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Super-marathon race increases serum and urinary nitrotyrosine and carbonyl levels

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Abstract

Background In normal conditions, proteins are not present in the urine, however, exercise of long duration could result in proteinurea. Increased levels of reactive oxygen and nitrogen species (RONS) are formed during exhaustive physical exercise and causes alterations to cellular proteins.

Materials In the present study serum and urinary nitrotyrosine and protein carbonyl levels were measured before and after each run of a 4-day super-marathon race.

Result Serum nitrotyrosine and protein carbonyl levels increased after the first (93 km) day running and reached a plateau on the second (120 km), third (56 km) and forth (59 km) days of the competition. A significant correlation was found between urinary and serum protein carbonyl and nitrotyrosine levels ($r = 0{\cdot}78$, $r = 0{\cdot}71$, respectively). A large percentage of urinary proteins were carbonylated and nitrated. Therefore, it appears that clearance of oxidized proteins in certain conditions occurs not only by the proteolytic pathways but also by filtration and urination.

Conclusion Data reveals that exhaustive aerobic exercise causes oxidative stress and increases the nitration and carbonylation of serum proteins. The presence of carbonyl and nitrotyrosine in proteins of the urine might reflect oxidative stress and could serve as a noninvasive diagnostic tool for exercise physiology.

Keywords Exercise, nitrotyrosine, oxidative protein damage, oxidative stress, reactive carbonyl derivatives.

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Introduction

Exercise increases the production of reactive oxygen and nitrogen species (RONS), which could induce adaptive mechanisms involving antioxidant and repair systems [1]. However, exercise at an unaccustomed intensity or duration might lead to the massive formation of RONS, which could result in oxidative damage even to trained individuals [2,3]. We have reported that 4-day-super-marathon running increases the levels of DNA damage, as assessed by the concentration of 8-hydroxyguanosine in the urine samples of well-trained individuals [3]. It is also known, that marathon racing often causes muscle damage, and proteins are mechanically and oxidatively damaged. It is important to note that oxidative damage takes place in a selective manner. For instance, oxidative protein damage is not always accompanied by lipid peroxidation and/or DNA damage, and the accumulation of the damage depends on the combined effectiveness of the antioxidant and repair systems [2,4]. Super-marathon racing without question results in very notable damage to muscular and, probably, other proteins as well.

Physical exercise increases blood flow and the shear-stress enhancing vascular function by production of nitric oxide (NO) [5]. The interaction between superoxide, which cannot be completely dismutased in aerobic conditions, and NO generates very reactive peroxynitrite. The tyrosine

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residues of proteins are readily nitrated by peroxynitrite, causing alteration of physiological function and rendering proteins to be degraded [6]. However, peroxynitrite has been suggested to be not the only generator of nitrotyrosine [7]. It has been shown that prolonged exercise increases urinary nitrate levels in humans [8]. Moreover, an increased dityrosine level was reported after exercise in rats, demonstrating that nitration of proteins occurs during exercise [9,10].

Protein carbonyls are used very often as a marker of oxidative modification of proteins [11–14]. An increased formation of protein carbonyl, as a result of 60 eccentric muscle actions, has been reported in blood samples of human subjects [15], and regular training has been shown to alter protein carbonyl levels in different tissues [16,17]. Exhaustive exercise, especially of long duration, could result in the appearance of proteins in urine samples of human subjects. We assumed that super-marathon running will cause proteinurea. Therefore, we have chosen super-marathon running to test whether running a distance of 328 km results in oxidative damage to proteins measured by carbonylation and nitration of serum and urinary proteins.

Methods

Subjects

Eight male super-marathon runners (age: 26–45 years) volunteered for the study, being informed of the aim and methods. The protocol of the study was reviewed and approved by the local Ethics Committee and was carried out according to the guidelines of the Declaration of Helsinki for Research on Human Subjects. The runners participated in the 7th Vienna–Budapest super-marathon. The daily running distance of the 4-day race comprised the following: 93, 120, 56 and 59 km, respectively. The athletes followed their own individual preparation programme and nutritional protocol to achieve their best results during the competition. None of them had taken antioxidants. In the last week, generally, the daily running distance was 15–25 km, which was the usual running distance 1 day before the competition. Venous blood was collected at rest, before and 1 h after finishing each daily running distance. After centrifugation, serum was immediately stored at –40 °C. The urine samples were collected in the last 12 h before the start of the competition and between each run. Collection of 24-h urine samples was impossible because of the schedule of the competition. Urine samples were centrifuged at 2000 g for 10 min and stored at –40 °C. The urine content of the competitors had changed during the 4-day race, possibly a result of the difference in the recovery period and dehydration; therefore the measured values were normalized to creatinine.

Determination of nitrotyrosine

The concentration of free nitrotyrosine in the serum and urine samples, a marker for peroxynitrite generation [18], was assayed by enzyme linked immunosorbent assay (ELISA); ELISA was conducted as described previously [19,20]. Briefly, 210-µL serum samples were added to ×4 volume of ethanol at 4 °C, vortexed and centrifuged at 3000 g for 10 min. The supernatant was evaporated under nitrogen and redissolved in 105 µL of ultra-pure water. Samples were then incubated overnight with antinitrotyrosine rabbit IgG and nitrotyrosine etylcholinesterase tracer in precoated (mouse antirabbit IgG) microplates followed by development with Ellman's reagent for 60 min. Serum nitrotyrosine concentration is expressed as nM L^{-1}.

Carbonyl assay

The modified method of Levine *et al.* [21] was used for the determination of protein carbonyl from serum [4]. The reliable determination of protein carbonyl by spectrophotometry requires nearly 800 µg of protein; an amount not present in the urine. Therefore, we obtained semiquantative data by Western blot as described previously [4]. In brief, the collected 12-h urine samples were centrifuged at 11 000 × g for 30 min. Then, proteins precipitated with trichloroacetic acid were suspended and incubated in a solution containing 10 mM of 2,4-dinitrophenylhydrazones (DNPH) for 1 h at 15 °C. The resulting protein hydrazones were pelleted in a centrifuge at 11 000 × g for 5 min. The pellets were washed three times with ethanol-ethyl acetate (1 : 1). The final precipitates were dissolved in 1 mL of 8 M urea and 5% 2-mercaptoethanol. The protein content was then remeasured. Duplicate polyacrylamide gel electrophoresis of derivatized proteins was carried out in 12% polyacrylamide gels containing 0·1% sodium dodecyl sulphate. After electrophoresis, the proteins were transferred to nitrocellulose membranes. Then the membranes were soaked in phosphate-buffered saline containing 3% skim milk, 0·05% Tween, and 0·05% sodium azide, and then treated with anti-DNPH antibody. After washing in buffer, without antibodies, the membranes were treated with secondary antibody and washed. The signals were visualized, then the coomasine blue protein stain and protein carbonyl lanes were subjected to densitometry. Data were expressed as the protein stain density per protein carbonyl stain density in arbitrary units and used for statistics [4].

Statistical analysis

The statistical significance was assessed by analysis of variance followed by Scheffé's post hoc test. The method of least squares was applied to determine the linear correlation coefficients. Significance was set at $P < 0{\cdot}05$.

Results

The 4-day super-marathon race resulted in an increased concentration of serum nitrotyrosine ($P < 0{\cdot}05$) levels

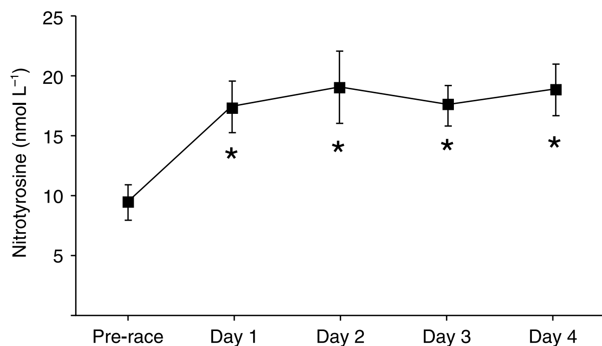

Figure 1 Super-marathon running increased the nitrotyrosine content in serum of runners detected by the ELISA method. Values are means ± SD of seven runners. $^{*}P < 0{\cdot}05$ vs. prerace.

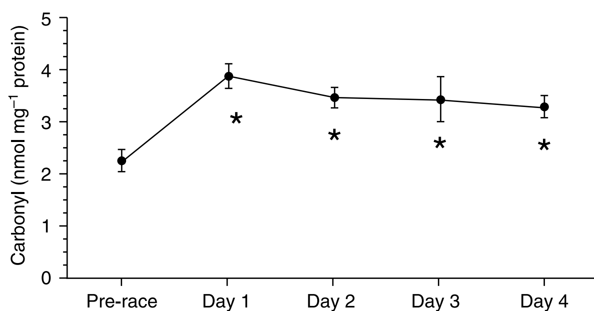

Figure 2 Protein carbonyl level in the serum of runners increased significantly after the first day of running and remained elevated throughout the race. Values are means ± SD of seven runners. $^{*}P < 0{\cdot}05$ vs. prerace.

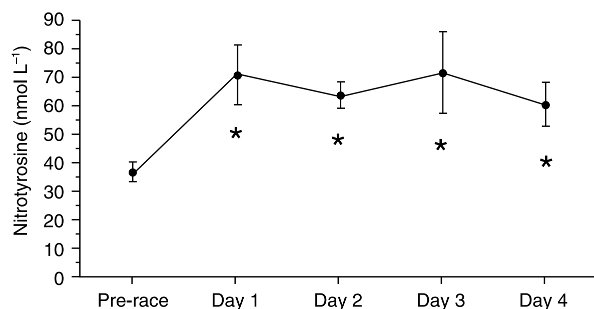

Figure 3 Urine nitrotyrosine levels increased after the first day of running and remained elevated during the 4-day race. The measured values were normalized to creatinine. Values are means ± SD of seven runners. $^{*}P < 0{\cdot}05$ vs. prerace.

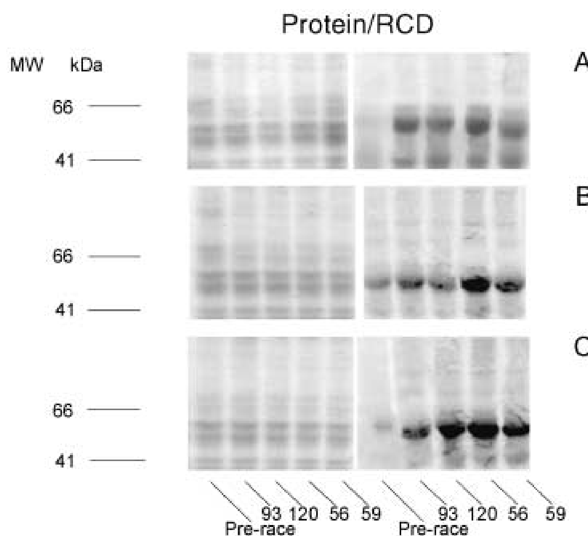

Figure 4 Protein stain (on the left) and reactive carbonyl derivatives (RCD) (on the right) from the urine samples of three runners are shown. Proteins are present on the prerace samples, however, the RCD content in those proteins is not as much as in the samples obtained after each running during the race. The numbers indicate the running distance of each day. On the left of the coomasine blue panel, molecular weight markers are displayed. Protein and carbonyl signals were detected only on the shown molecular weight region. Data of three runners are shown (A, B, C).

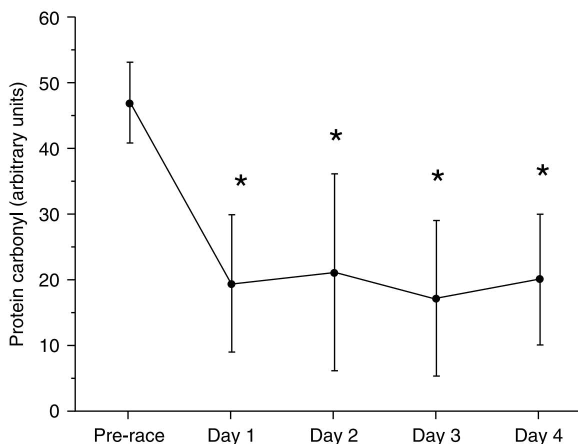

Figure 5 Western blot (shown in Figure 4) semiquantitative data of protein carbonyl are expressed in arbitrary units and are a result of protein stain density per anti-DNPH-signal density. The measured values were normalized to creatinine. Values are means ± SD of eight runners. $^{*}P < 0{\cdot}05$ vs. prerace.

(Fig. 1). The increase was not linear; significant elevation was apparent after the first day of the race. The nitrotyrosine level reached a plateau thereafter. The accumulation of protein carbonyl in the serum was very similar to the accumulation of nitrotyrosine with a marked increase on the first day, then with the plateau (Fig. 2). The nitrotyrosine concentration in urine increased after the first day of the race and thereafter remained at an elevated level (Fig. 3). The protein carbonyl content in the urine samples was detectable even on the first day, and then it increased because of the super-marathon (Fig. 4). A wide range of individual alterations were recorded, which might have resulted from genetic differences, the rate of individual fitness or the rate of dehydration, etc. However, it appears that the protein carbonyl level increased significantly on the first day of running and was elevated throughout the measured period. The protein carbonyl data of Western blot analysis is shown

in Fig. 5(a–d); the protein stain density was divided by the DNPH-signal density. This semiquantitative data shows decreased arbitrary units, meaning that the same level of protein contained a larger content of carbonyl groups. Significant relationship was found between serum and urinary protein carbonyl and nitrotyrosine levels ($r = 0{\cdot}78$, $r = 0{\cdot}71$, respectively).

Discussion

This is the first investigation, to our knowledge, measuring serum and urinary nitrotyrosine levels with exercise. Also carbonyl measurements from urine samples have not been reported to date. An increased appearance of nitrotyrosine in serum and urine, as a result of exhaustive exercise, supports our hypothesis. Nitration of proteins during exercise stress is the result of a significant increase in the formation of NO. Exercise has been shown to up-regulate the endothelial nitric oxide synthase (eNOS) gene expression and activity [22]. Moreover, during long-term aerobic exercise there is an increase in cytokine generation, which could induce iNOS and lead to increased levels of NO and formation of nitrotyrosine [23]. The patterns of change during the 4-day race are interesting, because the content of nitrotyrosine tended to plateau after the second day, in spite of the fact that the running distances were nearly three times that of the marathon. One possible explanation of this plateau is that the nitration of proteins was balanced by the degradation of nitrated proteins. Oxidative modifications of proteins results in a significant decrease in the physiological activity of modified proteins, and the alteration serves as a marking step for proteolytic degradation and might play a role in protein turnover [11,24]. If the generation rate of oxidative damage exceeds the rate of degradation, accumulation of oxidative damage occurs. The data from the present study indicate that exhaustive physical exercise results in accumulation of nitrotyrosine, and this might be compensated by the degradation of altered proteins as a result of adaptation. The urinary nitrotyrosine concentration can serve as a reliable marker of oxidative stress, as administration of vitamin C decreases the nitrrotyrosine level in urine [25]. However, it appears that free nitrotyrosine is not completely filterable into urine and this can explain the lower level of nitrotyrosine in urine than in blood [25]. In addition, a certain duration is necessary to filter and clear up nitrotyrosine, however, it is not known from our data how efficient this urinary clearing is. It appears that nitrotyrosine can be formed not only by the peroxinitrite and protein interaction but also by other nitrating agents including nitric oxide, nitrogen dioxide and myeloperoxidase [7,26]. Therefore, increased nitrotyrosine levels, as observed in the present study, are considered to be the result of nitrating stress on proteins [7].

The pattern of the protein carbonyl accumulation in serum proteins was similar to nitrotyrosine levels, suggesting that nitration and carbonylation of proteins took place in a similar fashion, though the underlying mechanisms are different. Moreover, it appears that the degradation of nitrated and carbonylated proteins are mainly executed by the proteasome enzyme complex; hence the similar accumulation pattern also can be explained by the efficiency of the same repair system, namely the proteasome complex [6,27].

The other novel finding of our investigation was that the protein carbonyl levels were measurable in urine samples of runners. To our surprise, in the control samples, the proteins and protein carbonyl levels were detectable in the urine even before the race. It is important to note that 1 day before the race, the average running distance of the athletes was more than 10 km. Therefore, data from the first day cannot be completely regarded as resting levels. After being aware of the pattern of protein carbonyl levels from the urine samples, we collected urine samples after 2 days of complete rest, 1 month after the race (the subjects had 3–5 light running training in this period). We could not detect proteins nor consequently protein carbonyl from any of these samples.

The protein carbonyl accumulation in the urine during the race showed individual differences. The samples obtained before the race contained significantly lower levels of protein carbonyl than those measured after running. The apparent proteins in urine were almost identical at the molecular weight with the carbonylated proteins. Hence, most of the Coomasine Blue-stained bands were carbonyl positive. Thus, the strong relationship between serum and urinary protein carbonyl levels suggests that the filtration of carbonylated proteins serves, at least in some part, as an alternative pathway to avoid the accumulation of oxidatively modified proteins.

The data for the present investigation reveal that a 4-day super-marathon race results in an increased accumulation of nitrotyrosine and protein carbonyl levels in the serum of runners. However, the pattern suggests that an adaptive mechanism takes place even during the race to prevent the linear accumulation of nitrated and carbonylated proteins. The adaptive mechanism might involve the urination of oxidized proteins. Measurement of urinary protein carbonyl might be a new method of assessing oxidative stress after extreme physical exercise.

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