

Research article

PLATELET ACTIVITY AND SENSITIVITY TO AGONISTS AFTER EXHAUSTIVE TREADMILL EXERCISE

Thomas Hilberg¹✉, Volker Schmidt¹, Wolfgang Lösche², Holger H.W. Gabriel¹

¹ Department of Sports Medicine, Friedrich-Schiller-University Jena, Germany.

² Centre of Vascular Biology and Medicine, Erfurt, Friedrich-Schiller-University Jena, Germany.

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ABSTRACT

The extent of platelet activation after exhaustive exercise remains under discussion. Previous studies have provided contrary data, probably due to differences in the methodologies and the enrolled subjects. In the present study a maximal treadmill exercise (TR) was used to test platelet activity and reactivity in 13 healthy non-smoking men. Blood samples were taken after a 30min rest, immediately before and after exercise, and 1h after completion of exercise. Platelets were analysed by whole blood flow cytometry either directly or after in vitro stimulation by incubating the blood samples for 10min with TRAP-6 (10 μ M) or ADP (5 μ M or 2,5 μ M). Binding of an anti-CD62P antibody or a PAC1 antibody directed against the activated fibrinogen receptor GPIIb/IIIa were used as a measure of platelet activation. Immediately after TR the percent CD62P positive platelets (%PC) unstimulated increased ($p < 0.01$) from 0.77 ± 0.06 to 1.12 ± 0.09 %PC and in PAC1 ($p < 0.05$) from 2.32 ± 0.54 to 3.83 ± 0.81 %PC (mean \pm SEM). After ADP-stimulation an increase from 4.18 ± 1.02 to 5.69 ± 1.40 %PC in CD62P ($p < 0.01$) and from 45.7 ± 3.4 to 57.9 ± 6.6 %PC in PAC1 ($p < 0.05$) after TR were detected. Using TRAP-6-stimulation only the increase of PAC1 ($p < 0.01$) after TR was different in comparison with the control experiment without exercise. Soluble CD62P in plasma as a marker of platelet and endothelial cell activation was also enhanced ($p < 0.05$) after TR. Although these results indicate that exhaustive exercise lead to a small platelet activation and an increase in platelet reactivity, it is rather doubtful that these changes alone implicate a prothrombotic situation in healthy young non-smokers.

KEY WORDS: Platelet activation, CD62P, PAC1, sCD62P, physical activity.

INTRODUCTION

It has been assumed that strenuous exercise augments the risk of vascular thrombotic events and primary cardiac arrest (Wang and Cheng, 1999). Although it is evidence-based that physical exercise alters platelet count and platelet functionality, the variability of methods, and exercise protocols used in these investigations makes an assessment of the dimension and importance of these changes difficult (Di Massimo et al., 1999; Möckel et al., 1999, Rauramaa et al., 1986; Wang et al., 1994). In addition these studies recruited not only healthy and trained subjects, but also patients e.g. with coronary heart disease (CHD) (Lindemann et al., 1999). In former years these changes in platelet activity after

strenuous exercise were investigated by measuring platelet aggregation and adhesivity, or indirectly by measuring plasma levels of platelet-released molecules such as β -thromboglobulin or platelet factor 4, which demonstrated platelet activation in vivo (Di Massimo et al., 1999; Wang et al., 1994; Weiss et al., 1998). The results of these studies led to the opinion that an increased platelet activity exists after strenuous exercise. In addition, several authors have underlined the link between the activation of platelets and cardiovascular complications and its role in the pathogenesis of coronary ischemia. However, other studies demonstrated that platelet activation does not always occur after exhaustive exercise e.g. in trained subjects (Davies et al., 1986; Fitzgerald et al., 1986;

Kestin et al., 1993). More recently flow cytometry has been used to study platelets *in vitro* and *ex vivo*. Measurements of the activation-dependent antigens on the platelet surface, e.g. P-selectin (CD62P), demonstrated *in-vivo* platelet activation in clinical situations with an increased risk for arterial thrombosis (Gawaz et al., 1996; Michelson et al., 2000; Neumann et al., 1996; Schmitz et al., 1998). There are few studies that have employed flow cytometry to investigate the influence of exercise on platelet function in healthy subjects as well as in patients with cardiovascular disease, in parts with contrary results (Kestin et al., 1993; Ley and Tedder 1995; Möckel et al., 2001; Pan et al., 1994; Wallen et al., 1999).

CD62P, that is stored in intracellular granules in platelets as well as in endothelial cells becomes not only surface-exposed upon cell activation, but is also released into the plasma. Thus soluble CD62P (sCD62P) is considered to be a marker of an *in vivo* activation of both platelets and endothelial cells (Fijnheer et al., 1997; Blann et al., 1997). Increased plasma levels of sCD62P have been reported in patients with cardiovascular disease (Ikeda et al., 1995; Ferroni et al., 1999) and also after repeated exercise (Kirkpatrick et al., 1997).

In the present study flow cytometry was used to investigate the changes of platelet function after a standardized exhaustive treadmill ergometer test in a group of trained healthy young men. Surface expression of CD62P and the activated glycoprotein (GP) IIb/IIIa complex were determined as measures of platelet activation. In addition, plasma levels of sCD62P were also tested before and after exercise as well as during a control experiment.

METHODS

Subjects

The study was comprised of 13 moderately trained healthy men. Average age of the subjects was 23 ± 0.6 years, the mean weight was 72.5 ± 2.5 kg and 1.81 ± 0.19 m mean height (body fat 8.9 ± 0.8 %). The mean of peak oxygen uptake (VO_2 peak) was 59.1 ± 1.8 ml \cdot kg⁻¹ \cdot min⁻¹ determined by the Oxycon beta - Jaeger (Hoechberg, Germany). The mean maximal power was 322 ± 16 W, [mean individual anaerobic threshold (IAT), 3.1 ± 0.2 W \cdot kg⁻¹, mean heart volume 12.0 ± 0.4 ml \cdot kg⁻¹]. A basic physical examination of the subjects, ECG and blood pressure measurements at rest, as well as a routine laboratory status and blood coagulation standard tests did not reveal pathological findings. Subjects did not use any medication 6 weeks prior to the study till the end. The study was approved by the Ethics Committee of the Faculty of Medicine of the

Friedrich-Schiller-University Jena. Written informed consent was obtained from each subject, prior to the start of the study.

Maximal exercise test

One to two weeks before the test program all subjects performed an incremental graded exercise on a cycle ergometer in a sitting position (step test, start 50 W, every 3 minutes an increase of 50 W until volitional exhaustion) to measure peak oxygen uptake (VO_2 peak) and the IAT. The VO_2 was measured at 30-second intervals using an open spirometric system (Oxycon beta - Jaeger). Capillary blood samples were obtained from the previously hyperemized ear lobe at rest, at the end of each level of exercise, and at the end of the 1st, 3rd, 5th, and 10th minute of the recovery period. Maximal lactate concentration measured by the EBIO plus (Eppendorf; Hamburg – Germany) was 11.1 ± 0.6 mmol \cdot l⁻¹. The IAT was determined according to the method of Stegmann et al. (1981).

Exercise program

The test program included a standardized treadmill ergometer test (TR) and a control experiment in randomised order. The participants had their last meal at least 10h before each test, with the exception of a small standardized breakfast in the morning. The TR started at an initial velocity of 2.0 m \cdot s⁻¹ with increments of 0.5 m \cdot s⁻¹ every 3 min until exhaustion (mean duration 20.2 ± 0.7 min) and a short rest (<15s) for ECG and blood sampling at the end of each step. At the control experiment (CO) the subjects were asked to sit for 20 min without any exercise; the other experimental procedure was identical to TR. All the exercise tests were done between 8 a.m. – 1 p.m. in the laboratory and each individual was tested at the same time of the day for TR or CO.

Analytical methods

Blood sampling and laboratory methods

Blood samples were taken by a clean venipuncture (20 gauge needle) from an antecubital vein under controlled venous stasis (< 30s) of 40 torr after 30 min rest, immediately before and after, and 60 min after exercise. The venipunctures taken immediately before the exercise were done while subjects were in a supine position, the other blood samples were taken from the subjects in reclined position. Samples were collected in the following sequence, after discarding the first 3 ml blood: 3 blood samples were anticoagulated by adding 10% 0.106 M trisodium citrate. The first sample was used for the assessment of sCD62P via ELISA (R&D Systems, Inc.; Minneapolis - USA) and other tests. The

sCD62P ELISA had an intra-assay coefficient of variation (CV) <6% and inter-assay CV <10%. The second sample was used for the assessment of the platelets count and hematocrit (Act-Diff - Coulter Electronics; Krefeld - Germany) and measurements by flow cytometry as explained in the following section and the last one for the assessment of other tests.

In addition, 9 ml of blood was added to 15 IE lithium-heparin/ml for the measurement of adrenaline and noradrenaline via high performance liquid chromatography (HPLC) as described by Foti et al. (1987). Immediately after taking the blood samples, tube 4 was rapidly put on ice and centrifuged after a few minutes at 4° with 2500 x g for 8 min. Multiple aliquots of plasma were snap-frozen and stored at -80°C until analysis. Changes in plasma volume were calculated for platelet count and sCD62P according to the method of Dill and Costill (1974).

Flow cytometric analysis

Direct immuno-fluorescence techniques were used for determination of changes in platelet activity and response to agonists. The following fluorescein-isothiocyanat (FITC) and phycoerythrin (PE)-conjugated monoclonal antibodies (mabs) were purchased from Coulter-Immunotech Diagnostics (Krefeld - Germany): anti-CD41-PE (clone P2), anti-CD42b-PE (clone SZ2), and anti-CD62P-FITC (Clone CLB-Thromb/6). The PAC1 antibody that recognises the activated form of the platelet GP IIb/IIIa was obtained from Becton Dickinson (Heidelberg - Germany; clone SP2).

Immediately after withdrawal of blood the platelet count in the samples was adjusted to approximately 20.000 platelets/ μ l with phosphate buffered saline (PBS) buffer (Life Technologies - Gibco BRL; Karlsruhe - Germany) including 0.5% bovine serum albumin (BSA) (Vitros Ortho - Clinical Diagnostics; Neckargemünd - Germany) and prewarmed to 37°C. Platelet stimulation was obtained by mixing 16 μ l of the diluted blood sample with 4 μ l of a 12.5 (PAC1-tubes) or 25 μ M (CD62P-tubes) solution of ADP (Sigma; Deisenhofen - Germany) or a 50 μ M solution of the thrombin receptor activating peptide (TRAP-6) (SFLLRN, Bachem; Heidelberg - Germany). After an incubation for 10 min at 37°C, the samples were incubated for further 5 min with either anti-CD41-PE (7.5 μ l) and anti-CD62P-FITC (4 μ l) or anti-CD42b-PE (7.5 μ l) and PAC1-FITC (10 μ l). After this incubation, 1 ml PBS buffer containing 0.5 BSA and 0.5% paraformaldehyde (Sigma; Deisenhofen - Germany) was added at 4°C for fixation of the platelets. In parallel, 20 μ l of unstimulated blood

samples were incubated with the labelled antibodies as described above. Unspecific binding of the fluorescence-labelled antibodies was determined by adding unlabelled anti-CD62P (4 μ l) or PAC1 (10 μ l) 5 min prior to adding the labelled antibodies (Schmitz et al., 1998).

Immediately after the immunolabeling procedure the samples were measured in a flow cytometer (Coulter® EPICS® XL-MCL™, Beckman Coulter GmbH; Krefeld - Germany; laser excitation 488nm, detection of FITC at 525nm and PE at 575nm). Data acquisition and analysis were performed with System II™ software (Beckman Coulter GmbH). Platelets were identified by their light scatter characteristics and the binding of the PE-labelled anti-CD41 or anti-CD42b antibodies and were analysed for the binding of the FITC-labelled anti-CD62P or PAC1. The data are given as percent positive cells and were corrected for the unspecific antibody binding.

Statistics

Results are reported as mean \pm SEM and the range unless otherwise stated. The data did not show normal distribution, which was tested by Kolmogorov-Smirnov-test. Therefore Wilcoxon-rank-test was used for testing the changes before and after exercise and to measure the differences between the exercise and control experiment. The level of significance was set at $p < 0.05$. Statistical analysis was done with SPSS-10.0 software.

RESULTS

The exercise (TR) caused a marked increase of the plasma levels of adrenaline and noradrenaline from 0.36 ± 0.05 to 1.70 ± 0.27 nmol \cdot l⁻¹ ($p < 0.01$) and from 1.48 ± 0.16 to 18.25 ± 1.68 nmol \cdot l⁻¹ ($p < 0.01$), respectively, immediately after exercise. The mean value of lactate after exercise was 10.8 ± 0.6 mmol \cdot l⁻¹.

Hematocrit changed from $40\pm 2\%$ immediately before exercise to $44\pm 3\%$ immediately after exercise and returned back to baseline 60 min after exercise ($39\pm 2\%$). Platelet count was significantly increased from $193\pm 14 \cdot 10^9 \cdot$ l⁻¹ immediately before exercise to $235\pm 17 \cdot 10^9 \cdot$ l⁻¹ ($p < 0.01$) immediately after exercise while remaining unchanged in the control experiment.

It is shown in Figure 1 that exercise resulted in a significant platelet activation as indicated by an increase of percent positive platelets for CD62P and PAC1 by a factor of 1.5 and 1.7, respectively. The increase was only seen immediately after exercise, and the values that were measured 1 hour after exercise were not significantly different from the

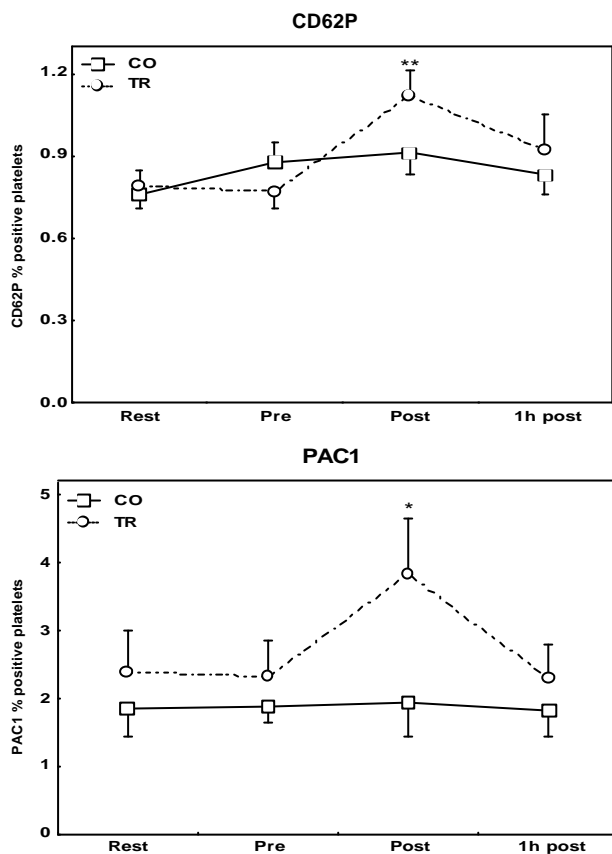


Figure 1. CD62P or PAC1, % positive platelets, unstimulated. Error bars indicate means \pm SEM. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, difference pre to post in treadmill exercise (TR) in comparison with the control experiment (CO).

pre-exercise values. No changes were seen in the control experiment.

Stimulation of platelets markedly enhanced the number of CD62P and PAC1 positive platelets (compare Figure 1 with Figure 2 and 3). It is also shown in Fig 2 and 3 that exercise augmented the effects of both ADP and TRAP-6. With ADP we observed approximately 1.3-fold higher number of CD62P and PAC1 positive platelets immediately after exercise (Figure 2).

When platelets were stimulated with TRAP-6, an enhancement of platelet activation due to exercise was only seen for PAC1 but not for CD62P (Figure 3). All exercise-induced changes of ADP and TRAP-induced platelet activation were abolished 1 h after exercise, and no changes were found in the control experiments.

Parallel to the exercise induced increase in the number of CD62P positive platelets, there was also an increase in the plasma level of sCD62P. The values measured immediately before and after exercise were 38.2 ± 2.9 and 46.3 ± 4.1 mg \cdot ml $^{-1}$ ($p < 0.05$) but remained unchanged in the control experiment.

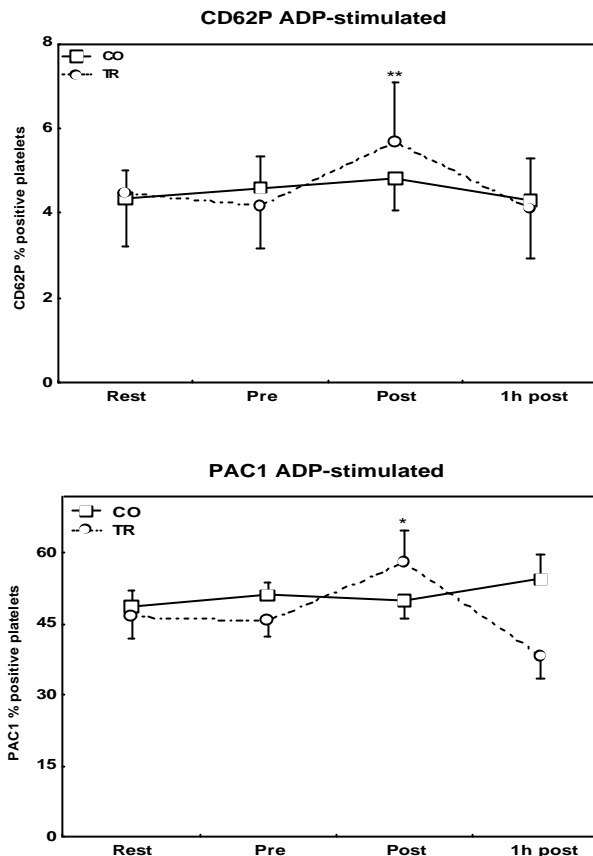


Figure 2. CD62P or PAC1, % positive platelets, ADP-stimulated. Error bars indicate means \pm SEM. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, difference pre to post in treadmill exercise (TR) in comparison with the control experiment (CO).

DISCUSSION

It has been postulated that exhaustive exercise increases thrombotic risk in different subjects caused by the increase of platelet count and -functionality after exercise. Different investigations have shown, that exhaustive exercise induces a change in platelet count in whole blood between 18-80% (El-Sayed et al., 2000; Wang et al., 1994). In the present study an increase of 22% in platelet cell count corrected, for plasma volume change, was observed. Although different laboratory methods and different exercise protocols did not lead to identical results it seems clear that, exhaustive exercise affects the platelet function in whole blood, e.g. platelets activity, aggregability and adhesivity (Bärtsch, 1999; Kestin et al., 1993; Wallen et al., 1999; Wang et al., 1994). Together with the changes in blood coagulation after exercise, these effects seem to increase the risk for cardiovascular events, particularly in subjects who are sedentary (Siscovick et al., 1984). However it is also well-known, that exercise is followed by an increase in fibrinolysis to counterbalance possible

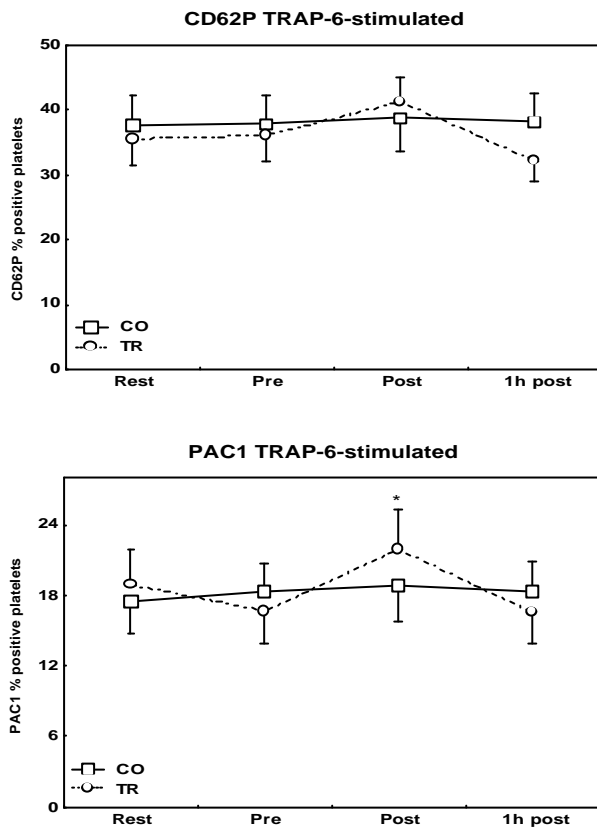


Figure 3. CD62P or PAC1, % positive platelets, TRAP-6-stimulated. Error bars indicate means \pm SEM. * denote $p < 0.05$, respectively, difference pre to post in treadmill exercise (TR) in comparison with the control experiment (CO).

effects in blood coagulation and changes in platelet function.

The changes of platelet aggregability and adhesiveness are dependent on the physical performance of the tested subjects and in addition also on exercise intensity as shown by Wang et al. (1994). Wang et al. (1994) showed that moderate exercise with a peak oxygen consumption of 50-55% for 30 min partly decreased platelet aggregation or adhesiveness, particularly in sedentary subjects in contrast to an exhaustive step test, which resulted in an increase of both. In another study Wang et al. (1999) investigated the mechanism of increasing platelet adhesiveness after strenuous exercise and found an increased platelet α_2 -adrenoceptor density which was accompanied by a decrease in affinity, thereby correlating with the rise of plasma catecholamine levels. Other studies have suggested that epinephrine may play a role in activating α_2 -adrenoceptors in human platelets to inhibit the adenylate cyclase system by coupling to a G_i protein and enhance the opening of GP IIb/IIIa binding sites for fibrinogen in the presence of ADP or other agonists (Aktories et al., 1981; Banga et al., 1986). Figures et al. (1986) have suggested that the

activation of platelet aggregation and the exposure of fibrinogen receptors by epinephrine requires additional agonists. This leads to the opinion that the assessment of the effect of epinephrine alone on platelets is not clear in detail.

In recent years only a few studies have used the flow cytometry technique to investigate changes of the surface of platelets due to exercise, in healthy subjects. These studies have, to a great extent, led to different results concerning platelet activity and -reactivity after exercise (Kestin et al., 1993; Möckel et al., 2001; Möckel et al., 1999; Wallen et al., 1999).

Platelet activity

In the present study, platelet activity was investigated flowcytometrically via changes of CD62P and PAC1 positive platelets. CD62P and PAC1 positive platelets were slightly but significantly increased immediately after exhaustive treadmill exercise compared to the control experiment (Figure 1). The changes after exercise are in line with the results of Möckel et al., (2001), who described an increase in CD62P after a triathlon competition, which included running as the last discipline; but the changes were distinctly smaller in the present study. In another study they also described an increase in platelet activity after aerobic as well as after anaerobic cycle exercise in trained athletes; Möckel et al., (1999) investigated only CD62P without stimulation and without a control group. These results are in contrast to the results of Kestin et al., (1993) who failed to demonstrate an increase after cycle ergometer testing of physically active subjects. Wallen et al., (1999) investigated the change in the fibrinogen binding index by measuring GPIIb/IIIa and GPIba positive cells after cycle exercise. The percentage of platelets positive for the markers and the mean fluorescence were multiplied to describe the binding index. After exhaustive exercise they found an increase in the fibrinogen binding index as well as in the control group. The reason for the discrepancies in the different study results is not clear, but it is possible that running, e.g. on a treadmill ergometer is a more intensive trigger to enhance platelet activity but this cannot clarify the differences between the results concerning only cycle exercise.

Platelet sensitivity to agonist

These tests were initiated to investigate platelet reactivity and to enhance the sensitivity of the test method; ADP and TRAP-6 were used for stimulation. The results after ADP stimulation are in line with the results without stimulation but the

results were clearer in detail (Figure 2). After exercise, significant increases of platelet hyper-reactivity in both markers were seen. These results are similar to the results after TRAP-6 stimulation, but a definitive statistically relevant difference in comparison with the control experiment was only seen in PAC1 positive platelets after exercise (Figure 3). Is it possible that the stimulation used was too intensive because the highest %PC was above 70%? This may have a negative and leveling influence on existing differences. Only Kestin et al. (1993) (CD62P, PAC1, GPIb, GPIV) and Wallen et al., (1999) (GPIIb/IIIa) investigated platelet hyperreactivity via flow cytometry but only after cycle exercise. Kestin et al., (1993) could show that, the changes in platelet reactivity to the agonist human α -thrombin are dependent on the performance of the subjects. Changes in platelet reactivity were, only observed in sedentary, but not in physically active men after cycle ergometer exercise. Wallen et al., (1999) investigated the change in fibrinogen binding index after maximal cycle exercise; after stimulation with thrombin. Compared to the control experiment a higher index was observed after exercise.

SCD62P: SCD62P was increased after exercise and the increase was significantly different compared to the control experiment. The determination of sCD62P as a marker of platelet activity is limited by the fact-, that endothelial cells release sCD62P as well (Ley and Tedder, 1995). Although Kirkpatrick et al. (1997) described that sCD62P after exercise may indicate progressive platelet activation, it is possible that the observed release after exercise is caused more by endothelial cells than by platelets.

CONCLUSION

After a standardized maximal step test on a treadmill ergometer, platelet activity and hyperreagibility is moderately increased in healthy non-smokers in comparison to a control experiment. In addition, this type of exercise also leads to an increase of sCD62P. It should be further investigated in future studies, if that release is primarily dependent on platelets or endothelial cells.

The results lead to the opinion that a small activation of platelets exists after maximal treadmill exercise but it is rather doubtful that, this small activation could indicate a prothrombotic situation in healthy young male non-smokers. However, the present work is limited by the fact that, the results are related to the healthy and moderately trained status of the included participants.

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AUTHORS BIOGRAPHY



Thomas HILBERG

Employment:

Senior Research Fellow,
Department of Sports Medicine,
Friedrich-Schiller-University
Jena, Germany

Degrees:

M.D., Ph.D.

Research interest:

Hemostasiology, Endothelial
function, Immunology.

E-mail: t.hilberg@ghz.de

**Volker SCHMIDT****Employment:**

Medical student, Department of
Sports Medicine, Friedrich-
Schiller-University Jena,
Germany

Research interest:

Platelets

Wolfgang LÖSCHE**Employment:**

Associate Professor, Centre of
Vascular Biology and Medicine, Erfurt, Friedrich-
Schiller-University Jena, Germany

Degrees:

M.D., Ph.D.

Research interest:

Platelets, Inflammation,
Thrombosis

E-mail: Wolfgang.Loesche@med.uni-jena.de

**Holger H.W. GABRIEL****Employment:**

Chair, Professor, Department of
Sports Medicine, Friedrich-
Schiller-University Jena,
Germany

Degrees:

M.D., Ph.D.

Research interest:

Public Health, Exercise
Immunology

E-mail: h.gabriel@ghz.de

✉ Dr. med. Dr. phil. Thomas Hilberg

Department of Sports Medicine, Friedrich-Schiller-
University Jena, Wöllnitzerstr. 42, D-07749 Jena
Germany