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ORIGINAL ARTICLE

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Plasma oxidative stress biomarkers, nitric oxide and heat shock protein 70 in trained elite soccer players

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Abstract The physiological response to the physical exercise involves a number of changes in the oxidative balance and in the metabolism of some important biological molecules, including nitric oxide (NO) and heat shock proteins (Hsp 70). With the aim to optimise previous laboratory diagnostic panels, we measured the plasma concentration of reactive oxygen metabolites (ROMs), total antioxidant status (TAS), glutathione reductase (GR) activity, and NO and Hsp 70 levels in 44 elite, antioxidant-supplemented and trained soccer players and in 15 sedentary controls. Although no statistically significant difference between athletes and controls was detected in the plasma level of ROMs and TAS, soccer players showed a significantly higher plasma GR activity, NO and Hst 70 levels than those of sedentary controls. These findings suggest that the measuring of relatively novel biomarkers in sport medicine, like GR, NO and Hsp 70, in addition to the well-known and reliable assays (d-ROMs test and TAS) may be useful to a clinician to better assess and evaluate the benefits of training and/or supplementation programs.

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Introduction

A regular training together with an adequate nutritional program is expected to protect athletes from oxidative stress, either by reducing reactive oxygen metabolites (ROMs) production during strenuous exercise or by increasing the level/activity of the antioxidant system before a competition (Alessio 1993). On the other hand, oxidative stress may enhance nitric oxide (NO) production (Quian 2002) while high-intensity exercise may increase the level of heat shock protein 70 (Hsp70) (Noble et al. 1999; Banfi et al. 2004). Due to the relative paucity of information on the above relationships, aimed at optimizing previous diagnostic panels, we have investigated the possible link among some recognized biochemical markers of oxidative stress—such as serum hydroperoxides (a class of ROMs), serum total antioxidant status (TAS) and glutathione reductase (GR) activity—Hsp70 and NO in a group of elite soccer players as recruited in a training program.

Methods

Subjects

Forty-four elite soccer players and 15 sedentary controls were enrolled in the study. The players—who had undergone six soccer matches per week for at least 1 year—were engaged in a scheduled program that consisted of a special training of 1 h and 45 min per day of exercise (from Monday to Friday) and a soccer match of 90 min twice a week. The controls were medical students with a sedentary lifestyle who did not practice any physical exercise regularly. All were healthy, with neither

family or personal history of diabetes, nor thyroid, hepatic and renal dysfunction. Special care was taken to exclude smokers, drinkers and anyone taking anabolic drugs. Both groups were similar in age, body mass index and waist/hip ratio. According to the findings of a detailed food frequency questionnaire, which were completed by all the participants, the quality, quantity and frequency of consumption of eggs, chicken, red meat, fish, vegetables, fruits, dairy products and soft drinks were similar in all subjects. However, instead of controls, athletes were taking as oral antioxidant supplementation vitamin C (250 mg per day) and vitamin E (200 IU per day). Informed consent was obtained from each participant.

Reactive oxygen species

Plasma ROMs (mainly hydroperoxides) were evaluated by performing the d-ROM test (Diacron International s. r. l., Grosseto, Italy) as previously reported (Trotti et al. 2002), on a common spectrophotometer (UVICAM, Cambridge, UK). The results of the d-ROMs test were expressed in arbitrary units called "Carratelli units" (CARR U), where 1 CARR U corresponds to 0.08 mg/100 ml H_2O_2 (Trotti et al. 2002). Reference values of healthy subjects were shown between 250 and 300 CARR U (Trotti et al. 2002).

Total antioxidant status

The plasma antioxidant capacity was measured with the commercially available kit TAS (RANDOX Laboratories Ltd, Ardmore, UK), as previously described (Erel 2004), using the above-mentioned spectrophotometer. The results of TAS were expressed as mmol/l, with the mean ± SEM normal value of 1.728 ± 0.004 mmol/l (Wang et al. 2001).

Glutathione reductase

The plasma level of GR was determined by a commercially available kit (RANDOX Laboratories Ltd, Ardmore, UK), according to the procedure of the manufacturer. A value of 10 U/l was considered as the detection limit.

Nitric oxide

Plasma NO was quantified by means of a commercially available kit (Nitric Oxide Colorimetric Assay, Oxis International Inc., Portland, OR, USA) that employs the NADH-dependent enzyme nitrate reductase for enzymatic reduction of nitrate to nitrite prior to quantity nitrite using Griess reagent.

Heat shock protein

The plasma level of Hsp70 in serum was detected by a commercially available sandwich ELISA (Stressgen Biotechnology, Victoria, BC, Canada). The amount of Hsp 70 in serum was estimated from the calibration curve which ranged from 0.78 to 50 ng/ml.

Statistical analysis

Means were compared by the unpaired *t* test or one-way analysis of variance (ANOVA). Data are presented as means ± SD. Correlations were assessed according the Tukey-Kramer multiple comparison tests and the Mann–Whitney non-parametric test (U-test). Differences were considered statistically significant at $P < 0.05$.

Results

Although no statistically significant difference between athletes and controls was detected in the plasma level of ROMs (232.38 ± 46.16 vs 221.07 ± 21.23 CARR U, respectively; $P = 0.47$) and TAS (1.33 ± 0.15 vs 1.30 ± 0.08 nmol/l, respectively; $P = 0.67$), soccer players showed a plasma GR activity significantly higher than that of sedentary controls (119.95 ± 12.50 vs 94.05 ± 5.91 U/l, respectively; $P < 0.0001$) (Table 1). Moreover, plasma concentration of Hsp 70 and NO was significantly increased in athletes (12.96 ± 5.65 ng/ml and 58.03 ± 15.20 μM, respectively) compared to controls (4.62 ± 1.62 ng/ml and 23.18 ± 4.84 μM, respectively ($P < 0.0001$) (Table 1).

Discussion

According to the above results, trained and antioxidant-supplemented elite soccer players did not significantly differ from controls when tested for common plasma biomarkers of both pro-oxidative status (d-ROMs test) and whole antioxidant capacity (TAS). This finding is in agreement with a previous study (Schippinger et al. 2002), thus suggesting that the antioxidant supplementation, together with the training program, although not

Table 1 Comparison of oxidative stress biomarkers found in our samples

	Controls	Soccer players	*P* value
ROMs (CARR U)	221.07 ± 21.23	232.38 ± 46.16	0.47
TAS (mmol/l)	1.30 ± 0.08	1.33 ± 0.15	0.67
GR (U/l)	94.05 ± 5.91	119.95 ± 12.50	< 0.0001
Hsp70 (ng/ml)	4.62 ± 1.62	12.96 ± 5.65	< 0.0001
NO (μM)	23.18 ± 4.84	58.03 ± 15.20	< 0.0001

ROMs reactive oxygen metabolites, *TAS* total antioxidant status, *GR* glutathione reductase, *Hsp70* heat shock protein 70, *NO* nitric oxide

able to increase TAS was at least effective in "containing" the production of peroxides in our athletes (Sacheck and Blumberg 2001). However, it has been previously reported also that soccer players showed significantly higher TAS compared to the controls (Brites et al. 1999). This conflicting result may probably reflect some differences between the two study populations of soccer players, including the duration and intensity of the exercise or training program, the age, the diet and health status of each individual, all these factors being able to diversely affect TAS.

Moreover, TAS, although reliable, is the only one among all the markers of the whole antioxidant defenses; in fact, also the activity of other enzymes, such as superoxide dismutase and catalases, may be enhanced by physical exercise (Metin et al. 2003; Tauler et al. 2002).

Due to the paucity of data on GR in sportsmen, we have decided to measure this enzymatic activity since it plays a significant role in antioxidant defenses by regenerating the biologically active reduced glutathione, in turn responsible for the maintenance of reduced pools of vitamin C and E (Husain et al. 2003). Our data evidenced that serum GR activity was significantly enhanced in the players compared to the controls. Therefore, it is conceivable that the specific training regimen, although not able to increase TAS for the above-reported reasons (Brites et al. 1999), was at least able to induce an up-regulation of GR in our soccer players.

In our study the soccer players showed also higher plasma levels of NO compared to the sedentary controls, in agreement with previous studies (Jay-Gerin and Ferradini 2000). In particular, NO concentration was shown to be significantly and persistently elevated after exercise in trained subjects (Maeda et al. 2001; Kingwell et al. 1997; Jungersten et al. 1997; Wang et al. 1997; Woodman et al. 1997), probably due to the changes in oxidative balance (Vassalle et al. 2003; Jableka et al. 2004) and/or to the exercise-related increased endothelial shear stress, a potent stimulus in the activation of NO synthase activity (Kingwell 2000; Maiorana et al. 2003; Green et al. 2004).

Finally, plasma levels of Hsp 70 were significantly higher in our soccer players compared to the controls, as expected. Indeed, not only the changes in temperature but also in pH and oxidative balance which globally occur during exercise are able to increase the release of this protein in the blood (Repetto et al. 1996), may be as a complementary protective mechanism (Moseley 2000).

Our data confirm that the inclusion of a moderate antioxidant supplementation in a training protocol may be useful in athletes, like soccer players, which are at high risk for oxidative stress. However, according to our findings, the effectiveness of such a protocol should be carefully evaluated by measuring more and different plasma biomarkers, sufficiently sensitive to the changes of oxidative balance and to the response of the body to oxidative stress. In this context, the measuring of relatively novel biomarkers in sport medicine, like GR, NO and Hsp 70, in addition to the well-known and reliable assays (d-ROMs test and TAS), can help the researcher to understand the relationships among these different bio-indicators in order to help the clinician to better assess and evaluate the benefits of training and/or supplementation programs.

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