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ORIGINAL ARTICLE

Role of Hsp72 and norepinephrine in the moderate exercise-induced stimulation of neutrophils' microbicide capacity

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Abstract The influence of a single session of moderate exercise (45 min at 55% of VO_2 max) performed by young sedentary men (23–25 years old) on the microbicidal capacity of neutrophils was compared by using both direct (killing of phagocytosed *Candida albicans*) and indirect (superoxide anion production measured by NBT reduction) techniques. In addition, the role of norepinephrine and heat shock protein (Hsp) 72 in the modulation of microbicide capacity of neutrophils was evaluated during the protocol of exercise and recovery period (24 h). No significant changes were found in the superoxide production after exercise. However, immediately after exercise there was an increase in the destruction of *C. albicans*, which remained higher than basal values 1 day later. This behaviour was similar to the changes found in the serum extracellular Hsp72 concentrations (an increase after exercise that remained higher than basal values 24 h later). In vitro, the raised physiological concentration of Hsp72 after exercise also increased the microbicide capacity of neutrophils with respect to controls and the values induced by the basal concentration of the protein. This indicates that Hsp72 is participating as a "stress mediator" of the stimulated microbicide activity during moderate exercise. However, norepinephrine is not mediating the increased killing of *C. albicans* during exercise.

Keywords Exercise · Stress · Immunology · Neutrophils · Hsp72 · Norepinephrine

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Introduction

The stimulation of innate immune response, such as neutrophils function, is particularly important because it constitutes the organism's first line of defence against pathogens. Neutrophils may thus impede the entry and maintenance of microorganisms during and after exercise (Ortega 1994). Although most studies have been performed on athletes, moderate exercise can also promote health in sedentary people who, in most cases, perform exercise in single sessions without training programs, for example during the weekend.

Some studies concluded that when moderate exercise is very prolonged the neutrophils' microbicide capacity (measured by the oxidative burst) in athletes is lower than the one observed after shorter duration, more intense and fatiguing exercise (Robson et al. 1999). Although prolonged exercise may induce temporary immunosuppression with a presumed increase of susceptibility for infection in athletes, recent studies suggest, however, that prolonged cycling at moderate intensity does not seem to seriously alter the function of cells of the first line of defence (Scharhag et al. 2005). Acute and short-duration exercise generally stimulates the innate cellular immune response (Ortega 1994), and this stimulation is usually mediated by stress hormones, above all in sedentary people (Ortega 2003). However,

the effects of single sessions of moderate exercise on the neutrophils' microbicidal activity in physically inactive subjects, as well as the neuroendocrine mediators of this effect, remain unclear. Studies using direct techniques such as killing of alive antigens have not always shown similar results to those using indirect techniques, such as the production of reactive oxygen species (ROS) (Smith and Pyne 1997). In addition, although the stress factors (i.e. glucocorticoids, catecholamines, GH, prolactin, etc.) are known to modulate neutrophil microbicide function during intensive exercise (Ortega 2003), less is known about moderate exercise, especially in sedentary people. Recently we found that norepinephrine is involved in the increased phagocytosis induced by a single session of moderate exercise (Ortega et al. 2005a). It has also been recently suggested that heat shock proteins may be involved in the modulation of innate response during stress; and catecholamines could be involved in the expression and release of these proteins (Fleshner et al. 2003). The concentration of this protein increases in the circulation during exercise (Walsh et al. 2001); it also enhances some aspects of innate immune response, such as pro-inflammatory cytokines production by monocytes (Asea et al. 2000), and facilitates the immune function during stress in the presence of bacterial and other pathogens (Fleshner and Johnson 2005). Thus, heat shock proteins have now been shown to act as initiators of the host's immune response, a process known as the chaperokine activity of Hsp72 (Asea 2005).

The first purpose of this study was to evaluate the influence of a single session of moderate exercise (45 min at 55% of VO_2 max) performed by young sedentary men on the microbicidal capacity of neutrophils, comparing the results obtained by indirect (superoxide anion production) and direct (killing of phagocytosed *Candida albicans*) techniques. The second purpose of the present investigation was to determine whether norepinephrine and/or Hsp72 are involved as a "stress mediator" in this influence.

Methods

Subjects

Ten healthy male volunteers aged between 23 and 25 years participated in the study after being informed of the protocol. They had to be physically inactive, having undertaken no exercise programme during the previous 24 months, healthy, non-smokers, and not heavy consumers of alcohol. The experiment was approved by the Ethical Committee of the University of Extremadura (Spain) according to the guidelines of the European Community Council Directives.

Physical exercise

The experiment started at 9.00 a.m. with the participants fasted and in repose for at least 1 h. Each subject exercised on a cycle ergometer (Ergometrix mod. Ergo-800S) for 45 min at an exercise intensity of 120 W, which gave an oxygen uptake (VO_2) of 1.875 ± 0.115 l min^{-1} corresponding to 55 ± 5% of maximal aerobic power (VO_2 max).

Isolation of neutrophils

Peripheral venous blood samples were drawn by antecubital vein puncture. The sampling was carried out before (basal state), immediately after, and 24 h after exercise. The blood was centrifuged in a density gradient (Histopaque, Sigma), and the neutrophils were harvested, washed twice in Hanks' medium (Sigma), counted, and adjusted to 10^6 cells ml^{-1} of medium. Cell viability was checked by the Trypan blue exclusion test, which gave 98% viable cells.

Serum

Serum used as the opsonin source in the microbicide test was obtained from venous blood taken from the volunteers in a basal state.

Microbicide capacity of neutrophils

Microbicidal capacity of neutrophils has been compared by using both direct (killing of phagocytosed *C. albicans*) and indirect (superoxide anion production after phagocytosis of inert particles) techniques. This capacity of neutrophils was evaluated before, immediately after, and 24 h after exercise.

Killing of phagocytosed *C. albicans* (ATCC 10321 from Microbiology Department of University of Extremadura) cultivated in agar was evaluated ex vivo using a technique previously described for isolated neutrophils (Ortega et al. 1993). *C. albicans* suspension, 0.5 ml (10^6 cells ml^{-1} Hanks' medium), and 50 µl serum were added to 0.5 ml neutrophil suspension (10^6 neutrophils ml^{-1}), followed by incubation in a thermostatic bath at 37°C for 50 min. Then, 1.5 ml of methylene blue (0.01%) that stained the dead *C. albicans* was added. The samples were then centrifuged at $300 \times g$ for 10 min, discarding two-thirds of the supernatant. The remainder was shaken and an aliquot taken for counting in a Neubauer haemocytometer

under phase contrast microscope. The numbers of phagocytosed and dead *C. albicans* by 100 neutrophils were counted (candidicide index).

The oxygen-dependent microbicide capacity of neutrophils was evaluated by means of the superoxide anion production. The assay was performed by the nitroblue tetrazolium (NBT; Sigma) reduction test. Neutrophil suspension, 250 µl (1×10^6 cells ml^{-1}), was incubated with 250 µl of NBT (1 mg ml^{-1} in PBS) and 25 µl of latex beads (1% in PBS) (stimulated samples), or 25 µl of PBS (non-stimulated samples). After 30 min of incubation, the reaction was stopped with HCl (0.5 N) and the samples were centrifuged (30 min, $600 \times g$). The intracellular reduced NBT (intracellular concentration of superoxide anion) was extracted with dioxan (Sigma). The absorbances of the supernatants at 525 nm were determined using the same amounts of Hanks' solution, NBT, and HCl or dioxan, respectively, as blank. The results are expressed as percentages of stimulation, calculated by giving the value 100 to the absorbance obtained in tubes without latex beads (non-stimulated samples).

Study of the variations in the heat shock protein (Hsp) 72 and norepinephrine concentrations

Changes in the blood concentrations of Hsp72 and norepinephrine were studied before, immediately after, and 24 h after exercise. For the Hsp72 assay, serum was obtained by centrifuging ($300 \times g$ for 20 min) 1 ml of blood from each participant. For the catecholamine assay, 40 µl stabilizing solution (900 mg of EGTA and 700 mg of glutathione in 10 ml of 0.1 M NaOH) was added to 2 ml of each blood sample before separation of the plasma. The plasma was then isolated by centrifugation as before. All plasma samples were stored at −30°C until assay.

Hsp72 was measured by ELISA (Stressgen) and norepinephrine was assayed by HPLC with electrochemical detection (Coulochem II), using a C18 column (Waters), at a working potential between 450 and 660 mV, flow rate of 1 ml min^{-1}, and a pressure of 200 bar (2×10^4 kPa) at most. An internal standard (dihydroxybenzylamine) was used to allow the determination of losses during assay (Chromosystems Instruments and Chemical).

In vitro study of the effect of the physiological concentrations of Hsp72 and norepinephrine on neutrophils' microbicidal capacity

In a second part of the study we evaluated the in vitro effect of the physiological concentrations determined before and after exercise of Hsp72 (5.12 vs 7.66 ng ml^{-1}) or norepinephrine (0.4 vs 0.9 ng ml^{-1}) on the microbicide capacity of neutrophils. In order to study which receptors were participating in the norepinephrine modulation we also added in the assays propranolol (a beta-blocker) and phentolamine (a alpha-blocker). Both were used at 10^{-5} M, an effective concentration for neutrophils as previously determined in a dose–response experiment (Ortega et al. 2005a). Therefore, microbicide capacity was evaluated as previously indicated, but in the presence of physiological (basal or after exercise) "stress factors" concentrations.

Statistical analysis

The variables were normally distributed. The parametric ANOVA-Scheffe F test was used for comparisons, taking $P < 0.05$ as the minimum significance level. Values are given as means (SEM).

Results

Figure 1 shows the results corresponding to neutrophils' microbicide capacity. There was an increase ($P < 0.05$) in the destruction of *C. albicans* (candidicide index) immediately after our protocol of exercise, which remained higher than basal values 1 day later (Fig. 1a). However, there were not significant changes in the variations of NBT (Fig. 1b).

This moderate exercise increased the norepinephrine ($P < 0.05$) and Hsp72 ($P < 0.001$) concentrations. However, while Hsp72 remained higher ($P < 0.001$) than basal values 24 h later, the norepinephrine concentration returned to basal values 1 day later (Table 1).

In vitro incubation of isolated neutrophils with the Hsp72 serum post-exercise concentration increased their microbicide capacity with respect to the control values ($P < 0.01$) and the values obtained with the Hsp72 basal concentration ($P < 0.05$) (Fig. 2). In vitro incubation of isolated neutrophils with the basal or post-exercise plasma concentrations of norepinephrine increased ($P < 0.05$) the microbicide capacity with respect to the control values (in the absence of norepinephrine). This stimulation disappeared in the presence of both propranolol (beta-blocker) and phentolamine (alpha-blocker). However, there were no significant differences in the candidicide index between the values found with the post-exercise and basal concentrations of norepinephrine (Fig. 3).

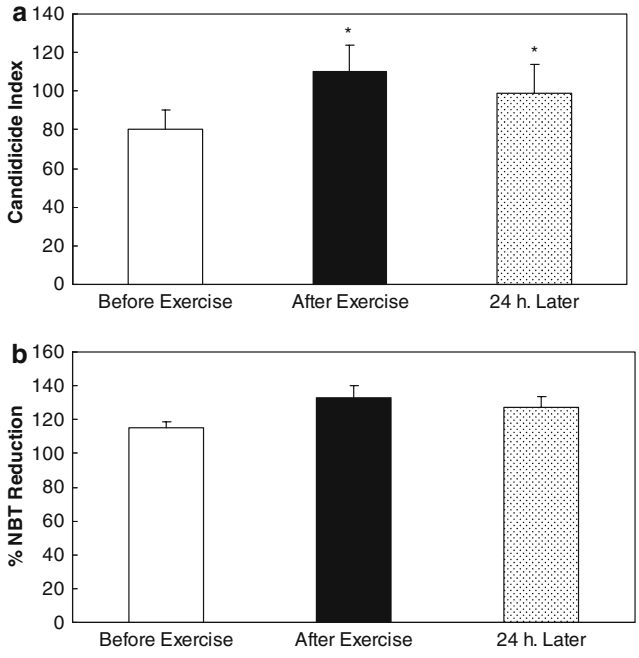

Fig. 1 Influence of exercise on neutrophils' microbicide capacity evaluated by direct (candidicide index) (**a**) or indirect techniques (% NBT reduction) (**b**). Each value represents the mean (SEM) of ten experiments performed in duplicate. $^{*}P < 0.05$ with respect to basal values (before exercise)

Table 1 Changes induced by moderate exercise on the Hsp72 and norepinephrine blood concentrations

	Hsp72 (ng ml^{-1})	NE (ng ml^{-1})
Before exercise	5.12 (0.52)	0.40 (0.08)
Immediately after exercise	7.66 (0.64)***	0.90 (0.04)*
24 h after exercise	6.59 (0.93)***,••	0.45 (0.05)•

Each value is the mean (SEM) of ten determinations performed in duplicate

$^{*}P < 0.05$, $^{***}P < 0.001$ with respect to basal values

$^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$ with respect to the values obtained immediately after exercise

Discussion

Earlier studies in our lab showed that a single session of moderate exercise performed by sedentary men stimulates the adherence, chemotaxis, and phagocytosis of neutrophils (Ortega et al. 1993). However, some studies showed that moderate exercise enhances neutrophil oxidative activity (Smith et al. 1990), while others found a temporary suppression (Macha et al. 1990; Pyne et al. 1996). Thus, the microbicidal activity, the last and probably the most decisive stage of the phagocytic process, has yielded the most controversial results, above all when it is evaluated by using indirect techniques, such as ROS production (Smith and Pyne 1997; Pyne et al. 2000). In this investigation moderate exercise did not induce significant changes in the superoxide anion production (measured by the NBT reduction). We could only conclude that a single session of moderate exercise does not significantly change the oxygen-dependent microbicidal capacity because an increase in the whole microbicidal capacity of neutrophils after exercise was demonstrated by direct techniques (killing of *C. albicans*). These results are indicative of the controversies when the microbicide capacity of neutrophils during exercise is measured by indirect techniques, confirming that these methods are not always enough. In addition, the microbicide capacity remained stimulated at least 1 day after the exercise, confirming an important role of phagocytes in avoiding the "open window" situations after a single bout of moderate exercise in sedentary people (Ortega et al. 2005b). In contrast to what is found after intense exercise, it is possible that these changes in neutrophil

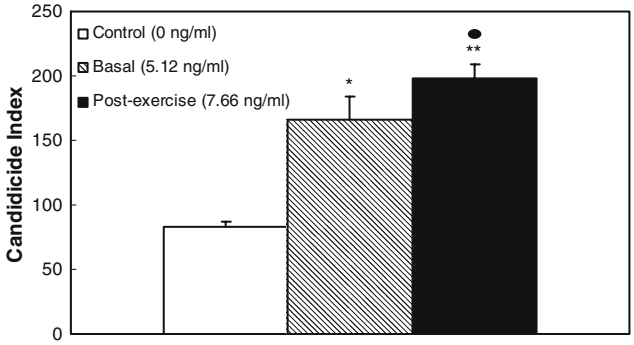

Fig. 2 In vitro effect of the Hsp72 physiological concentrations on neutrophils' microbicide capacity against *C. albicans*. Each value represents the mean (SEM) of ten assays performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to control values. $^{\bullet}P < 0.05$ with respect to basal concentration-induced values

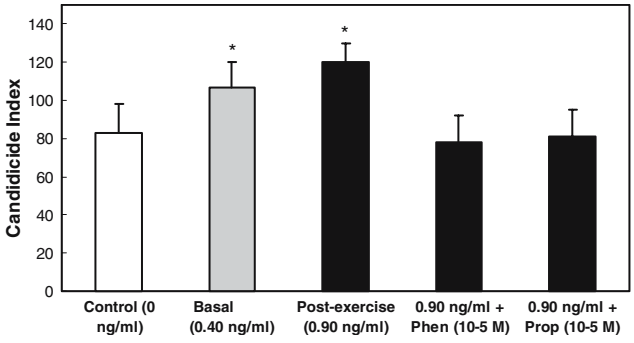

Fig. 3 In vitro effect of the norepinephrine physiological concentrations on neutrophils' microbicide capacity against *C. albicans*. Each value represents the mean (SEM) of ten assays performed in duplicate. $^{*}P < 0.05$ with respect to control values (0 ng ml^{-1}). *Phen* phentolamine, *Prop* propranolol

function observed with moderate exercise may be due to entry of more mature cells, which were previously adhered to the vascular endothelium at rest, into circulation (Gabriel and Kindermann 1998), and this will result in a higher microbicide capacity.

Recently, we found that norepinephrine participates as a "stress mediator" in the increased phagocytic capacity (Ortega et al. 2005a) and the number (Ortega et al. 2005b) of neutrophils from sedentary men during a bout of a similar (55% VO_2 max) moderate exercise. In the present investigation, norepinephrine plasma concentration also increased during moderate exercise, and the physiological norepinephrine concentrations stimulate the neutrophils' microbicide capacity through both alpha- and beta-receptors. However, norepinephrine is not involved in the increased killing of *C. albicans* induced by the exercise, as similar results were found after incubating neutrophils with the norepinephrine basal or post-exercise concentrations. In addition, while norepinephrine returned to basal values 24 h after exercise, neutrophils' microbicide capacity still remained stimulated.

Searching for other possible "stress mediators" we considered heat shock proteins (Hsp), which may have an important role as activators of the innate immune response (Wallin et al. 2002). The 70-kDa Hsp (Hsp70) family includes a constitutive 73-kDa protein (Hsp73) and a highly stress-inducible 72-kDa protein (Fleshner et al. 2003). It has been demonstrated that these extracellular heat shock proteins (Hsp) 72 are released during exercise-induced stress in several tissues (Campisi et al. 2003a) and in blood (Walsh et al. 2001) in a duration and intensity-dependent way (Fehrenbach et al. 2005). In addition, exogenous Hsp70 stimulates phagocytosis, processing and antigen presentation by macrophages (Wang et al. 2006), and Hsp70 also has marked effects on cytokine production by monocytes through TLR2, TLR4, and CD14 receptors (Asea et al. 2000, 2002). As TLR2 and CD14 are also present on the surface of human neutrophils (Kurt-Jones et al. 2002; Power et al. 2004), it is reasonable to think that extracellular Hsp70 has effects also on neutrophils. In this investigation we have observed an increased in Hsp72 serum concentration even after a single bout of 45 min of moderate exercise, which remained elevated at least during 1 day. This role was confirmed in vitro, as the post-exercise Hsp72 concentration induced higher neutrophil candidicide capacity than the basal one. To our knowledge this is the first demonstration that physiological Hsp72 concentrations stimulate neutrophils' microbicidal capacity. In the field of the "danger signal theory for the immune system" (Matzinger 2002), Hsp72 has been considered as a possible "danger signal" during stress (Campisi et al. 2003b). The results presented here may contribute to confirm this hypothesis.

Some investigations support the idea that moderate exercise enhances neutrophils' respiratory burst, reflecting an improved responsiveness of neutrophils following exercise of moderate intensity, thereby increasing the individuals' resistance to infection (Peake 2002). A somewhat different interpretation is given by Niess et al. (1999) who conclude that the increased production of ROS by immunocompetent cells in the inflammatory response to exercise may exert damaging effects. Although free radicals are indispensable for phagocytic cells to fulfil their microbicidal task correctly, any excess production may contribute to the harmful effects associated with physical exercise, such as muscle damage and aging process, above all after intense exercise (Suzuki et al. 1996). In the present investigation we have found that a single bout of moderate exercise performed by sedentary people did not maintain elevated levels of superoxide anion but, however, increased the neutrophils' microbicide activity against *C. albicans*. This means that moderate exercise may improve the innate function of neutrophils to destroy pathogens without an excess of ROS production, which is associated with different exercise-induced pathologies. Norepinephrine, and above all extracellular Hsp72, seems to be involved in the stimulation of the neutrophils' microbicide capacity. Norepinephrine could serve as a signal that mediates the elevation of Hsp72 in the circulation during exercise because it has been recently reported that Hsp72 increases in blood via alpha-adrenergic receptor-mediated signalling pathway, and this elevated Hsp72 can facilitate innate immunity (Johnson et al. 2005). Preliminary results in our laboratory showed that Hsp72 stimulates neutrophils' chemotaxis. Other investigation supports that increased neutrophils' apoptosis coincided with induction of Hsp72 following transmigration through endothelial barriers (Hennigan et al. 1999), and Hsp induction in neutrophils may provide the danger signals required to generate a more effective macrophages response, a mechanism recently reported for regulating the extent of the immune response to invading microbes (Zhen et al. 2004). Results from the present investigation confirm that Hsp72 is involved in neutrophils activation to carry out the phagocytic process during exercise.

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