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The effect of prolonged intense physical exercise of special forces volunteers on their plasma protein denaturation profile examined by differential scanning calorimetry

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ABSTRACT

The human blood plasma proteome profile has been an area of intensive investigation and differential scanning calorimetry (DSC) has come forward as a novel tool in analyzing plasma heat capacity changes to monitor various physiological responses in health and disease. This study used DSC to assess potential alterations in the plasma heat capacity profile of albumin and globulins during extremely demanding physical exercise. We monitored the changes in denaturation profiles of those plasma proteins for five consecutive days of an extraordinary exercise training schedule in 14 young male Special Forces volunteers, as well as after a 30-day recovery period. The major effect of the prolonged intense exercise was the continuous upward shift of the albumin peak by 2°–3 °C on the initial days of exercise, with a tendency to plateau circa the 5th day of exercise. In addition, some redistribution of the denaturational enthalpy was observed upon exercise, where the globulins peak increased relative to the albumin peak. Noteworthy, the alterations in the plasma proteome denaturational profiles were not persistent, as virtually full recovery of the initial status was observed after 30 days of recovery. Our findings indicate that 5 days of exhaustive physical exercise of highly trained individuals enhanced the thermal stability of plasma albumin shifting its denaturational transition to higher temperatures. We surmise that these effects may be a result of increased blood oxygenation during the prolonged intense exercise and, consequently, of albumin oxidation as part of the overall adaptation mechanisms of the body to extreme physical and/or oxidative stress.

1. Introduction

Although the long-term beneficial effects of regular physical activity on chronic diseases, i.e., on the incidence of coronary artery disease, hypertension, dyslipoproteinemia, and obesity, as well as on the general health status and life expectancy, are established (Rigla et al., 2000), however the systemic physiological responses to acute demanding exercise and during the recovery period are not well understood. These responses are complex and involve a wide range of metabolic, immunological and hormonal changes. Indeed, acute prolonged physical exercise affects whole-body metabolic and immunologic homeostasis and changes in various blood variables such as glucose, total protein,

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albumin, uric acid, calcium, phosphorus, creatinine, bilirubin, and alkaline phosphatase have been reported following prolonged exercise, associated with multiple physiological responses to a demanding exercise (Carraro et al., 1990; Kratz et al., 2002a). Elucidating the complex processes that determine the human body's ability to adapt to prolonged intense exercise stimuli is of particular importance in order to better understand and improve physical performance.

The human blood plasma proteome profile has been an area of intensive investigation, aiming at the discovery of specific biomarkers for various physiological alterations in health and disease. Indeed, blood plasma has the potential to serve as a convenient specimen for disease diagnosis and monitoring of various physiological responses. Within this context, differential scanning calorimetry (DSC) has recently come forward as a novel tool in biomedicine and physiology research. DSC analyzes plasma (or serum) heat capacity changes in the temperature range of protein thermal denaturation; Modifications of the plasma heat capacity profiles detected by DSC are considered to be associated with either alterations in plasma protein content, changes in the protein structure by covalent modifications, or with changes in their specific denaturation temperature due to a ligand binding.

More specifically, a DSC thermogram signature of blood plasma has been established for healthy individuals, characterized by major peaks which are attributed to the major protein fractions in the plasma, i.e., albumin, globulins, immunoglobulins G (IgGs), fibrinogen, and transferrin. Other low abundance proteins also contribute to the signature heat capacity profile, though to a lesser extent due to their low plasma concentrations. In particular, albumin is the most abundant protein in plasma, making up more than 50% of total plasma protein content (Anderson et al., 2002). It has been associated with the protein balance of the body and with changes in skeletal muscle mass, while it acts as transport and redox system and its redox state may be affected by physical exercise (Imai et al., 2002; Visser et al., 2005). Globulins are another major blood protein fraction and have also been shown to be influenced by prolonged exercise (Fragala et al., 2017; Kratz et al., 2002b). In addition to exercise-induced changes in the concentrations of these major plasma protein components, alterations of the signature plasma thermogram as a result of physical exercise, mainly attributed to the protein fractions of albumin and globulins, have also been reported in studies using DSC analysis for the evaluation of the beneficial or detrimental effects of physical activity (Michnik et al., 2013, 2014, 2017). However, potential plasma heat capacity changes during extremely demanding exercise have not yet been investigated. A model of such an extreme exercise is the so-called "Hell Week" military training of the Basic Underwater Demolition School (BUD/S) of the Hellenic Navy Special Operations Forces (Navy SEALS) and it is a challenge to characterize the plasma proteome denaturation profiles of Special Forces volunteers during one of their most demanding physical training courses.

The aim of the present study was to assess the alterations in the plasma heat capacity profile of albumin and globulins during a prolonged intense physical exercise compared to a control condition profile, assessed before that intense exercise training, as well as after a 30-day recovery period. We monitored the changes in denaturation profiles of those plasma proteins for five consecutive days of an extraordinary exercise training schedule in young males, setting the hypothesis that their heat capacity profiles would be changed during the exercise period, thus indicating their potential role as mediators of exercise-induced adaptations to the extremely demanding stimuli.

2. Materials and methods

2.1Ethical approval

All the volunteers provided an informed consent to participate in this study, which has been approved by the Ethics Committee of the Harokopio University of Athens.

2.2. Subjects

Fourteen healthy men (age 22.7 ± 1.7 years, height 180.4 ± 1.7 cm, body mass 70.2 ± 2.7 kg, body mass index 23.7 ± 0.6) participated in the study.

2.3. Exercise training program

This study was conducted during the most demanding military training week (Hell Week) of the Basic Underwater Demolition School (BUD/S) of the Hellenic Navy Special Operations Command (Navy SEALS). Hellenic Navy Special Operations Command (SOC) is the elite unit of the HN Special Operations Forces (SOF) community. HN SOC mission is to conduct unconventional warfare and amphibious operations in and out of Greek national territory area as NATO's member.

HN Navy SEALs (**SE**a **A**ir **L**and) officially known as O.Y.K, are expertly trained to achieve the impossible, to deliver highly specialized, intensely challenging warfare capabilities that are beyond the means of standard military forces. Their mission is not limited to direct action warfare, but includes special reconnaissance and counterterrorism. All HN Navy SEALs must successfully attend the Basic Underwater Demolition School (BUD/S) of the Hellenic Navy Special Operations Command.

BUD/S is a 32-week extremely intensive and high-risk training schedule with very low ratio of success (approx. 15%) and has been described as ''brutal''. This military training program develops the O.Y. K. candidates' mental and physical stamina and assesses them in physical conditioning, water competency, teamwork and mental tenacity. Physical conditioning utilizes running, swimming and calisthenics. Furthermore, special training objects as combat diving, unconventional warfare techniques, amphibious operations, use of man-portable medium range weapon systems and standard explosives are included. In particular, during the "Hell Week" candidates participate in 5 consecutive days of continuous training course characterized by extreme mental and physical fatigue. The candidates did not sleep at all during the entire week and remained wet during this period while walking and running more than 300 km as well as doing physical training for more than 20 h per 24 h.

2.4. Blood sampling and plasma analyses

Blood samples were withdrawn 7 days prior to, on each of the five consecutive days of the "Hell Week", as well as 30 days after its completion, always at the same time of the day for all participants (08.00–08.30 a.m.). The participants were seated quietly for 10 min and 10 ml of blood were collected into commercially available anticoagulant-treated tubes. Plasma was separated from blood after centrifugation at 4000 rpm for 10 min at 4 °C, stored frozen in 0.4 ml aliquots at −80 °C and only thawed once for analysis. Plasma protein concentrations were determined by the Bradford assay (Bio-Rad).

2.5. DSC analyses

The DSC analyses were performed using a Nano DSC instrument (TA Instruments). Briefly, 2 heating scans were made for each sample at 1 K/min scan rate in the range 1–105 °C. The first scans displayed the thermal denaturation heat capacity profiles of the native samples, while subsequent scans displayed thermograms typical of irreversibly denatured samples, with no detectable thermal events. The second scans, which originated from the same sample, with the same chemical composition, but displayed no thermal events, were used as a baseline and were subtracted from the first scans. Such approach is typically used for samples with complex composition, undergoing irreversible thermal transformations and was described in our previous work (Tenchov et al., 2017). The complex heat capacity profiles have been deconvoluted using Gaussian curves.

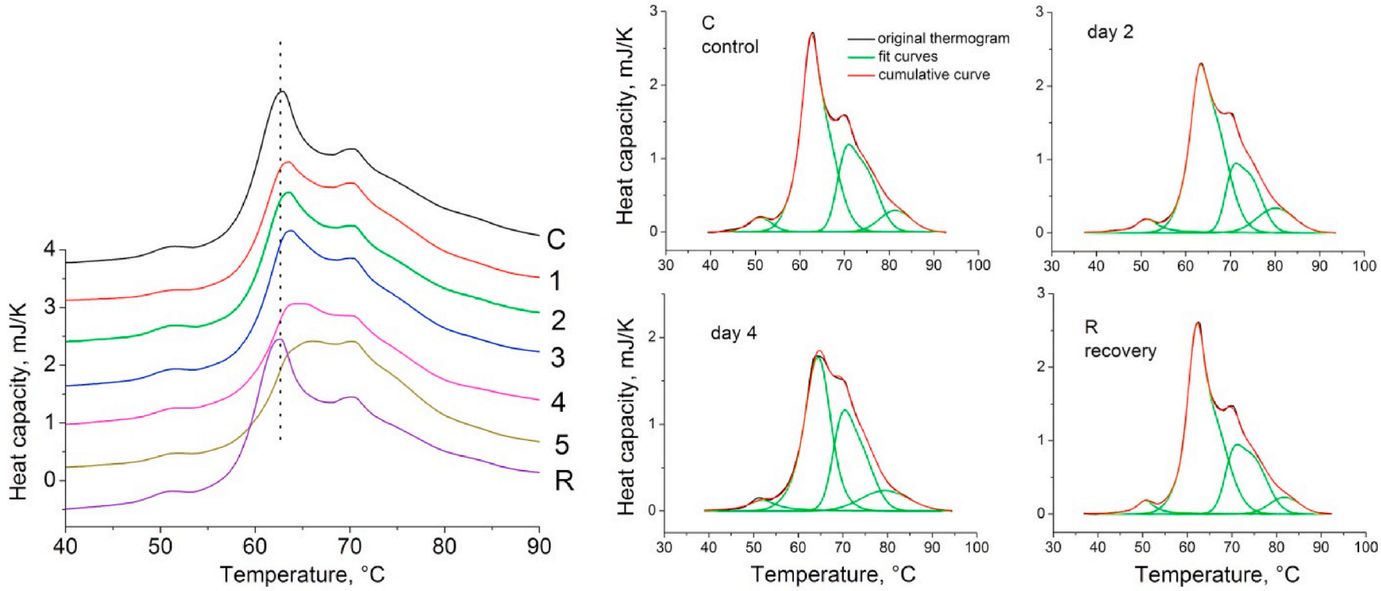

Fig. 1. *Left panel:* representative heat capacity profiles of blood plasma of one volunteer during five days of extreme physical exercise (designated with numbers 1–5). Blood samples were taken on each day of exercise. The top thermogram (C) corresponds to a plasma sample taken before exercise. The bottom thermogram (R) corresponds to a blood sample taken after 30 days of recovery. *Right panel:* Deconvolution of representative heat capacity profiles with Gaussian curves.

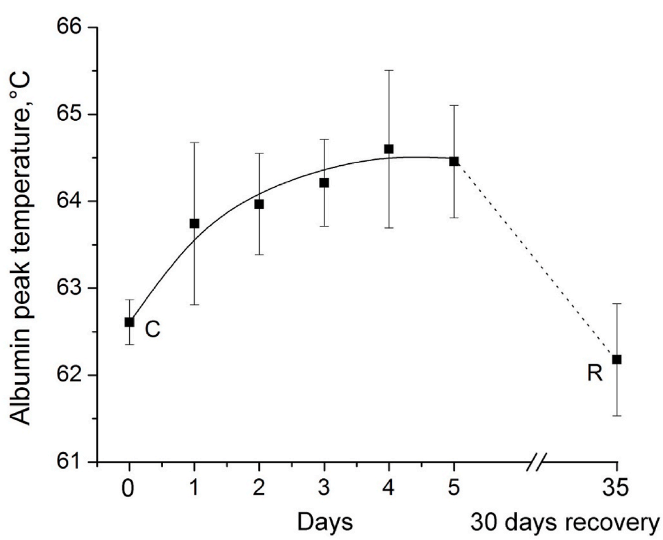

Fig. 2. Shift of the average albumin peak temperature during 5 days of exercise and after 30 days of recovery. The first point (C) is from a thermogram recorded before exercise and the last point (R) – after recovery (mean ± SD.; n = 14).

3. Results

No significant or consistent changes were observed in the protein concentration of each plasma sample at any time point measured ($p > 0.05$). In order to obtain a reference point and to determine how exercise alters plasma thermal behavior, the heat capacity profiles of plasma samples before exercise were recorded (Fig. 1, top thermogram in each panel). These profiles displayed the typical signature DSC pattern of healthy plasma proteome, with dominant peaks at 63 °C and 72 °C, resulting from denaturation of the most abundant albumin and globulin fractions, respectively. Two minor fibrinogen peaks were observed at ~52 °C and 82 °C, which are usually attributed to IgG and transferrin components (Garbett et al., 2009).

The major effect of the prolonged intense exercise was the continuous upward shift of the albumin peak – by 2–3 °C (Figs. 1 and 2). The temperature increase was well expressed on the initial days of exercise, with a tendency to plateau circa the 5th day (Fig. 2).

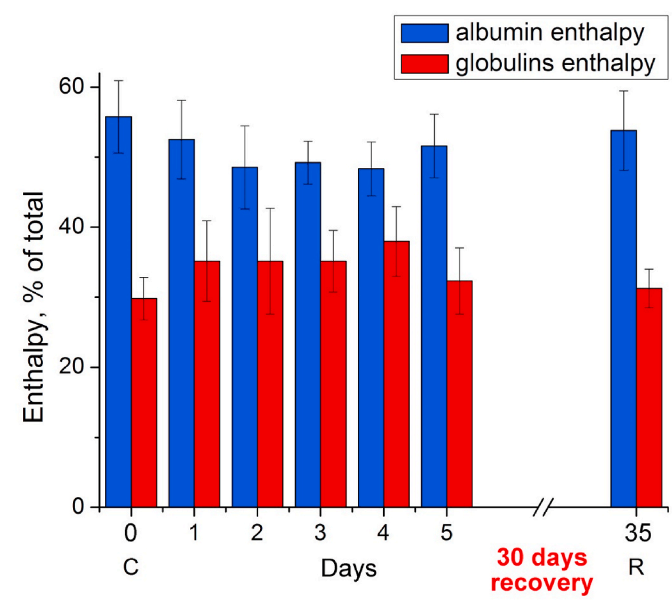

Fig. 3. Alterations of the average albumin and globulins peak enthalpies (as percentage of the total blood plasma protein denaturation enthalpy) over time. The first point (C) is from a thermogram recorded before exercise and the last point (R) – after 30-day recovery (mean ± SD.; n = 14).

In addition, some redistribution of the denaturational enthalpy was observed upon exercise – where the globulins peak increased relative to the albumin peak, particularly during the initial four days of exercise (Fig. 3). The thermograms of blood samples collected 30 days after the completion of the "Hell Week" revealed virtually total return to the initial signature pattern (Fig. 1, bottom profile in each panel; Figs. 2 and 3).

4. Discussion

Our study examined the changes in the denaturation profiles of plasma albumin and globulins during a prolonged intense physical exercise of Special Forces volunteers, in order to reveal the potential involvement and interactions of those major plasma proteins in the adaptation process to an extremely demanding physical training schedule. Our findings demonstrate a continuous upward shift of the albumin peak, while some redistribution of the denaturational enthalpy was observed upon exercise where the globulins peak increased relative to the albumin peak. It is interesting to note that modifications of plasma DSC transition observed during the exercise period in this study may not indicate the maximum potential effects of that extremely demanding exercise on plasma denaturation transition, because of the already high training status and physical performance of the participants before the "Hell Week". Also, noteworthy, the alterations in the plasma proteome denaturational profiles were not persistent, as virtually full recovery of the initial status was observed after 30 days of a recovery period.

The increase of the plasma albumin thermal stability during exercise is a likely result of oxidation. Indeed, it is now well documented that exhaustive exercise induces production of reactive oxygen species (ROS), potentially due to the increased uptake of oxygen from the active skeletal muscles, resulting in increased oxidative stress (Pingitore et al., 2015) and it has been reported that oxidation enhances human serum albumin thermal stability (Sancataldo et al., 2014; Gorobets et al., 2019). More specifically, it is known that human serum albumin possesses a free thiol group in Cys34 (the other 34 Cys residues form intramolecular disulfide bonds), which is involved in the heterogeneity of albumin isoforms, and may function as an extracellular antioxidant by scavenging ROS (Imai et al., 2002; Halliwell, 1988). However, under oxidative stress conditions, albumin is oxidized and Cys34 forms disulfide bonds and albumin dimers. Thus, the oxidation caused by free radicals may affect the molecular conformation and structure (Lamprecht et al., 2008a) manifested, inter alia, as an upward shift of the albumin denaturation temperature. A number of studies (Imai et al., 2002; Lamprecht et al., 2008b; Spanidis et al., 2017) have reported the association between exercise and the oxidation of albumin, thus the determination of albumin dimer levels, as reflected by denaturation temperature increase, may be a good indicator of exercise-induced oxidative stress. We have previously observed similar effects on albumin oxidation in an *in vitro* model (unpublished data).

In this study, the albumin thermal stabilization was observed during the initial days of exercise and then it tended to plateau (Fig. 2). While there were not any systematic trends in the denaturation parameters of globulins over time, nevertheless the change in the albumin/globulins enthalpy ratio took place only during the initial 3–4 days of exercise (Fig. 3). The plateau of the thermodynamic parameters of blood plasma heat capacity profiles is supposedly due to a rapid protective adaptation process during the exercise period. It has been established that the exercise-induced increase in ROS activates adaptive responses through various intracellular signaling pathways. ROS increases have been reported to cause cellular damage, but also to play an important role in the modulation of cell signaling involved in exercise training adaptations at the cellular level, such as the activation of redox-sensitive signal transduction pathway and the enhancement in gene expression of antioxidant enzymes (Gomez-Cabrera et al., 2008). Indeed, exercise training-induced up-regulation of endogenous antioxidants has been established to reduce the risk of cellular injury during exhaustive exercise (Powers et al., 2016; Kojda and Hambrecht, 2005). Moreover, other studies have reported several positive aspects of ROS generation in physical performance and their role in cell signaling and adaptation to regular exercise training (Kojda and Hambrecht, 2005; Steinbacher and Eckl, 2015).

It should be mentioned that exercise-induced oxidative stress has long been considered as harmful to muscle fibers, causing fatigue and muscle soreness. However, recent studies show that exercise-induced oxidative stress may be beneficial and even necessary for exercise-induced adaptations, such as skeletal muscle hypertrophy and mitochondrial biogenesis (Powers et al., 2016). Therefore, it can be assumed that assessing plasma albumin redox state may help to understand the optimum oxidative stress for exercise-induced adaptations of skeletal muscle.

5. Conclusions

Our findings indicate that 5 days of exhaustive physical exercise of highly trained individuals enhanced the thermal stability of plasma albumin and shifted its denaturational transition to higher temperatures. This effect was consistently observed for virtually all sets of blood plasma samples studied. We surmise that the observed effects are a result of enhanced blood oxygenation during the prolonged intense exercise, and consequently, of plasma protein oxidation. These effects might be part of the overall protective, adaptation mechanisms of the body to extreme physical and/or oxidative stress.

CRediT authorship contribution statement

Stamatis Mourtakos: Conceptualization, Data curation, Methodology, Formal analysis, Roles/. **Anastassios Philippou:** Conceptualization, Project administration, Supervision, Writing – review & editing. **Anastasios Papageorgiou:** Conceptualization. **Peter Lembessis:** Writing – review & editing. **Stella Zaharinova:** Investigation, Methodology, Resources. **Yozlyam Hasanova:** Software. **Rumiana Koynova:** Data curation. **Fragiskos Bersimis:** Data curation. **Boris Tenchov:** Visualization, Methodology. **Nikolaos Geladas:** Formal analysis, Resources. **Emmanuel Mikros:** Resources. **Lampros S. Sidossis:** Validation. **Michael Koutsilieris:** Supervision, Project administration, Resources.

Declaration of competing interest

The authors declare no conflict of interest.

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