Influence of an Acute Exercise on Neutrophil and Platelet Adhesion, Nitric Oxide Plasma Metabolites in Inactive and Active Subjects

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In this work we studied the influence of an acute exercise either on nitrite/nitrate plasma levels or on neutrophil and platelet adhesion in inactive and active subjects. Twelve healthy subjects (6 inactives and 6 actives) exercised on a racing cycle ergometer performing stepwise increases in intensity until reaching, within 5 min, a heart rate of 150 beats × min^{-1} which represents an oxygen consumption of about 75 % of the individual maximum rate of oxygen uptake. From peripheral venous blood samples (drawn from all subjects before, immediately after the end of exercise, and 1 hour later) neutrophils and platelets were isolated to test plate adhesion, and nitrite/nitrate concentrations were measured in the plasma. Immediately after the acute exercise, in active subjects we observed a significant decrease in the percentage of neutrophil adhesion (7.96 ± 2.38 vs. 14.10 ± 3.14), associated with an increase in nitrite/nitrate plasma levels (81.38 ± 10.76 vs. 41.08 ± 8.13 μmol × l^{-1}), restored by a 40 min pre-incubation with N^G-nitro-L-arginine methyl ester (L-NAME). In unstimulated platelets we observed a significant lower percentage of platelet adhesion in active subjects compared to inactives after exercise. With thrombin or adenosine 5′-diphosphate as agonists platelet adhesion did not result significantly different in active subjects compared to inactives. In conclusion, our data show that physical exercise can induce changes in some cell activities, even if transient, and favour the generation of nitric oxide. The lower adhesion of neutrophils and platelets induced by regular exercise could be an important goal in the prevention of vascular and inflammatory diseases.

Key words: Physical exercise, nitric oxide, cell functions.

Abbreviations

NO:	nitric oxide
cGMP:	cyclic guanosine monophosphate
$\dot{V}O_2$max:	maximum rate of oxygen uptake
MAP:	mean arterial blood pressure
fMLP:	formylmethionylleucylphenylalanine
PGI_2:	prostacyclin
NOS:	nitric oxide synthase
COX:	cyclooxygenase
L-NAME:	N^G-nitro-L-arginine methyl ester
EDTA:	ethylenediaminetetraacetic acid
THR:	thrombin
ADP:	adenosine 5′-diphosphate

Introduction

Previously we observed a significant decrease in neutrophil adhesion in professional players during the sports season [6] and in active subjects after an acute exercise [7]. Other authors referred that physical exercise induces quantitative and qualitative functional changes in neutrophils [14] and platelets [17]. We hypothesized a role for NO since it is known [8] that NO inhibits cellular adhesion. Some authors [20] observed that an hemodynamic shear stress elevated NO and prostacyclin (PGI_2) production from vessel preparations, and others [3] showed NO increases in exhaled air during exercise.

The purpose of this work was to evaluate if physical exercise could have affected NO production, determined as nitrite/nitrate plasma levels, in inactive and active subjects. We hypothesized a possible role not only by endothelial cells [20] but also by neutrophils and/or platelets as sources of NO. In fact it is known [2] that physical exercise can upregulate the enzyme producing NO, nitric oxide synthase (NOS), and that the constitutive form of the enzyme is present in the platelets [21]. For this reason we evaluated if cell adhesion decreased in relation to NO production in our experimental conditions and if this decrease was reversed *in vitro* in the presence of L-NAME, a non-selective inhibitor of NOS.

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Materials and Methods

Subjects

Eight inactive and seven active healthy male subjects, aged 21 to 25 years, participated in the study after being informed that it could cause stress. However, one of the actives and two of inactives had abnormal values of some haematological parameters and were excluded from the study, thus samples from six inactives and six actives were taken into account. All inactive subjects, recruited from medical students, devoted less than 3 h × week^{-1} to exercise-related activities and were non-smokers. The active subjects (members of the Research Center of Physical Fitness and Sports, Verona) engaged in regular exercise at least for the last two years and were investigated after an extensive training period of one month consisting of running (66.9 ± 17.3 km × week^{-1}), swimming (11.1 ± 3.4 km × week^{-1}), and cycling (246.4 ± 116.1 km × week^{-1}). None had any symptom of infection or illness, in particular any platelet or coagulation disorder, or had taken any pharmacological treatment known to influence platelet responsiveness in the previous 2 months. In addition, in order to avoid the influence of food on nitrite/nitrate evaluation, all subjects received for 1 week before the day of exercise a limited nitrate diet (the diet excluded food items that contain a high concentration of nitrate, i.e. cured meat, fruit, and particularly green leafy vegetables).

Exercise protocol

On the day of the test a standardized exercise schedule was undertaken by each participant. The study was performed at 8 a.m. in the fasting state. Peripheral venous blood samples were drawn by antecubital venipuncture immediately before exercise. Each subject exercised on a racing cycle ergometer performing stepwise increases in intensity until reaching, within 5 min, a constant heart rate of 150 beats × min^{-1} which represents an oxygen consumption of about 75% of the individual maximum rate of oxygen uptake ($\dot{V}O_2$max). This exercise intensity was then maintained for 10 min after which 40 ml of blood were collected. This model of a single submaximal exercise was considered sufficient to induce changes in leukocyte population as previously reported by other authors [10]. One hour after the end of the exercise another blood sample was taken from each subject. Moreover, in order to assess aerobic capacity, heart rate, mean arterial blood pressure (MAP), and $\dot{V}O_2$max were determined before exercise. MAP was calculated using the following formula: 1/3 (systolic blood pressure – diastolic blood pressure) + diastolic blood pressure.

Separation of neutrophils

The neutrophils were prepared from ethylene-EDTA anticoagulated blood (fractionated by different centrifugations over Percoll gradients) and used at the concentration of 4×10^6 neutrophils × ml^{-1} for adhesion test.

Neutrophil adhesion test

Neutrophil adhesion was evaluated as previously described [4], in presence or absence of formylmethionylleucylphenylalanine (fMLP) 10^{-7} mol × l^{-1} as stimulant, by measuring the membrane enzyme acid phosphatase. The p-nitrophenol production was measured spectrophotometrically at 405 nm with a microplate reader (Reader 400, SLT Labs Instr.). The percentage of cellular adhesion was calculated on a standard curve obtained with known numbers of neutrophils. The potent non-selective NOS inhibitor L-NAME was tested for its capacity to modulate neutrophil adhesion *in vitro* and was incubated at the final concentration of 300 μM for 40 min with the cells before the adhesion test.

Separation of platelets

A volume of 10 ml of blood was added to 1.66 ml of an anticoagulant solution (15 g/L citric acid, 25 g/L sodium citrate), and platelet-rich plasma was obtained by centrifugation at 300 g for 10 min. Washed platelets were obtained by further centrifugation (700 g for 15 min). The platelet suspensions (100×10^6 platelets × ml^{-1}) were kept at room temperature and utilized within one hour: ten minutes before use the cells were warmed up to 37 °C.

Platelet adhesion test

Platelet adhesion was evaluated as previously described [5] in 96-well microtiter plates coated overnight with 0.2 mg/ml human fibrinogen type I, with 5 μmol × l^{-1} ADP or 0.05 U/ml thrombin as agonists and L-NAME (at the final concentration of 300 μM) added to each well. The p-nitrophenol produced by the reaction was measured with a microplate reader (Reader 400, SLT Labs Instr.) at 405 nm against a platelets free blank. The percentage of adherent cells was calculated on the basis of a standard curve obtained with a defined number of platelets of the same subject.

Quantification of nitrite/nitrate

Nitrite/nitrate concentrations in the plasma were measured using a simple two-step procedure (Cayman Chemical Co.): the reaction employs an enzymatic conversion of nitrate to nitrite by the nitrate reductase enzyme, followed by diazotization of the total nitrite formed. The absorbance was measured at 540 nm using a microplate reader (Reader 400, SLT Labs Instr.).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by Student's t-test (for comparison between inactives and actives) and by repeated measures analysis of variance (ANOVA) in order to identify differences between the dependent variables for comparison between and within subjects when three times were compared: p values < 0.05 or less were considered as significant.

Results

Anthropometric and performance data of all subjects are summarized in Table **1** and clearly indicate that the physical characteristics of the subjects selected were homogeneous within the groups: Heart rate at rest was significantly lower ($p < 0.05$) in actives than in inactives, whereas $\dot{V}O_2$max was significantly higher ($p < 0.005$).

Table 1 Anthropometric and performance data of subjects included in our study. Data are expressed as mean ± SD

	Inactives	Actives
Age (years)	21 ± 2.40	22.5 ± 2.11
Height (cm)	179 ± 2.30	181 ± 1.40
Body weight (kg)	76.5 ± 7.30	72.3 ± 3.21
$\dot{V}O_2$max (ml/kg/min)	53.1 ± 5.39	65.2 ± 3.41**
HR (beats/min)	68 ± 8.16	58 ± 6.00*
MAP (mm Hg)	102 ± 8.16	93 ± 6.00

*p < 0.05,
**p < 0.005 actives vs. inactives
$\dot{V}O_2$max = maximum rate of oxygen uptake
HR = heart rate
MAP = mean arterial blood pressure

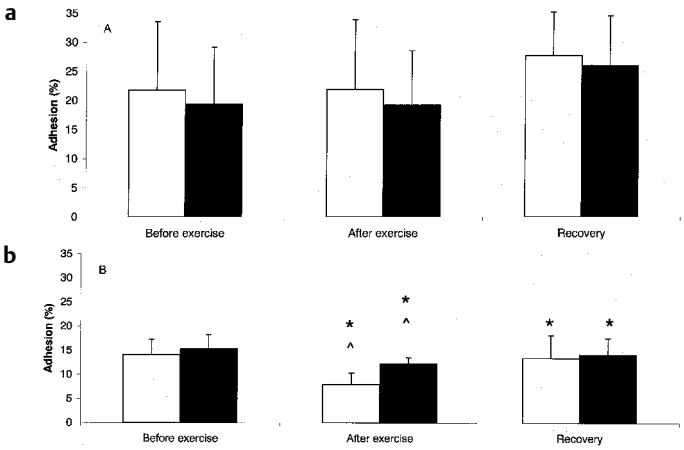

Fig. 1 The percentage of fMLP-stimulated neutrophil adhesion in inactive (**a**) and active subjects (**b**) in absence (□) and presence (■) of L-NAME. The values are expressed as mean ± SD. *p < 0.05 inactives vs. actives; ^p < 0.005 (ANOVA test).

The percentage of fMLP-stimulated neutrophil adhesion in inactive and active subjects is shown in Fig. **1**. In inactives (Fig. **1 a**) we did not observe any significant change in neutrophil adhesion in relation to the acute exercise. In the members of the Research Center of Physical Fitness and Sports (Fig. **1 b**) immediately after the acute exercise we observed a significant decrease in this neutrophil function (7.96 ± 2.38 vs. 14.10 ± 3.14, p < 0.005) even if from our data 1 h seems to be sufficient time to recover the percentage of cellular adhesion. Forty min pre-incubation of stimulated-neutrophils with L-NAME 300 μM restored, *in vitro* the percentage of adhesion decreased by the acute exercise (12.30 ± 1.23 vs. 7.96 ± 2.38, p < 0.005). Moreover after exercise we observed a significantly lower percentage of adhesion in the active subjects compared to the inactives (7.96 ± 2.38 vs. 21.86 ± 12.05, p < 0.05). Without the stimulant fMLP we did not detect any adhesion (data not shown), and the addition of the NOS inhibitor did not affect this situation.

The significant decrease in neutrophil adhesion observed immediately after the end of exercise, which was observed only in active subjects, was associated to significantly higher nitrite/nitrate plasma levels (81.38 ± 10.76 vs. 41.08 ± 8.13 μmol × l^{-1}, p < 0.001) that resulted higher in these subjects compared to inactives also before exercise (44.31 ± 7.12 vs. 25.08 ± 6.44 μmol × l^{-1}, p < 0.001) (Fig. **2**).

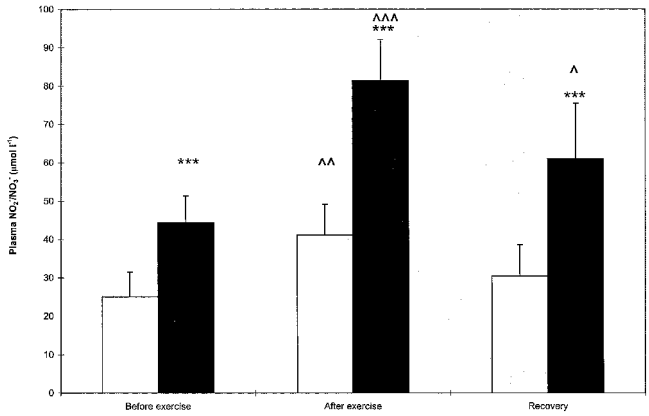

Fig. 2 Plasma nitrite/nitrate in inactive (□) and active subjects (■). The values are expressed as mean ± SD. ***p < 0.001 inactives vs. actives; ^p < 0.05, ^^p < 0.005, ^^^p < 0.001 (ANOVA test).

Table **2** shows platelet adhesion to fibrinogen-coated wells. In unstimulated platelets (resting) we observed a significant lower percentage of adhesion in active subjects compared to inactives after exercise (6.24 ± 2.05 vs. 3.88 ± 1.80, p < 0.05). In the presence of L-NAME, unstimulated platelets of actives showed

Table 2 Platelet adhesion to fibrinogen-coated wells in inactive and active subjects evaluated before (1), immediately after the end of exercise (2), and 1 h later (3). Data are expressed as mean percentage of adhesion ± SD in absence (resting) and presence of adenosine 5′-diphosphate (ADP) or thrombin (THR) agonists

		Without L-NAME	With L-NAME
Inactives			
1	Resting	4.49 ± 1.72	5.86 ± 2.07
	ADP	13.76 ± 3.58	14.73 ± 4.03
	THR	14.43 ± 2.04	15.36 ± 3.13
2	Resting	6.24 ± 2.05	5.55 ± 1.97
	ADP	13.87 ± 4.63	13.73 ± 4.48
	THR	14.32 ± 3.61	14.57 ± 4.16
3	Resting	5.56 ± 2.25	6.33 ± 3.07
	ADP	12.79 ± 4.85	13.51 ± 5.41
	THR	14.12 ± 3.44	13.73 ± 3.64
Actives			
1	Resting	3.16 ± 1.15	2.84 ± 1.20*
	ADP	16.59 ± 2.59	15.40 ± 4.39
	THR	17.51 ± 2.34*	17.77 ± 3.00
2	Resting	3.88 ± 1.80*	2.29 ± 1.11**
	ADP	15.60 ± 2.44	14.21 ± 3.15
	THR	17.51 ± 2.63	16.98 ± 2.03
3	Resting	3.44 ± 1.46	2.71 ± 1.19*
	ADP	15.67 ± 3.43	14.83 ± 4.79
	THR	18.12 ± 3.35	17.88 ± 4.38

*p < 0.05, **p < 0.01 actives vs. inactives

a significantly lower percentage of plate adhesion before, immediately after the exercise, and 1 h later. In the presence of THR or ADP as agonists platelet adhesion did not result significantly different in active subjects compared to inactives in our experimental conditions.

Discussion

In this paper we observed, at first, that resting plasma nitrite/nitrate (the major stable end products of NO metabolism) were higher in active subjects than in inactives. Furthermore we found that a single acute exercise was sufficient to elicit an elevation of nitrite/nitrate levels in both groups, even if transiently, as they decreased 1 h after the end of the acute exercise.

Considering the role of the diet as not relevant since a dietary restriction was adopted in both groups and that NO levels evaluated in both groups at rest are to be considered physiological, we conclude that physical fitness and formation of NO at rest are positively linked to each other, as was also observed by other authors [13].

As we already in a previous paper [7] observed some changes in neutrophil adhesion in members of the Research Center of Physical Fitness and Sports, with our experiments in this study we tried to understand what kind of tissue or cell could have taken part in this increase observed in actives at rest and in both groups after the acute exercise.

Other authors [2] found an upregulation, following hemodynamic shear stress induced by exercise, in NOS of endothelial and muscle cells where cNOS and iNOS are present. Moreover, the acute exercise could stimulate NOS in neutrophils *in vivo*. We observed a decrease in neutrophil plate adhesion only in active subjects, an effect reversed *in vitro* after 40 min pre-incubation with L-NAME. It is known [8] that NO inhibits adhesion in neutrophils and other cells. On the other hand, L-NAME being a non-selective inhibitor, it is not easy to evaluate the role of neutrophil cNOS and iNOS.

In every case, from our data the role of NO on neutrophil adhesion is evident, in relation to an acute exercise, in subjects engaged in regular exercise.

We can discuss the importance of the amount of NO or the influence of exercise (acute and chronic) on the neutrophil adhesion. From our data it is clear that the NO increase observed after the acute exercise in inactive subjects (about 40 μmol/L) is not sufficient to induce any change in neutrophil adhesion. On the other hand, it is not easy to understand whether *in vivo* the higher NO levels (about 80 μmol/L) observed following the acute exercise in subjects engaged in regular exercise could have directly induced some changes in neutrophil molecules of adhesion or whether the physiological changes induced by the acute exercise elicited an initial activation of neutrophils leading to the decrease in plate adhesion observed in the presence of the stimulant fMLP.

Since adherent properties of granulocytes depend on many factors [18] such as cellular metabolic activity, complement activation, divalent cations, hormones, cytokines, NO and prostacyclin, we think that this latter hypothesis could be more acceptable on the basis of our data and as also stated by other authors [9,11,12]. All these considerations could also help us to explain the data concerning platelets since in platelets physiology various mediators can alternatively promote or inhibit platelet adherence [1,16]. Kedziora et al. [15] observed, after submaximal physical exercise, changes in the activities of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase with beneficial effects on the extent of lipid peroxidation.

When comparing the platelet adhesion between inactive and active subjects, in the absence of agonists (resting) we observed a significant decrease in adhesion after the acute exercise in subjects engaged in regular exercise: no changes were evident comparing this parameter before and after the acute exercise in the same individuals. It seems that a regular moderate exercise, rather than an acute exercise, could have influenced platelet adhesion capacity. Moreover, considering the paradoxical results observed in platelet adhesion of active subjects in the presence of L-NAME, we can only hypothesize that either NO had a minimum role in our experimental conditions or L-NAME was not active as NOS inhibitor. This could be related to the concentration of L-NAME or to the fact that cellular esterases did not hydrolyze L-NAME to N^{ω}-nitro-L-arginine (L-NNA), a more potent cNOS inhibitor [19]. In any case the reason for this decrease is at present unknown, and further experiments are needed.

Different considerations should be made when ADP and THR agonists are present. Platelet adhesion to fibrinogen depends on the expression of surface activated GPIIb/IIIa, and this is the result of the activating process in response to specific agonists [1]. We put the agonists in the plate *in vitro* in contact with platelet taken *ex vivo*, and in these experimental conditions the effects of chronic exercise on platelet adhesion were cancelled. This is difficult to explain, since it is likely that different cellular events can happen *in vitro*, the knowledge of which is beyond the aim of this paper. In any case these results strengthen our hypothesis that a chronic moderate exercise can induce some changes in platelet activity.

In conclusion, these data show that an acute exercise *in vivo* could favour the generation of NO, but more important we observed that the chronic exercise can prime some changes in neutrophil and platelet function, that subsequently an acute exercise can modify in a significant way, particularly evident in our experiments with neutrophils.

In addition, if it is true that acute physical exercise can induce changes in some cell activities, it is also true that these are transient.

From a clinical point of view the decrease in platelet and neutrophil adhesion induced by a regular moderate exercise could be an important goal in the prevention of vascular and inflammatory diseases, but also in the improvement of pharmacologically treated diseases.

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