Oxidative Stress Effects on Endothelial Cells Treated with Different Athletes' Sera

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ABSTRACT

CONTI, V., G. CORBI, G. RUSSOMANNO, V. SIMEON, N. FERRARA, W. FILIPPELLI, F. LIMONGELLI, R. CANONICO, C. GRASSO, P. STIUSO, A. DICITORE, and A. FILIPPELLI. Oxidative Stress Effects on Endothelial Cells Treated with Different Athletes' Sera. *Med. Sci. Sports Exerc.*, Vol. 44, No. 1, pp. 39–49, 2012. **Purpose**: Exercise training is a nonpharmacological intervention that improves cardiovascular function and enhances endothelial homeostasis in patients with cardiovascular diseases. However, the amount of benefit achieved varies widely depending on the type and duration of exercise. Moreover, data about the long-term effects of physical activity are scarce. **Methods**: In this study, endothelial cells, exposed or not to oxidative stress, were conditioned with sera from athletes regularly participating in sports classified as "aerobic" (triathlon), "mixed aerobic–anaerobic" (soccer), and "anaerobic" (sprint running). **Results**: Functional and hemodynamic variables did not differ between groups of athletes, whereas there were dramatic changes in serum markers for oxidative stress. Lipid peroxidation assessed by the thiobarbituric acid reactive substances assay and catalase activity were the lowest and nitric oxide availability was the highest in sera of triathletes. Endothelial cells cultured in serum from triathletes (T-endothelial cells) had the highest survival, evaluated by viability assay, BrdU incorporation, and senescence-associated β galactosidase assays, and preserved the endothelial appearance before and after stress in contrast to the cells grown in sera from the other athletes. T-endothelial cells also had the highest catalase messenger RNA expression and, after stress, the highest catalase activity of all the endothelial cells. Moreover, poststress activity of Sirt1, a NAD^+-dependent deacetylase involved in cellular stress resistance and a key regulator of longevity, was significantly increased in T-endothelial cells. **Conclusions**: Different types of exercise training induced different molecular effects in terms of survival, morphology, and antioxidant system efficiency. The *in vitro* technique used herein may help to shed light on the molecular basis of effects of long-term physical activity in humans. **Key Words:** EXERCISE TRAINING, SIRT1, CATALASE, ENDOTHELIAL CELLS

The beneficial effects of physical activity are well known. Exercise training is a nonpharmacological intervention that improves endothelial function in patients with such cardiovascular syndromes as hypertension, atherosclerosis, and CAD (24). However, the amount of benefit achieved varies greatly depending on the type and duration of exercise (37).

In humans, most of the research in this area is based on aerobic exercise protocols. Typical protocols have included submaximal- or maximal-effort aerobic exercise, and most studies have reported the effects of short bouts (≤2 h) of moderate-duration exercise. Very few protocols have included much longer exercise times (12).

At present, it is well established that acute exercise increases free radical production, which may or may not result in oxidative stress. In humans, exercise-induced oxidative damage is evaluated from the extent of lipid peroxidation measured with the thiobarbituric acid reactive substances (TBARS) assay. On the other hand, serum superoxide dismutase, catalase (Cat), glutathione peroxidase, and glutathione reductase levels provide a measure of antioxidant activity (36). High concentrations of reactive oxygen species result in damage to DNA, proteins, and lipids, which can cause cell and tissue impairment (8).

Endothelial oxidative stress is associated with impaired function, and it is a key feature of the onset and evolution of cardiovascular diseases.

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The effects of exercise on clinical outcomes may be partially related to a direct and independent positive effect of physical training on endothelial dysfunction in the conduit arteries (7) or in the peripheral microcirculation (26,31). Regular physical activity is associated with increased nitric oxide (NO)–dependent vasodilation because it restores the balance between NO production and NO inactivation by free radicals (18,35). We previously showed that moderate and prolonged training restored antioxidant defense in the aged rat heart by increasing NAD^+-dependent deacetylase Sirt1 activity, which suggests the importance of choosing a specific exercise training protocol that is known to produce beneficial effects (11).

Few studies have examined the effects of different types of exercise training on endothelial cells in humans. Moreover, little is known about the effects of different exercise training programs on endothelial cells undergoing oxidative stress.

Therefore, the aims of this study were to 1) compare oxidant and antioxidant responses in sera collected from athletes participating in three very different specialties and 2) investigate the effects of these sera on endothelial cells exposed or not exposed to oxidative stress. The sport specialties we evaluated are the triathlon, soccer, and sprinting. The triathlon is a three-event endurance sport in which athletes compete sequentially in swimming, cycling, and running. The energetic mechanism used in this sport is mainly aerobic (28). Soccer is a high-intensity, intermittent, noncontinuous exercise.

The average aerobic energy yield during a game is around 80% of the individual maximum (9). Hence, soccer is considered a mixed aerobic–anaerobic sport. In sprint runners, the energetic mechanisms that support speed events are mainly anaerobic (95%) (3).

METHODS

Ethical approval. The study was approved by the Ethics Committee of the Second University of Naples, where participants were recruited and human experimentation was conducted according to the Declaration of Helsinki guidelines. The athletes gave their written informed consent after medical staff explained the purpose, possible risks, and stress associated with the study.

Study population. Ten triathletes (T, mean age = 29.8 ± 8.7 yr), 15 soccer players (S, mean age = 26.0 ± 0.6 yr), and 10 sprinters (Sp, mean age = 31.3 ± 6.4 yr) were recruited from the Sport Medicine Service of the Second University of Naples. All subjects voluntarily participated in the study and were competing athletes.

The subjects were male and had regularly exercised for a minimum of 5 yr. T were training for 14.80 ± 4.64, S for 8.86 ± 1.57, and Sp for 11.14 ± 3.24 $h \cdot wk^{-1}$. All recruited athletes were healthy and had normal thyroid, hepatic, and renal function and no familial or personal history of diabetes, dyslipidemia, or other metabolic disorders.

None of the athletes were taking any drug or following a special diet. Particular care was taken to exclude subjects who were taking anabolic drugs, vitamins, or antioxidant supplements and smokers. Athletes underwent a physical examination upon entering the study. Because acute exercise is known to affect circulating markers of oxidative stress and antioxidant capacity, athletes refrained from training for about 15 d. Then, blood samples were collected, and the athletes underwent a treadmill stress test and spirometry. All measurements were carried out at a constant room temperature (22°C–25°C).

Blood samples. Subjects fasted for at least 12 h before blood collection. Blood samples (20 mL) were obtained between 8:00 and 9:00 a.m. from an antecubital vein. They were collected in tubes containing K_3EDTA, an anticoagulant, for evaluation of routine biochemical parameters and in a 5-mL glass tube to obtain serum for molecular analyses. Blood was centrifuged (1500*g* for 10 min at 4°C), and plasma or serum was collected and stored at −80°C until analyzed.

Spirometry and treadmill stress test. All tests were performed under the direct supervision of the exercise test laboratory medical staff. The spirometry test was carried out with a spirometer (FlowScreen II; VIASYS, Hoechberg, Germany). Spirometer calibration and measurements were carried out according to the European Respiratory Society/American Thoracic Society criteria (27). For the treadmill stress test, subjects were connected to a treadmill-for-stress test system (Cardiotread; Cardioline, Milan, Italy) and began symptom-limited exercise testing according to the standard Bruce protocol (5). HR was continuously monitored, and blood pressure (BP) was measured at each exercise stage. The maximum predicted HR was calculated with the formula 220 bpm minus age. An adequate HR response to exercise was defined as ≥85% of the maximum predicted HR. The Bruce protocol increases treadmill speed and slope incrementally at approximately 3-min intervals. After maximal exercise effort, the athletes were immediately seated and remained connected to the electrodes for at least 5 min while continuous HR data were recorded by the treadmill, whereas HR and BP were manually recorded.

Cell culture and treatments. Human endothelial cells line EA.hy-926 (ECs) (American Type Culture Collection, Mannassas, VA) and primary human umbilical vein ECs (pECs) were used for the *in vitro* experiments. The EA.hy-926 were grown in a Dulbecco modified Eagle medium containing 10% fetal bovine serum (FBS), 100 U of penicillin, and 100 $\mu g \cdot mL^{-1}$ of streptomycin at 37°C in 5% CO_2.

pECs, kindly provided by Dr. G. Cobellis (Department of Pathology of the Second University of Naples), were isolated from human umbilical veins as described by Jaffe et al. (20) and cultured in an endothelial growth medium, containing FBS at a concentration of 2% and bovine brain extract (with FGF-2 at a concentration of 100–500 $pg \cdot mL^{-1}$). The cells were subcultured by trypsinization, seeded on cell culture dishes coated with 0.1% gelatin and growth in an atmosphere of 5% CO_2 at 37°C.

In both ECs and pECs, we conducted pilot experiments to identify the concentration of hydrogen peroxide (H_2O_2 =

100–750 μM) that effectively induced a significant decrease in the survival of control cells, and we chose 500 μM. Moreover, in a preliminary experiment, we evaluated the effect of oxidative stress 12, 24, 48, and 72 h after treatment of 500 μM of H_2O_2. Finally, we chose the time of 48 h as representative of the most relevant changes.

The cells were cultured in a medium supplemented with the athletes' sera (10%) or FBS (10%) as a control (FBS ECs, FBS-ECs/pECs) and were exposed or not to oxidative stress. In detail, the cells were seeded and cultured for 48 h in a medium supplemented with T (triathlon-conditioned ECs, T-ECs and T-pECs), S (soccer-conditioned ECs, S-ECs and S-pECs), and Sp (sprinter-conditioned ECs, Sp-ECs and Sp-pECs) sera. Then, the culture medium was aspirated, and ECs were exposed to 500 μM of H_2O_2. Four hours after H_2O_2 exposure, the growth medium was replaced with a fresh medium containing FBS.

Serum NO availability. Serum NO availability was determined by two indirect methods, Griess and ion chromatography assays.

About the Griess method, serum aliquots (100 μL) were incubated for 10 min at room temperature with 500 μL of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 5% hydrochloric acid). Optical density was measured at 550 nm with a spectrophotometer. The amount of nitrites (NO_2^-) was calculated from a standard curve generated using serial dilutions of sodium nitrite. The results were reported as nanomole per milligram of protein. The analyses by ion chromatography (17) were carried out in the sera diluted 1:4 with Milli-Q (Millipore, Palo Alto, CA) water by using an Agilent chromatographic system (Agilent Technologies, Wilmington, DE). The filling of the column was an ion exchanger based on styrene-divinylbenzene with quaternary amine in the chlorine form, 10 mm (150 × 33 mm). Detection of nitrate/NO_2^- anions was carried out by absorbance at 212 nm using ultraviolet-visible detection. All chemicals were of analytical grade. Sodium nitrite, sodium nitrate, o-phosphoric acid 85%, and sodium perchlorate monohydrate were purchased from Sigma (Milan, Italy). NO_2^- and nitrate stock solutions were diluted in Milli-Q water to obtain the required concentrations. The eluent was 0.02 M of sodium perchlorate monohydrate at pH 3.9. The flow rate was 0.2 mL·min^{-1}. The results were reported as NO_2^- plus nitrate (μM). All data are the means ± SD of three independent measurements.

Serum lipid peroxidation. Peroxidative damage to cellular lipid constituents was determined with the TBARS method. The assay was performed in 10 μL of serum. The chromogen (TBARS) was quantified using a spectrophotometer at a wavelength of 532 nm with 1,1,3,3-tetramethoxyprophane as a standard. The amount of TBARS was expressed as nanomole per microgram of protein. All data are the means ± SD of three independent experiments.

Sirt1 activity. Crude nuclear samples were extracted by suspending 1 × 107 cells into 1 mL of lysis buffer (10 mM of Tris HCl at pH 7.5, 10 mM of NaCl, 15 mM of $MgCl_2$, 250 mM of sucrose, 0.5% NP-40, 0.1 mM of EGTA). Cells were spun through 4 mL of sucrose cushion (30% sucrose, 10 mM of Tris HCl at pH 7.5, 10 mM of NaCl, 3 mM of $MgCl_2$) at 1300*g* for 10 min at 4°C. The isolated nuclei were suspended in 50–100 μL of extraction buffer (50 mM of HEPES KOH at pH 7.5, 420 mM of NaCl, 0.5 mM of EDTA Na_2, 0.1 mM of EGTA, 10% glycerol). After centrifugation at 15,000 rpm for 10 min, the protein concentration of the crude nuclear extract without protease inhibitor was determined by the Bradford method. Sirt1 activity in the nuclei was determined using the CycLex Sir2 Assay Kit (Ina, Nagano, Japan).

The reaction was carried out by simultaneously mixing fluorescent-labeled acetylated peptide as substrate, 10 μL of the sample, trichostatin A, NAD, and lysyl endopeptidase. The fluorescence intensity at 440 nm was measured 60 min after reaction onset. Values are reported as relative fluorescence per microgram of protein (arbitrary unit). All data are the means ± SD of three independent experiments.

NAD^+/NADH ratio quantitative determination. NAD^+/NADH was quantified using the EnzyChrom™ NAD^+/NADH Assay Kit (BioAssay Systems, Hayward, CA) on the basis of an alcohol dehydrogenase cycling reaction in which NADH reduces a formazan reagent. First, 105 cells were washed with cold phosphate-buffered saline. The pellet was then homogenized with 100 μL of either NAD^+ or NADH extraction buffer. Extracts were heated at 60°C for 5 min, and then, 20 μL of assay buffer and 100 μL of the opposite extraction buffer were added. Samples were spun at 14,000 rpm for 5 min, and the supernatant was used for the NAD^+/NADH assay. The assay was performed using a clear-bottomed 96-well plate. First, 80 μL of working reagent was added to 40 μL of sample.

The optical density was read at 565 nm at time zero and after 15-min incubation at room temperature. Delta Optical density values were used to determine the sample NAD^+/NADH concentration from the standard curve. All data are the means ± SD of three independent experiments.

Cat activity. Cat activity in the cell lysates and serum was determined using the Cayman Catalase Assay Kit (Cayman Chemical, Ann Arbor, MI). Samples were previously diluted with buffer (1:2 for cell lysates; 1:10 for serum). All samples (10 μL) were incubated for 20 min in the presence of 3.5 mM of H_2O_2 at room temperature. This method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The reaction was quenched by the addition of potassium hydroxide. The formaldehyde produced was measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald; Cayman Chemical, Ann Arbor, MI) as the chromogen. The absorbance was read at 540 nm using a plate reader. One unit of Cat activity is defined as the amount of enzyme that leads to the formation of 1.0 nmol of formaldehyde per minute at 25°C. The values were reported as unit per microgram of protein. All data are the means ± SD of three independent experiments.

RNA isolation and real-time quantitative polymerase chain reaction analysis. Cat messenger RNA (mRNA) levels were quantified by SYBR Green real-time polymerase chain reaction (Roche Diagnostics, Indianapolis, IN). Total cellular RNA was isolated from ECs using the RNA extraction kit (Roche Diagnostics, Milan, Italy). One microgram of RNA was reverse transcribed using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche Diagnostics, Milan, Italy). A reaction without reverse transcriptase served as a negative control.

The nucleotide sequences of the polymerase chain reaction primers were specific and lacked dimerization and were as follows: Cat, forward 5′CCAGAAGAAAGCGGTCAAGAA-3′ and reverse 5′-TGGATGTGGCTCCCGTAGTC-3′. The human housekeeping gene *GAPDH* was measured to control for the amount of input mRNA using the following primer sequences: forward 5′-CAGCCGCATCTTCTTTTGC-3′ and reverse 5′-CCATGGTGTCTGAGCGATGT-3′. The expression of genes was quantified by the 7500 Sequence Detection System 1.4 Software (Applied Biosystems, Foster City, CA). The data were expressed as relative mRNA expression. All data are the means ± SD of three independent experiments.

Viability assay (MTT). To evaluate cell viability in the presence or absence of stress induction, ECs and pECs were seeded (3.5×10^3 and 5×10^3 per well, respectively) in 96-well plates and cultured in the athletes' sera at 37°C. Cell viability was determined by the CellQuanti-MTT assay (BioAssay Systems), which measures the amount of formazan produced. The amount of formazan is directly proportional to the number of living cells. The data were expressed as a percentage of viable cells relative to the FBS-ECs (control). All data are the means ± SD of three independent experiments.

Senescence associated β galactosidase activity. Cultured cells were washed in phosphate-buffered saline (pH 7.4) and fixed with 2% formaldehyde and 2% glutaraldehyde for 10 min at room temperature. After being washed twice with phosphate-buffered saline, the cells were incubated at 37°C in freshly prepared staining buffer (40 mM of citric acid/sodium phosphate (pH 6.0), 0.15 M of NaCl, 2 mM of $MgCl_2$, 5 mM of potassium ferrocyanide, 1 mg·mL^{-1} of X-gal (5-bromo-4 chloro-3-indolyl β-D-galactoside)).

At the end of 4 h of incubation, senescence-associated β galactosidase (SA-βgal) rate was obtained by counting four random fields per dish assessing the percentage of SA-βgal–positive cells from 100 cells per field.

BrdU incorporation assay. DNA synthesis was assessed using a BrdU (5-bromo-2-deoxy-uridine) Labeling and Detection Kit (Roche Diagnostics, Milan, Italy). The assay was performed according to the manufacturer's instructions. The results are expressed as a percentage of BrdU incorporation.

Cat activity inhibition. To investigate if the Cat activity could influence the changes in survival and senescence of the cells conditioned with athletes' sera, we inhibited Cat activity by the use of 3-amino-1,2,4-triazole (ATZ) purchased from Sigma at a concentration of 10 mM for 3 h.

Statistical analysis. Data were analyzed using a one-way ANOVA, followed by Bonferroni *post hoc* analyses or paired and unpaired *t*-tests, as appropriate. A multivariate analysis was performed to assess correlations among variables. All values are reported as means ± SD. A P value of <0.05 was considered statistically significant. All data were analyzed with the SPSS 15.0 statistical software (SPSS, Chicago, IL).

RESULTS

Study population. The study population consisted of three groups of athletes participating in physical training designated "aerobic" (T), "mixed aerobic–anaerobic" (S), and "anaerobic" (Sp). We evaluated biochemical and molecular variables in the sera of each group. The clinical features and functional data determined by treadmill stress and spirometry tests and information about training durations are shown in the Supplemental Digital Content Table http://links.lww.com/MSS/A107 (Table: Main clinical and functional findings of the three groups of athletes). There were no differences among groups in terms of age, body mass index, forced vital capacity, forced expiration volume in 1 s, FEV1/FVC, HR, or systolic or diastolic BP. The multivariate analysis did not show any correlation between age and the other parameters. Training time (number of training sessions per week multiplied by hours of training per day) was significantly higher in T than in S ($P = 0.01$).

Serum concentrations of oxidative stress markers and NO bioavailability. As shown in Figure 1, serum lipid peroxidation levels (TBARS) differed significantly among the three groups. In particular, lipid peroxidation levels were higher in S (2.11 ± 0.38) than in T (0.88 ± 0.19, $P < 0.0001$)

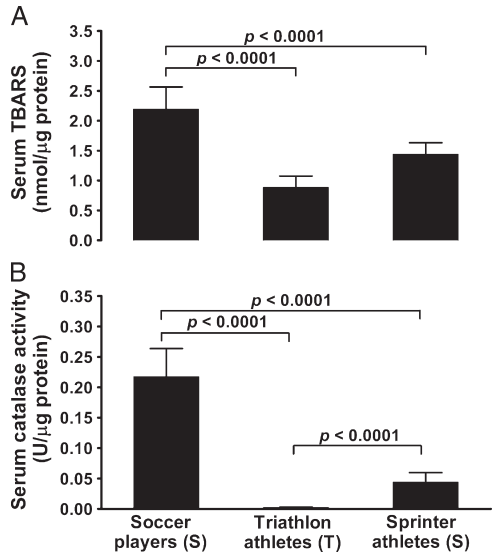

FIGURE 1—Serum concentrations of oxidative stress markers in the three groups of athletes. A, Serum lipid peroxidation levels assessed using the TBARS method in the three groups. S versus T, $P < 0.0001$; S versus Sp, $P < 0.0001$. B, Differences in Cat activity among the three groups of athletes determined by colorimetric assay. S versus Sp, $P < 0.0001$; S versus T, $P < 0.0001$; T versus Sp, $P < 0.0001$.

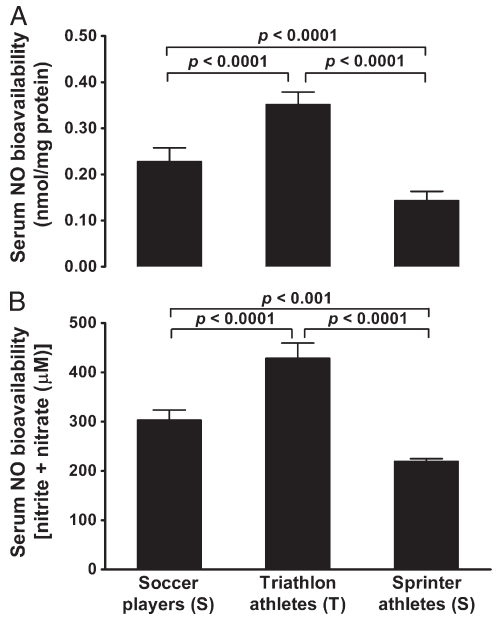

FIGURE 2—Triathlon serum has the highest NO bioavailability levels. The figure shows differences in NO bioavailability, assayed by the Griess method (A) and ion chromatography (B), in the serum of the three groups of athletes. A. T versus S, $P < 0.0001$; S versus Sp, $P < 0.0001$; T versus Sp, $P < 0.0001$. B. T versus S, $P < 0.0001$; S versus Sp, $P < 0.001$; T versus Sp, $P < 0.0001$.

and Sp (1.44 ± 0.20, $P < 0.0001$). TBARS levels were the lowest in T but did not differ significantly from levels in Sp (Fig. 1A).

Cat activity differed among the three groups. It was higher in the S serum (0.22 ± 0.05) than in the Sp (0.04 ± 0.02, $P < 0.0001$) and T sera ($P < 0.0001$). Cat activity was lowest in the T serum (0.002 ± 0.0001) and significantly higher in Sp than in T ($P < 0.0001$) (Fig. 1B).

Two indirect measures of NO bioavailability were performed. The Griess method (Fig. 2A) showed a higher bioavailability of NO in the T serum (0.35 ± 0.03) than in the S (0.23 ± 0.03, $P < 0.0001$) and Sp sera (0.14 ± 0.02, $P < 0.0001$). Moreover, it was higher in the S than in the Sp serum ($P < 0.0001$). The ion chromatography technique (Fig. 2B), by using to detect in nitrites and nitrates concentration, confirmed that NO in the T serum (428.72 ± 30.45) was higher than that in the S (303.37 ± 20.01, $P < 0.0001$) and Sp sera (219.77 ± 4.94, $P < 0.0001$). Moreover, it was higher in the S than in the Sp serum ($P < 0.001$).

Oxidative stress induced an increase of Sirt1 activity but not of the NAD^+/NADH ratio in T-ECs and pECs. As shown in Figure 3, in the EA.hy-926 EC line (ECs), before stress induction, Sirt1 (NAD^+-dependent deacetylase) activity was higher in Sp-ECs than in T-ECs ($P < 0.0001$) and S-ECs ($P < 0.0001$). A significant difference was also found between S-ECs and T-ECs ($P < 0.001$). After stress induction, Sirt1 activity was lower in Sp-ECs than in T-ECs ($P < 0.0001$) and S-ECs ($P < 0.0001$). No difference was found between S-ECs and T-ECs. Stress induction produced the opposite effect in T-ECs and Sp-ECs compared with the baseline: Sirt1 activity was increased in T-ECs ($P < 0.05$) and decreased in Sp-ECs ($P < 0.005$) versus baseline, whereas Sirt1 activity remained stable in S-ECs (Fig. 3A). In HUVEC (pECs) (Fig. 3B), before stress induction, Sirt1 activity was higher in Sp-pECs than in T-pECs ($P < 0.001$) and S-pECs ($P < 0.05$). A significant difference was also found between S-pECs and T-pECs ($P < 0.05$).

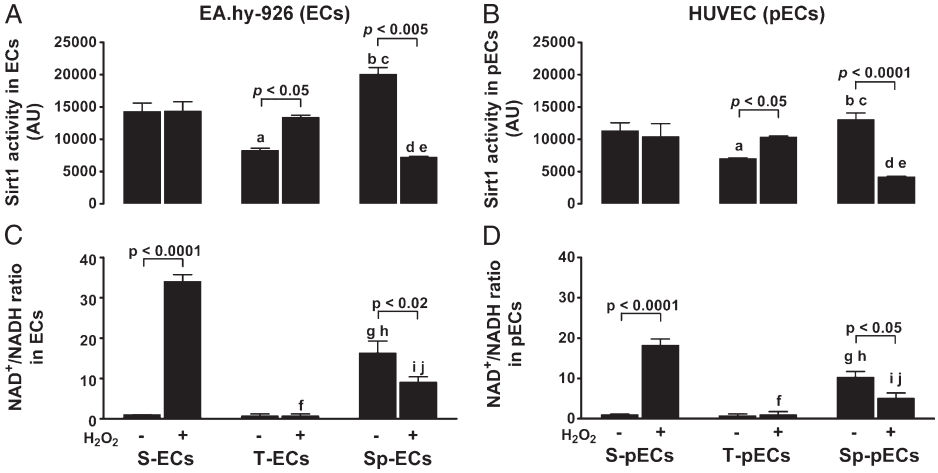

FIGURE 3—Oxidative stress increased Sirt1 activity in T-ECs and T-pECs without any change in the NAD^+/NADH ratio. Sirt1 activity in ECs (A) and pECs (B) in the three groups (+) or (−) H_2O_2: a) S versus T (−) H_2O_2 (ECs, $P < 0.001$; pECs, $P < 0.05$); b) S versus Sp (−) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.05$); c) T versus Sp (−) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.001$); d) S versus Sp (+) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.05$); e) T versus Sp (+) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.001$). The NAD^+/NADH ratio in ECs (C) and pECs (D) in the three groups (+) or (−) H_2O_2: f) S versus T (+) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.0001$); g) S versus Sp (−) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.001$); h) T versus Sp (−) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.001$); i) S versus Sp (+) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.0001$); j) T versus Sp (+) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.01$). ECs, EA.hy-926; pECs, HUVEC; S, soccer players' serum; T, triathletes' serum; Sp, sprinters' serum; (+), with; (−), without.

Before stress, the NAD^+/NADH ratio was higher in Sp-ECs than in S-ECs ($P < 0.0001$) and T-ECs ($P < 0.0001$); no difference was found between S-ECs and T-ECs. After stress, the NAD^+/NADH ratio was highest in S-ECs and lowest in T-ECs, whereas the ratio was intermediate in Sp-ECs. Stress dramatically increased the NAD^+/NADH ratio in S-ECs ($P < 0.0001$), whereas there was no change in T-ECs and a decreased NAD^+/NADH ratio in Sp-ECs ($P < 0.02$) (Fig. 3C). In primary cells, the NAD^+/NADH ratio was higher in Sp-pECs than in S-pECs ($P < 0.001$) and T-pECs ($P < 0.001$); no difference was found between S-pECs and T-pECs. After stress, the NAD^+/NADH ratio was highest in S-pECs and lowest in T-pECs, whereas the ratio was intermediate in Sp-pECs. As observed in the EC line, stress increased the NAD^+/NADH ratio in S-pECs ($P < 0.0001$), whereas there was no change in T-pECs and a decreased NAD^+/NADH ratio in Sp-ECs ($P < 0.05$) (Fig. 3D).

In ECs, after stress induction, T serum induced an increase in Cat activity without any change in mRNA expression. As shown in Figure 4, Cat mRNA expression was higher in T-ECs than in S-ECs ($P < 0.0001$) and Sp-ECs ($P < 0.0001$), and also, after stress induction, Cat mRNA expression remained significantly higher in T-ECs than in S-ECs ($P < 0.0001$) and Sp-ECs ($P = 0.001$). Within groups, Cat mRNA in S-ECs was significantly increased after stress induction ($P < 0.002$), whereas it remained stable in T-ECs and Sp-ECs (Fig. 4A). In primary cells (Fig. 4B), mRNA expression of T-pECs was higher than that of S-pECs ($P < 0.0001$) and Sp-pECs ($P < 0.001$). After stress, mRNA expression was again higher in T-pECs than in S-pECs ($P < 0.001$) and Sp-pECs ($P < 0.01$). Within groups, Cat mRNA in S-pECs was significantly increased after stress induction ($P < 0.02$), whereas it remained stable in T-pECs and Sp-pECs.

Before stress induction, Cat activity was higher in Sp-ECs than in S-ECs ($P < 0.01$) and T-ECs ($P < 0.01$). After stress, it was lower in Sp-ECs than in S-ECs ($P < 0.02$) and T-ECs ($P < 0.01$), whereas there was no difference between T-ECs and S-ECs (Fig. 4C). Notably, after stress induction, Cat activity was enhanced in T-ECs ($P < 0.01$), whereas mRNA remained at baseline level. In contrast, both Cat activity and mRNA were enhanced in S-ECs ($P < 0.05$ and $P < 0.002$, respectively). No changes were observed in Sp-ECs (Figs. 4A, C). In primary cells (Fig. 4D), before stress induction, Cat activity was higher in Sp-pECs than in S-pECs ($P < 0.05$) and T-pECs ($P < 0.05$). After stress, it was lower in Sp-pECs than in T-pECs ($P < 0.01$). As observed in EA.hy-926 cells, in HUVEC, after stress induction, Cat activity was enhanced in T-pECs ($P < 0.05$), whereas mRNA remained at baseline level. In contrast, both Cat activity and mRNA were enhanced in S-pECs ($P < 0.05$ and $P < 0.02$, respectively). No changes were observed in Sp-pECs (Figs. 4B, D).

ECs conditioned with T serum showed increased survival and proliferation rate with a decrease in the senescence level. Survival significantly differed among cells conditioned with serum from groups of athletes (Fig. 5). In particular, T-ECs' survival (by MTT assay) was greater than that of S-ECs ($P < 0.0001$) and Sp-ECs ($P < 0.0001$). This pattern was maintained after oxidative stress induction, where

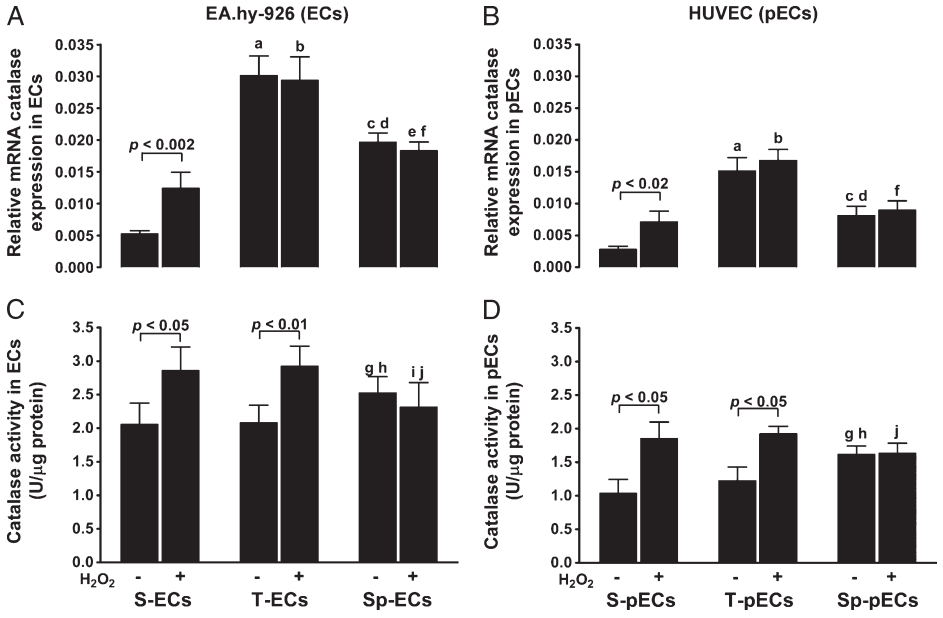

FIGURE 4—In ECs, T serum induced an increase in Cat activity after stress induction without any change in mRNA expression. Cat mRNA expression in ECs (A) and pECs (B) in the three groups (+) or (−) H_2O_2: a) S versus T (−) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.0001$); b) S versus T-ECs (+) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.001$); c) S versus Sp (−) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.001$); d) T versus Sp (−) H_2O_2 (ECs, $P < 0.0001$; pECs, $P < 0.001$); e) S versus Sp (+) H_2O_2 (ECs, $P = 0.001$); f) T versus Sp (+) H_2O_2 (ECs, $P = 0.001$; pECs, $P < 0.01$). Cat activity in ECs (C) and pECs (D) in the three groups (+) or (−) H_2O_2: g) S versus Sp (−) H_2O_2 (ECs, $P < 0.01$; pECs, $P < 0.05$); h) T versus Sp (−) H_2O_2 (ECs, $P < 0.01$; pECs, $P < 0.05$); i) S versus Sp (+) H_2O_2 (ECs, $P < 0.02$); j) T versus Sp (+) H_2O_2 (ECs, $P < 0.01$; pECs, $P < 0.01$).

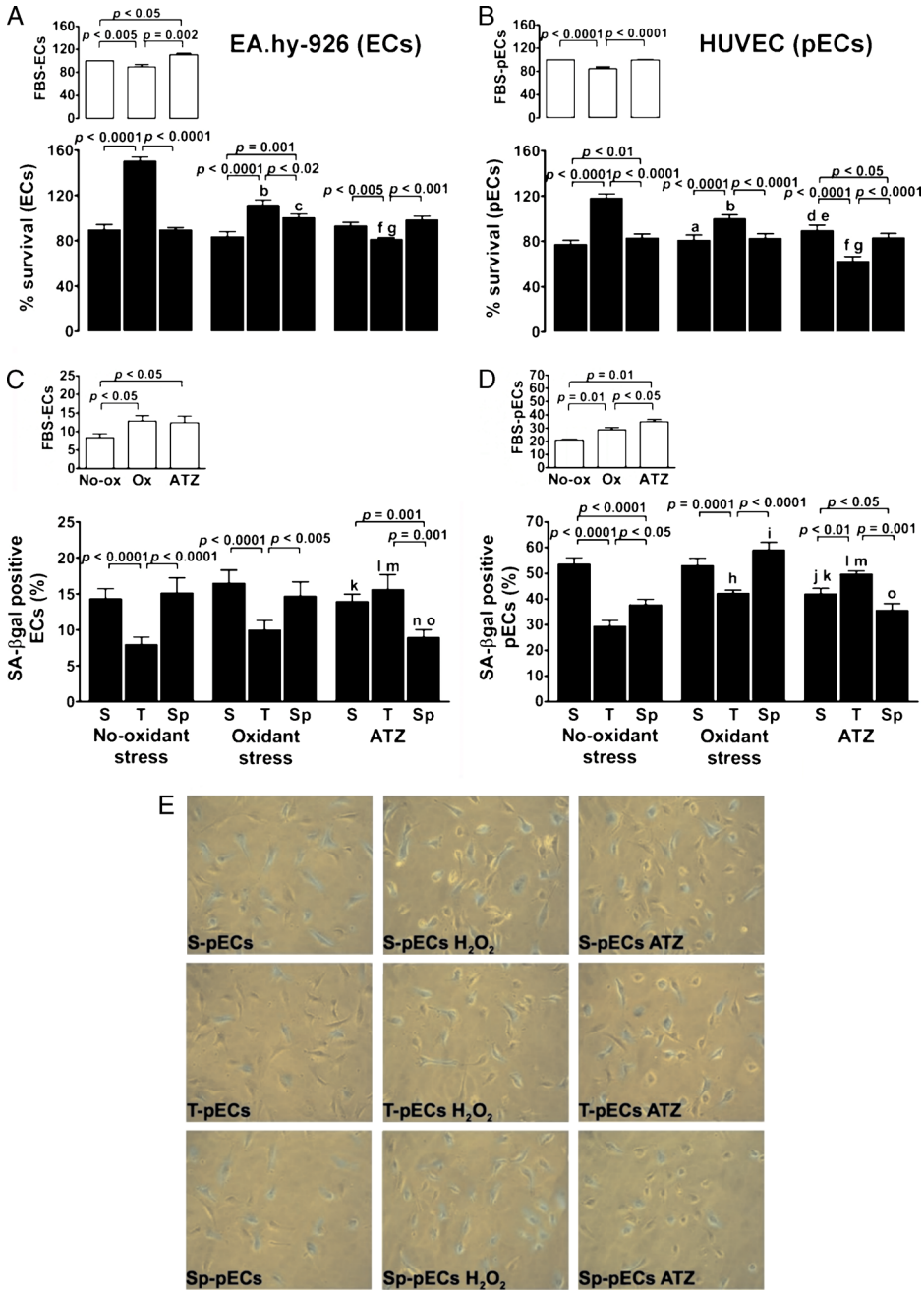

FIGURE 5—Survival and SA-βgal of ECs conditioned with athletes' serum. Difference in survival assessed with the MTT assay in ECs (A) and pECs (B) (+) or (−) H_2O_2 and after addition of ATZ: a) S (−) H_2O_2 versus S (+) H_2O_2 (pECs, $P < 0.05$); b) T (−) H_2O_2 versus T (+) H_2O_2 (ECs, $P < 0.0001$; pECs, $P = 0.001$); c) Sp (−) H_2O_2 versus Sp (+) H_2O_2 (ECs, $P < 0.02$); d) S (−) H_2O_2 versus S (+) ATZ (pECs, $P < 0.05$); e) S (+) H_2O_2 versus S (+) ATZ (pECs, $P < 0.05$); f) T (−) H_2O_2 versus T (+) ATZ (ECs, $P < 0.0001$; pECs, $P < 0.0001$); g) T (+) H_2O_2 versus T (+) ATZ (ECs, $P < 0.005$; pECs, $P < 0.0001$). Survival data are shown as a percentage of the reference condition, FBS, which is 100%. SA-βgal assay in ECs (C) and pECs (D) (+) or (−) stress induction and after addition of ATZ: h) T (−) H_2O_2 versus T (+) H_2O_2 (pECs, $P = 0.001$); i) Sp (−) H_2O_2 versus Sp (+) H_2O_2 (pECs, $P = 0.001$); j) S (−) H_2O_2 versus S (+) ATZ (pECs, $P < 0.05$); k) S (+) H_2O_2 versus S (+) ATZ (ECs, $P < 0.05$; pECs, $P < 0.005$); l) T (−) H_2O_2 versus T (+) ATZ (ECs, $P = 0.002$; pECs, $P < 0.01$); m) T (+) H_2O_2 versus T (+) ATZ (ECs, $P < 0.01$; pECs, $P < 0.05$); n) Sp (−) H_2O_2 versus Sp (+) ATZ (ECs, $P < 0.05$); o) Sp (+) H_2O_2 versus Sp (+) ATZ (ECs, $P < 0.005$; pECs, $P < 0.0001$). SA-βgal staining of pECs conditioned with athletes' sera (E), (+) or (−) H_2O_2 and after addition of ATZ, were shown. The figure shows representative data from three independent experiments.

survival was greater in T-ECs than in S-ECs ($P < 0.0001$) and Sp-ECs ($P < 0.02$) (Fig. 5A). In primary cells, we obtained comparable data in respect to the EC line. T-pECs' survival was greater than that of S-pECs ($P < 0.0001$) and Sp-pECs ($P < 0.0001$). After oxidative stress induction, T-pECs' survival was again higher than that of S-pECs ($P < 0.0001$) and Sp-pECs ($P < 0.0001$) (Fig. 5B). Consistent data were found in both ECs and pECs in a stable endothelial monolayer (seeding the cells in number of 10×10^3 per well) (data not shown).

In regard to the effects of athletes' sera on EC senescence (by SA-βgal staining), T-ECs and T-pECs showed the lowest levels of senescence both before and after stress induction.

As shown in Figure 5C, T-ECs exhibited a senescence rate lower than S-ECs ($P < 0.0001$) and Sp-ECs ($P < 0.0001$). After stress, T-ECs showed a lower senescence rate in respect to S-ECs ($P < 0.0001$) and Sp-ECs ($P < 0.005$). In primary cells (Fig. 5D), the senescence rate of T-pECs was lower than that of S-pECs ($P < 0.0001$) and Sp-pECs ($P < 0.05$). After stress induction, the senescence rate of T-pECs was lower than that of S-pECs ($P = 0.0001$) and Sp-pECs ($P < 0.0001$).

SA-βgal staining in primary ECs conditioned with athletes' sera, with or without stress induction, showed that the pECs conditioned with T serum preserved the endothelial morphological appearance. On the contrary, both the S-pECs and the Sp-pECs were characterized by an enlarged and flattened morphology, and the emergence of an endogenous SA-βgal activity (Fig. 5E).

Moreover, before stress, the proliferation rate (by the BrdU incorporation assay) in T-ECs was higher than that in S-ECs and Sp-ECs (both, $P < 0.0001$), and no difference was found among groups after stress (Fig. 6A). In HUVEC, comparable data were found (Fig. 6B). The BrdU incorporation was higher in T-pECs than in S-pECs and Sp-pECs (both, $P < 0.0001$), and after stress, T-pECs showed higher values of proliferation than Sp-pECs ($P = 0.001$). Fluorescence images of primary cells conditioned with athletes' sera, with or without stress induction, were shown in Figure 6C.

Inhibition of Cat activity by ATZ abrogates the T serum protective effect. To investigate the involvement of Cat on survival and senescence in the ECs conditioned with athletes' sera, we inhibited its activity by ATZ, a well-known inhibitor of Cat activity. As shown in Figure 5, we found that ATZ caused a survival decrease only in ECs

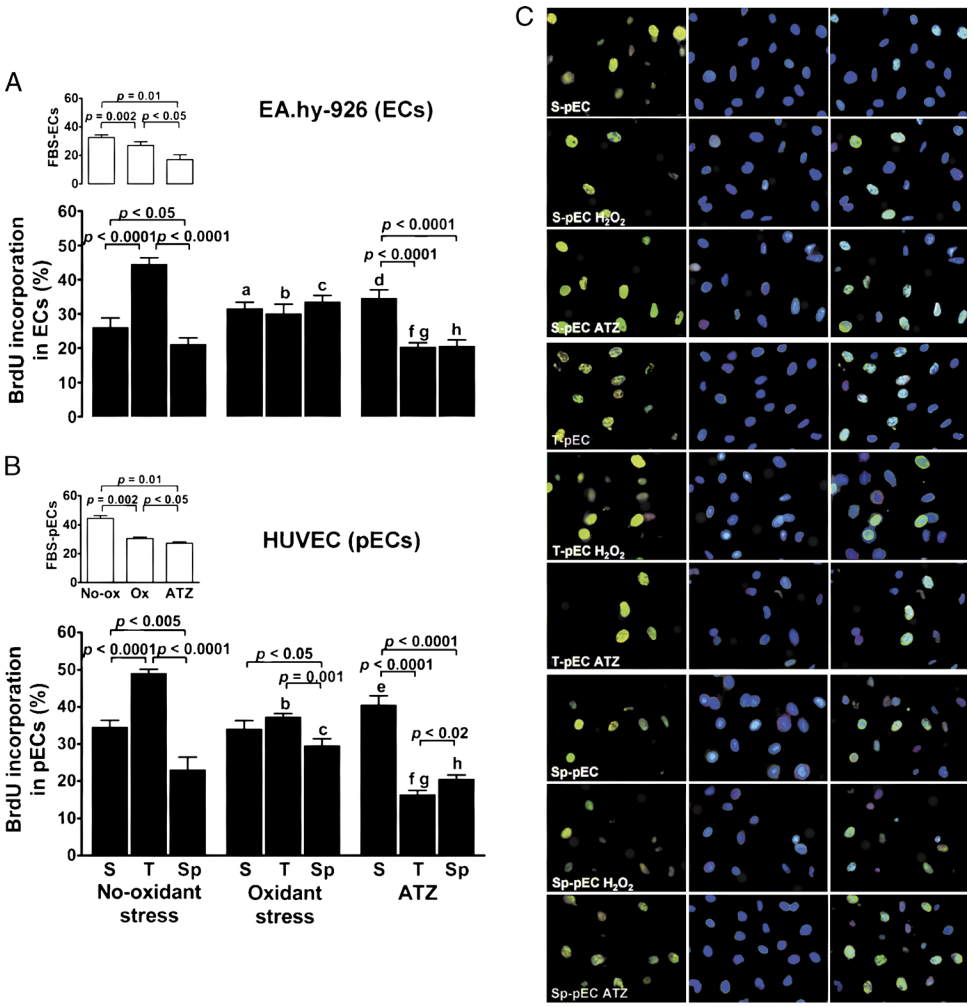

FIGURE 6—5-bromo-2′-deoxy-uridine detection. Differences in BrdU incorporation in ECs (A) and pECs (B) (+) or (−) stress induction and after addition of ATZ: a) S (−) H_2O_2 versus S (+) H_2O_2 (ECs, $P < 0.05$); b) T (−) H_2O_2 versus T (+) H_2O_2 (ECs, $P < 0.01$; pECs, $P < 0.0001$); c) Sp (−) H_2O_2 versus Sp (+) H_2O_2 (ECs, $P = 0.002$; pECs, $P < 0.05$); d) S (−) H_2O_2 versus S (+) ATZ (ECs, $P < 0.05$); e) S (+) H_2O_2 versus S (+) ATZ (ECs, $P < 0.05$); f) T (−) H_2O_2 versus T (+) ATZ (ECs, $P < 0.0001$; pECs, $P < 0.0001$); g) T (+) H_2O_2 versus T (+) ATZ (ECs, $P = 0.005$; pECs, $P < 0.0001$); h) Sp (+) H_2O_2 versus Sp (+) ATZ (ECs, $P < 0.0001$; pECs, $P = 0.001$). Fluorescence images of BrdU-labeled pECs conditioned with athletes' sera (C), (+) or (−) H_2O_2 and after addition of ATZ were shown. The figure shows representative data from three independent experiments.

(both primary and cell line) conditioned with serum from T (Figs. 5A, B).

On the contrary, under ATZ, both T-ECs and T-pECs showed an increase in senescence rate in respect to baseline ($P = 0.002$ and $P < 0.01$, respectively). Moreover, the senescence rate in T-ECs was higher in respect to that in Sp-ECs ($P = 0.001$), and it did not differ from S-ECs (Fig.5C). In HUVEC, the senescence rate of T-pECs was higher than that of S-pECs ($P < 0.01$) and Sp-pECs ($P = 0.001$) (Fig. 5D). The βgal staining in primary ECs showed that ATZ addition abrogated the positive effect on endothelial appearance in T-pECs demonstrating a typical senescent phenotype (Fig. 5E).

Finally, both T-ECs and T-pECs showed a significant (both, $P < 0.0001$) BrdU incorporation decrease in presence of ATZ (Figs. 6A, B). A representative image of BrdU-labeled primary ECs conditioned with athletes' sera was shown in Figure 6C. A treatment with ATZ induced a drastic decrease in BrdU-positive cells in primary ECs conditioned with T serum (Fig. 6C).

DISCUSSION

In this study, we investigated the effects of sera collected from three groups of athletes, i.e., T, S, and Sp, on human ECs. In 2008, Allard et al. (2) examined the effects of sera from calorie-restricted subjects on cultured cells and demonstrated that this *in vitro* technique enabled them to predict the effectiveness of calorie restriction in humans. Calorie restriction and exercise training share various features, but although the molecular calorie restriction effects were largely investigated, little is known about long-term exercise training effects in humans.

To avoid confounding factors related to the acute effects of exercise, all experiments were conducted after a period of 15 d of detraining. Several reports, including a study by Slentz et al. (34), indicate that this period is insufficient to lose the exercise adaptation in humans.

We found no difference in functional and hemodynamic variables among the athlete groups recruited in this study, whereas there were dramatic changes in serum oxidative stress markers. In particular, mixed aerobic–anaerobic training (soccer) was characterized by higher serum levels of lipid peroxidation compared with both aerobic (triathlon) and anaerobic (sprint run) specialties. Moreover, Cat activity was higher in the serum of soccer players Lipid peroxidation and antioxidants were considered as biomarkers of tissues damage, and enzymes inhibiting peroxidation can be called preventive antioxidants. In particular, Cat removes H_2O_2 when present in a high concentration, and serum Cat activity was identified as a marker of stress consequent to the finding that its levels are high in pathological conditions such as acute pancreatitis and respiratory distress syndrome (13,23). In this study, the high Cat activity in S could be due to increased oxidative stress (as shown by the TBARS assay), and we suppose that enhanced levels of TBARS in the serum of S induce higher serum Cat activity in the effort to reduce oxidative stress.

Various studies have investigated changes in pro-oxidant and antioxidant production in the sera of athletes, most of which compared athletes to sedentary subjects. Few studies compared athletes engaged in different sports. For instance, Kostaropoulos et al. (22), by comparing long-distance with short-distance runners, highlighted that chronic exercise provokes an increase in antioxidant defense that enhances protection against reactive oxygen species.

However, the results are variable probably because of differences in the type and duration of exercise (12). Some studies evaluated oxidative stress in T, often with conflicting results. In fact, some authors found an increase in oxidative stress (29), whereas others reported no persistent oxidative stress and no DNA damage (38). Only few studies have compared aerobic and anaerobic exercise. Bloomer and Smith (4) found no significant differences in induced patterns of oxidative stress, whereas Selamoglu et al. (33) reported differences in the antioxidant profile between the two types of exercise. However, to our knowledge, no studies have investigated the molecular mechanisms responsible for the effects of long-term exercise on oxidative stress.

In this study, we found that NO levels were higher in sera from T than in sera from S and Sp. The beneficial effects of exercise training have been documented in animals and humans, and often, these effects are associated with an increase in NO bioavailability. Yang et al. (40) showed that training induces a significant enlargement of collateral vessels in striated ischemic muscle and that these effects were lost upon inhibition of NO production. Eksakulkla et al. (10) suggested that physical activity could improve endothelial dysfunction in aged rats by increasing NO bioavailability.

In ECs, both primary and cell line, we analyzed some indicators of cellular health, such as survival, proliferation, and senescence. Survival and proliferation rates were higher in ECs supplemented with T serum than in the others. Moreover, the T serum inhibited the senescent phenotype as judged by SA-βgal staining. It is well known that changes in many functional aspects of cell behavior, including proliferation, are closely related to NO bioavailability (16). Moreover, it has been demonstrated that NO production is strongly limited in senescent ECs (32).

The highest survival and proliferation rates and the lowest SA-βgal associated senescence observed in ECs conditioned with T serum, both at baseline and after stress induction, may depend, at least in part, on high NO bioavailability in the serum of T. Therefore, we suggest that the high NO bioavailability in serum from athletes undergoing aerobic training produces favorable effects on human ECs.

Moreover, we found that conditioning with athletes' sera induced changes in the mRNA and activity levels of molecules important for the maintenance of the intracellular redox state. In particular, after stress induction, Sirt1 activity was significantly increased in ECs conditioned with T serum

versus baseline, in contrast to S-ECs, where levels did not change, and Sp-ECs, where Sirt1 activity was significantly decreased. Sirt1 is a NAD^+-dependent deacetylase that produces protein deacetylation by consuming NAD^+.

Several studies have demonstrated that Sirt1 extends the lifespan of many organisms by increasing cellular stress resistance (1,6). The requirement of NAD^+ for Sirt1 activity implies that Sirt1 effectiveness depends on the cellular metabolic state. It has also been suggested that the beneficial effects of calorie restriction may depend on the regulation of NAD metabolism or on Sirt1 activity or both (21). The NAD^+/NADH ratio plays a crucial role in controlling the intracellular redox state, and it is known to fluctuate in response to changes in cellular metabolism (25). Moreover, it has been demonstrated *in vitro* that H_2O_2 accelerates cellular senescence via decrease in Sirt1 function, caused in turn by NAD^+ depletion (14). Several studies have suggested that the ability to regulate the NAD^+/NADH ratio is reduced also under pathological conditions characterized by high oxidative stress, such as diabetes and Parkinson disease (19,15). We found that, after stress, the NAD^+/NADH ratio remained constant in T-ECs, whereas it increased in S-ECs and decreased in Sp-ECs. Taking into account both the Sirt1 activity and the NAD^+/NADH ratio, we suggest that Sirt1 is more efficient in ECs conditioned with T serum because these have the same NAD^+/NADH ratio after stress as at baseline.

We previously demonstrated that prolonged aerobic exercise training restores antioxidant efficiency in aged rats by inducing Sirt1 activity (30,11). Here, we show that Sirt1 activity is increased in human ECs conditioned with serum from individuals on a long-term aerobic exercise program. Moreover, ECs conditioned with the serum of T had the highest mRNA levels of Cat, which is an important Sirt1 target involved in the stress response. After stress, Cat activity was significantly increased in those ECs, whereas mRNA levels remained unchanged. On the contrary, Cat mRNA expression but not Cat activity was increased in S cells. These findings suggest that the serum collected from T is able to induce a signaling in the cells that guides to an optimal adaptation of the Cat. Indeed, ATZ, a Cat activity inhibitor, caused the abrogation of T serum protective effect in regard to survival, proliferation, and senescence. Therefore, the addition of ATZ demonstrated that indicators of function and health in the cells conditioned with the serum of athletes undergoing aerobic training could be influenced by Cat activity. This finding is in agreement with Xu et al. (39), who previously demonstrated that the increased H_2O_2 generation guides to an increase in endothelial senescence levels, which could be attenuated by Cat treatment in a dose-dependent manner.

A possible limitation of this study is that the molecular differences observed in the cells conditioned with athletes' sera could depend on training duration. However, training duration changed significantly only between S and T, whereas the molecular effects differed among all the three groups and with respect to all the variables considered. Another limitation of this study is the possibility that the athlete's sport selection and individual response to exercise were genetically influenced. This aspect is not addressed in the current article owing, in part, to the small cohorts studied. It would be interesting to perform future studies to quantify the genetic contribution to athletically favorable blood profiles.

Aerobic exercise training improves endothelial function, and it is generally used as a cardiac rehabilitation tool, especially in patients with CAD (26). Despite its proven effectiveness, optimal exercise training remains a topic of continuing study. Further investigations using *in vitro* experiments similar to the one used in this study could be helpful in determining the most effective exercise protocols for health enhancement and rehabilitation of patients with cardiovascular and other chronic degenerative conditions. In conclusion, we demonstrate that different types of exercise training induce different molecular effects.

Moreover, the *in vitro* technique used in this study may be useful to investigate the circulating factors involved in the response and in the activation of molecules responsible for the effects of long-term physical activity in humans.

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The authors declare no conflicts of interest.

The results of the present study do not constitute endorsement by the American College of Sports Medicine.

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