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RESEARCH ARTICLE

Growth-differentiation factor-15, endoglin and N-terminal pro-brain natriuretic peptide induction in athletes participating in an ultramarathon foot race

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Abstract

We investigated the actions of growth-differentiation factor (GDF)-15, endoglin and N-terminal pro-brain natriuretic peptide (NT-pro-BNP) in 15 male athletes who participated in the ultradistance foot race of the 246 km 'Sparthathlon'. Measurements were performed before (phase I), at the end of the race (phase II) and 48 h post-race (phase III). GDF-15 and endoglin serum concentrations were determined with enzyme-linked immunosorbent assay and NT-pro-BNP plasma levels by electrochemiluminescence. GDF-15 levels were increased from phase I (563.9 ± 57.1 pg ml^{-1}) to phase II (2311.1 ± 462.3 pg ml^{-1}) and decreased at phase III (862.0 ± 158.0 pg ml^{-1}) ($p < 0.0002$). NT-pro-BNP levels followed a similar pattern to that of GDF-15 from 38.1 ± 4.8 pg ml^{-1} at phase I to 1280.6 ± 259.0 pg ml^{-1} at phase II and 89.8 ± 13.6 pg ml^{-1} at phase III ($p < 0.0001$) and at the same time points, endoglin levels were 4.7 ± 0.2 ng ml^{-1} at phase I, 5.8 ± 0.2 ng ml^{-1} at phase II and 4.3 ± 0.2 ng ml^{-1} at phase III ($p < 0.002$). These findings indicate that circulating GDF-15, endoglin and NT-pro-BNP levels reflect a transient endothelial dysfunction in these athletes who participated in a foot race consisting of continuous, prolonged and brisk exercise.

Keywords: *Exercise; endothelium; endoglin; NT-pro-BNP; GDF-15*

Introduction

The Spartathlon ultradistance foot-race (246 km) is one of the most strenuous and exhausting physical ordeals. It not only tests the resilience and character of the runner but also submits the athlete to a huge physical stress measured through a variety of extreme changes in biological and biochemical markers (Bartzeliotou et al. 2007, Margeli et al. 2005). In the physiologically hypertrophied athletes' hearts, exercise increases plasma concentrations of N-terminal pro-brain natriuretic peptide (NT-pro-BNP) and the cardiac troponins I and T compared with sedentary people (Scharhag et al. 2005). BNP and its cleaved inactive fragment, NT-pro-BNP, are mainly synthesized in response to increases in myocardial wall stress (Scharhag et al. 2005). In a recent study of our group it has been demonstrated that the biomarker of choice for the detection of cardiac injury and necrosis, troponin T, was not detected in athletes during the 'Spartathlon' foot race, in contrast to NT-pro-BNP, which reached a maximum value at the end of the race, tending to decrease at 48 h post-race (Bartzeliotou et al. 2007).

Growth-differentiation factor (GDF)-15 is a stress-responsive member of the transforming growth factor (TGF)-β cytokine superfamily. In animal models, GDF-15 is induced in the heart in response to

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ischaemia–reperfusion injury, pressure overload and heart failure, possibly via proinflammatory cytokine and oxidative stress-dependent signalling pathways (Kempf et al. 2006, Xu et al. 2006).

Endoglin is an accessory protein of the TGF-β receptor system expressed on the surface of endothelial cells. Endoglin is highly expressed on endothelial cells in healing wounds, developing embryos, inflammatory tissues and solid tumours (Cruz-Gonzalez et al. 2008). It is a marker of activated endothelium, while its vascular expression is limited to the proliferating cells. Endoglin plays a role in vascular integrity and endothelium functioning, whereas soluble endoglin acts as an antiangiogenic protein that interferes with the binding of TGF-β to its receptor (Garcia-Pozo et al. 2008). Mice lacking one or both copies of the endoglin gene cannot survive; their vessels form but are dilated and fragile, and easily ruptured (Bourdeau et al. 1999). They die from defective vascular development, as the loss of endoglin causes poor vascular smooth muscle development and arrested endothelial remodelling (Bourdeau et al. 1999, Li et al. 1999).

Recent studies have highlighted important functional roles of GDF-15 in the heart (Kempf et al. 2007a), where it is highly expressed in the infarcted myocardium (Kempf et al. 2006) and also in atherosclerotic plaques (Eggers et al. 2008). In view of endoglin's role in angiogenesis, its stimulation by ischaemic processes and its identification as an endothelial cell marker (Garcia-Pozo et al. 2008), we hypothesized that it would be important to study it during endothelial stress induced by strenuous exercise (Bartzeliotou et al. 2007). Hence, the purpose of the present study was to examine circulating levels of GDF-15, endoglin and NT-pro-BNP in 15 athletes who participated in the ultradistance foot race of 246 km between Athens and Sparta, called 'Sparthathlon'.

Materials and methods

Human subjects and exercise protocol

Fifteen male healthy, endurance-trained runners who participated in the 2006 'Spartathlon' race in Greece were studied. 'Spartathlon' is an ultradistance foot race of continuous, moderate intensity exercise, during which runners attempt to cover the 246 km distance from Athens to Sparta. The ambient daily temperatures during running were maximum 36°C and minimum 5°C, and the mean daytime relative humidity was 60–85%. The study was approved by the Ethics Committee of the Harokopio University of Athens and performed according to the Declaration of Helsinki. All experimental procedures conformed to the National Health and Medical Research Council guidelines for experimentation with human subjects. All subjects were informed of the procedures and purposes of the study, and written informed consent was obtained prior to their participation in the study. The mean ± SD age of the subjects was 41.2 ± 1.2 years, ranging from 33–52 years, and they finished the race in less than 36 h, the time limit according to the organizers. The athletes consumed water, electrolytes and carbohydrates *ad libitum*, available at 75 checkpoints throughout the race.

Sampling and analysis of blood samples

A 10 ml blood sample was drawn from an antecubital vein the day before the race (phase I), within 15 min of the end of the race (phase II) and 48 h after the end of the race (phase III). Four millilitres of the sample was immediately transferred to non-additive tubes to obtain a serum sample and it was allowed to clot at room temperature for 30 min. The remaining 6 ml of the sample were immediately distributed and put into EDTA-coated tubes to obtain plasma samples. Serum and plasma samples were separated from whole blood by centrifugation at 1500*g* at 4°C for 10 min. Finally, serum and plasma were aliquoted to cryovials, frozen at –80°C until assayed.

Biochemical analyses

The quantitative determination of human soluble GDF-15 was conducted in serum using the DuoSet kit enzyme-linked immunosorbent assay (ELISA) human GDF-15, purchased from R&D Systems Inc., Minneapolis, USA, according to the manufacturer's general protocol for DuoSet kits. The intra- and interassay precision coefficient of variation for GDF-15 ranged between 6.5–7.5% and 8.5–9.5%, respectively, at different levels. The detection limit for GDF-15 was 2.01 pg ml^{-1}.

The quantitative determination of human soluble endoglin was conducted in serum by solid-phase ELISA techniques, using commercially available kits purchased from R&D Systems Inc. The intra- and interassay precision coefficient of variation for endoglin ranged between 2.8–3.2% and 6.3–6.7%, respectively, at different levels, according to the manufacturer. The detection limit for this assay was < 0.007 ng ml^{-1} and the appropriate range of measurements was from 0.007 to 10 ng ml^{-1}. Every sample quantified by ELISA was run in duplicate, measurements differed by < 10%, and the mean value was calculated and used for statistical analysis.

Plasma levels of NT-pro-BNP levels were quantitatively determined on the Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics, Mannheim, Germany), using an electrochemiluminescence immunoassay (ECLIA) technique.

Statistical analysis

Data are presented as mean ± SE, and the level of statistical significance was set at $p < 0.05$. ANOVA repeated measures test was used to analyse time-course changes, while pair-observations *t*-test was used for point-to-point changes. All statistical procedures were performed using the STAT-GRAPHICS PLUS version 5.1 for Windows program (Graphic Software System).

Results

The biochemical parameters examined, expressed as mean ± SE, and the level of statistical significance before, at the end and 48 h post-race are presented in Table 1. The measurements were performed before (phase I), at the end (phase II) and 48 h post-race (phase III). GDF-15 levels at phase I were 563.9 ± 57.1 pg ml^{-1} and increased significantly at phase II (2311.1 ± 462.3 pg ml^{-1}) ($p < 0.001$), while at phase III they decreased (862.0 ± 158.0 pg ml^{-1}) (Table 1, Figure 1). GDF-15 was increased by fourfold at the end of the race, showing a remarkable decline at 48 h post-race, without returning to normal ($p < 0.02$).

Endoglin presented a similar profile to that of GDF-15 at the time points examined, reaching a maximum value at the end of the race (phase II 5.8 ± 0.2 ng ml^{-1}) ($p < 0.001$), with an increase of 1.2-fold, and returning to pre-race levels at 48 h after the end of the race ($p > 0.210$) (Table 1, Figure 2).

NT-pro-BNP levels have also been performed in the athletes of 2006 'Spartathlon' race and confirmed previous data of our group (Bartzeliotou et al. 2007), before (phase I), at the end (phase II) and at 48 h post-race (phase III). NT-pro-BNP concentration at phase I (38.1 ± 4.8 pg ml^{-1}) was significantly increased by 34-fold at phase II (1280.6 ± 259.0 pg ml^{-1}) ($p < 0.0006$) and subsequently decreased at phase III (89.8 ± 13.6 pg ml^{-1}) but without reaching the pre-race levels ($p < 0.001$) (Table 1).

Discussion

An elevated level of GDF-15 seems to reflect several underlying conditions, acute and/or chronic, associated with adverse cardiovascular outcomes, providing prognostic information beyond clinical characteristics and conventional risk markers, such as the electrocardiogram and serial cardiac troponin measurements (Eggers et al. 2008). Under baseline conditions, GDF-15 a marker of oxidative stress is weakly produced in most tissues. However in response to pathological or environmental stress, GDF-15 production may sharply increase (Kempf et al. 2007b). The link between stress, inflammation, obesity and coronary heart disease is interleukin (IL)-6 (Yudkin et al. 2000), which is dramatically elevated in

Table 1. Serum and plasma biochemical parameters (mean ± SE) measured in athletes who successfully completed the 2006 'Spartathlon' foot race.

	Phase I	Phase II	Phase III	*p*-Values
GDF-15 (pg ml^{-1})	563.9 ± 57.1	2311.1 ± 462.3	862.0 ± 158.0	0.001[a], 0.02[b]
sEndoglin (ng ml^{-1})	4.73 ± 0.19	5.80 ± 0.24	4.35 ± 0.22	0.001[a], 0.21[b]
NT-pro-BNP (pg ml^{-1})	38.1 ± 4.8	1280.6 ± 259.0	89.8 ± 13.6	0.0006[a], 0.001[b]

Pair observations *t*-test: [a]phase I vs phase II; [b]phase III vs phase I.
GDF, growth-differentiation factor, NT-pro-BNP, N-terminal pro-brain natriuretic peptide.

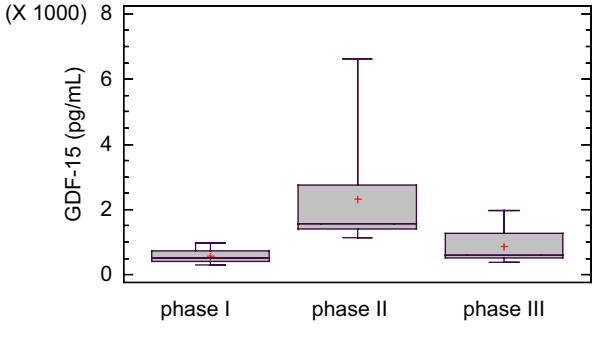

Figure 1. Serum concentrations of growth-differentiation factor (GDF)-15 are depicted as box plots before (phase I), at the end (phase II) and at 48 h after (phase III) the 'Spartathlon' race. Boxes represent the interquartile range, lines inside boxes represent the median value, whiskers represent 5th and 95th percentiles ($p < 0.0002$) (ANOVA repeated measures).

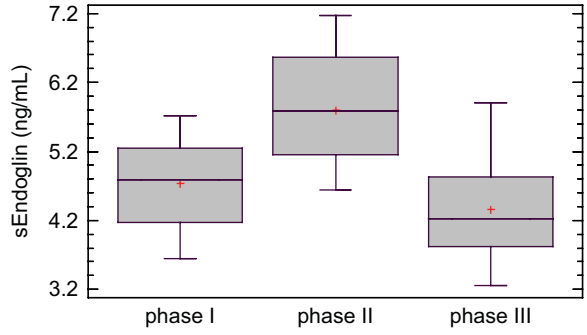

Figure 2. Serum concentrations of soluble endoglin are depicted as box plots before (phase I), at the end (phase II) and at 48 h after (phase III) the 'Spartathlon' race. Boxes represent the interquartile range, lines inside boxes represent the median value, whiskers represent 5th and 95th percentiles ($p < 0.002$) (ANOVA repeated measures).

the 'Spartathlon' model of exercise (Margeli et al. 2005, Goussetis et al. 2009). Markedly elevated levels of GDF-15 were noted in athletes after their brisk competition in the 'Spartathlon' race, especially at the end of the race, while at 48 h post-race GDF-15 levels remained slightly elevated compared with those before the race, indicating that myocardium and vessel wall of 'Spartathlon' athletes tend to maintain a protracted mild state of activation. Kempf et al. 2007b reported that GDF-15 concentrations were significantly higher in heart failure patients than in healthy control individuals, and they proposed to use 1200 pg ml^{-1} as the upper limit of the reference interval in elderly individuals. They also mentioned that in apparently healthy elderly individuals, GDF-15 levels were not significantly correlated to NT-proBNP concentrations (Kempf et al. 2007b). Elevated levels of NT-proBNP and GDF-15 were related to a higher risk of adverse outcomes, associated with markedly higher mortality rates, in patients with chest pain and unstable coronary artery disease (Eggers et al. 2008). It has been reported that these biomarkers are also increased in the presence of renal dysfunction (Eggers et al. 2008). In order to elucidate any possible involvement of renal dysfunction in the athletes of 'Spartathlon' race, we measured cystatin C, a marker of glomerular filtration rate (GFR), and found that it remained constant (phase I: 0.58 ± 0.02 mg l^{-1}; phase II: 0.62 ± 0.06 mg l^{-1}; and phase III: 0.02 mg l^{-1}, $p > 0.780$). NT-proBNP, a marker of systolic dysfunction and decreased left ventricular ejection fraction, provides information about cardiac cell damage and ventricular wall stress. In the 'Spartathlon' athletes, NT-proBNP was significantly increased by 34-fold at the end of the race, possibly due to the increased myocardial stress, and fell abruptly at 48 h post-race (Bartzeliotou et al. 2007). Both NT-pro-BNP and GDF levels remain slightly increased at 48 h post-race, compared with those before the race, presenting a similar fluctuation profile during the 'Spartathlon' race. This transient increase of NT-proBNP in the 'Spartathlon' study seems to support a concept of cardiac fatigue due to the exercise-associated myocardium stress, without signs of cardiac injury, evident from the cardiac troponin T concentrations that were less than the lower reference limit at all time points examined (Bartzeliotou et al. 2007). Strenuous endurance exercise in runners induces a significant elevation of biochemical cardiac-specific markers, which may reflect transient subclinical myocardial damage, but can also reflect a physiological reparative or adaptive process (Vidotto et al. 2005).

In a mouse ischaemia model, decreased tissue levels of endoglin were found to be associated with impaired angiogenesis (Jerkic et al. 2006, Düwel et al. 2007). Elevated levels of serum endoglin in cancer patients correlate positively with neoangiogenesis and tumour metastasis indicating poor prognosis (Cruz-Gonzalez et al. 2008). After an acute coronary event, compensatory mechanisms such as angiogenesis and endothelial regeneration occur, predominantly in response to tissue hypoxia resulting from myocardial ischaemia. The process of angiogenesis is coordinated by vascular endothelial growth factor (VEGF), endoglin and hypoxia inducible factor-1 (HIF-1). In patients with acute myocardial infarction, changes in serum soluble endoglin levels may reflect the effectiveness of angiogenesis in ischaemic/necrotic myocardium and its relation with endothelial function, supporting the hypothesis that reduced serum endoglin levels may be a marker of impaired endothelial function (Cruz-Gonzalez et al. 2008). It has been demonstrated that early changes in serum endoglin may predict mortality after acute myocardial infarction (Cruz-Gonzalez et al. 2008). The early decrease of soluble endoglin may represent impaired endothelial function, which precedes subsequent adverse clinical outcome (Cruz-Gonzalez et al. 2008). In the study conducted in athletes participating in the 'Sparthathlon', an increase in endoglin levels was observed at the end of the race (1.2-fold), compared with the levels before the race, suggesting that this index of endothelial stress, endoglin, was strongly affected at the end of the race, while 48 h post-race it regained the values observed before the race, suggesting that the activated endothelium surpasses the acute phase and reaches the resolving phase. It seems that the increase of endoglin at the end of the brisk exercise, supplies a possible protection of the heart against infarction and of the vessels against harmful alterations or against a possible rupture.

In athletes who participated in the 'Spartathlon' race and finished on time, this prolonged exercise induced an inflammatory response and strongly affected the indices of oxidative, endothelial and ventricular wall stress, causing an increase of GDF-15, endoglin and NT-pro-BNP levels. GDF-15, which is strongly upregulated in cardiac myocytes by various stressors, including reactive oxygen species, nitrosative stress and inflammatory cytokines, was extremely elevated at the end of the race compared with healthy controls. In these athletes endoglin, a hypoxia-inducible protein, is increased compared with healthy controls, even before the race, presenting a peak value at the end of the race and regaining the same levels as those before the race later on, contrary to patients with acute myocardial infarction who present lower endoglin levels than healthy controls. Given that endoglin, NT-pro-BNP and GDF-15 were significantly increased during the 'Spartathlon' race and tended to regain normal values at 48 h post-race, we can speculate that they could act as protectors for heart, tissues and vessels, indicating a transient endothelial dysfunction in these athletes. Whether or not these changes have long-term negative or protective effects on the vasculature remains to be further elucidated.

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