ($\mu\text{g/mg}$ protein) derived from juvenile (J, filled squares) and adult (A, filled circles) as a function of time (hours) following exercise (n=7, data is shown as mean \pm S.E.M.; * significant difference as compared to control RBC, $p < 0.05$).

effect of exercise in both groups. Significantly lower levels of sialic acid content were seen in the juvenile group 24 h and 48 h after exercise (Fig. 4).

Discussion

Many studies on RBC adhesion to endothelial cells have been performed in the diseases such as thalassemia, sickle cell anemia, uremia, and diabetes mellitus (Howard and Gilladoga 1989, Wautier *et al.* 1994, Wick *et al.* 1987). We were unable to find reports examining the effect of acute exercise on red blood cell adhesion to endothelial cells.

Here we found multiple changes in the RBC-EC adhesion parameters as a result of exercise. We also found differences in the adhesive properties of RBC that change with the age of the rat (Fig. 1). Notably, the EC used in this study did not originate from the experimental animals and the differences observed, i.e. more RBC-EC adhesion when RBC from adult rats were examined; may be attributed to the RBC age. RBC-EC adhesion changed in response to exercise regardless of the RBC source; however, the magnitude of response to exercise was greater in RBC taken from juvenile rats (Fig. 1). Interestingly, this increased adhesion was transient,

returning to baseline by 48 h (Fig. 1). The molecular basis of this increased adhesion is not known, but differential expression of adhesion molecules with age is one possibility. Indeed, RBC express a relatively large number of known adhesion receptors including CD44 and VLA-4 that mediate, for example, adhesion to components of the extracellular matrix (Telen 2000).

Surprisingly RBC from adult rats were more resistant to protein oxidation, and there was no significant increase in their membrane protein oxidation after the exercise. It is not clear why this is so, but the higher basal protein oxidation may further limit the exercise-induced oxidation.

We were unable to corroborate previous reports of increased plasma fibronectin and fibrinogen levels after exercise (Schuit *et al.* 1997, DuBose *et al.* 1989, El Sayed *et al.* 2000, Li-Saw-Hee *et al.* 2001). Despite this difference with previously published observation, we feel that our results are valid and attributable solely to changes in RBC membranes. Our protocol involved dilution of blood with PBS by a factor of approximately 50, considerably eliminating the contribution of plasma proteins to adhesion.

Sialic acid residues are believed to provide repulsive electrostatic forces to RBC membranes (Hadengue *et al.* 1996, 1998). A significantly lowered

sialic acid residue content was observed in RBC from juvenile animals after 24 h of exercise. Thus, a mechanistic correlation between the adhesion of RBC to EC and the RBC membrane sialic acid content cannot be drawn from our data.

Red blood cell adhesion may initiate or promote intravascular occlusion leading to ischemic tissue and organ damage, retinopathy, dermal ulcers, strokes and other pathological symptoms based on vascular occlusion. RBC adherence is dependent on EC surface molecules and may be modulated by local hemodynamic factors. The data obtained in our study showed that acute exercise in untrained rats increases membrane protein oxidation in RBC membranes and increases RBC adhesion to EC. This effect lasted for at least 48 h after exercise. Oxidative damages probably play a key role in RBC-EC adhesion.

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Reprint requests

A.Temiz Artmann, Aachen University of Applied Sciences, Dept. Cell Biophysics and Bioengineering, Ginsterweg 1, D-52428 Jülich, Germany. Fax: +49-2461993273. E-mail: Temiz@fh-aachen.de, aysegul.temiz@deu.edu.tr