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Changes in oxidative stress markers and NF-κB activation induced by sprint exercise

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Abstract

This study was aimed to investigate changes in blood markers of oxidative damage induced by short-term supramaximal anaerobic exercise and to determine whether oxidative stress was associated to activation of the redox-sensitive transcription factor nuclear factor-κB (NF-κB). Both a single Wingate test (WAnT) test and series of four WAnTs separated by 60 min rest intervals were carried out by eight professional cyclists. Leukocyte 8-OH-2-deoxyguanosine levels were significantly elevated 24 h after both exercise protocols. A significant decrease in blood reduced glutathione (GSH) concentration was observed immediately after and at 15, 60 and 120 min of the single WAnT, followed by a return to basal value after 24 h. This decrease was parallel to a significant increase of the oxidised/reduced glutathione (GSSG/GSH) ratio, to an activation of NF-κB and to a significant decrease in the protein level of its inhibitor IκB. GSH concentration and the GSSG/GSH ratio changed significantly for the first three of the WAnTs series and normalised thereafter. A significant activation of NF-κB and a decrease in the IκB protein level were also detected. We conclude that short-term supramaximal anaerobic exercise induces oxidative stress, as evidenced by non cumulative damage to macromolecules and changes in the glutathione status. Our data also indicate that high intensity anaerobic work gives rise to an activation of the transcription factor NF-κB accompanied by a degradation of IκB.

Keywords: *Oxidative stress, glutathione, NF-κB, anaerobic, sprint exercise*

Introduction

Although regular exercise training is indeed associated with numerous health benefits, many studies have reported that physical exercise increases the production of reactive oxygen species (ROS), thereby inducing oxidative stress [1]. The majority of these studies utilised aerobic exercise as the fundamental cause of elevated levels of ROS [2–4]. However, there are few data on the effects of short-term anaerobic exercise, especially in humans. During prolonged submaximal aerobic exercise, the increase in ROS production is largely due to a disturbance in electron transport leading to an increased leakage of superoxide radicals [5,6]. It has been suggested that oxidative stress specific to anaerobic exercise may be mediated through various other pathways such as proton accumulation due to lactic acidosis [7], autooxidation of catecholamines [8], catabolism of purines to xanthine and urate [9] and a transient and acute muscular deoxygenation, which resembles the ischemia-reperfusion syndrome [10]. Factors such as prostanoid metabolism, phagocytic respiratory burst activity, disruption of iron-containing proteins, or alteration of calcium homeostasis could also be involved [11].

Enhanced production of ROS causes cellular damage represented by modifications to various macromolecules, including proteins, lipids and

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nucleic acids and also induces change on the antioxidant defense system. Moreover, ROS have an important modulating function in gene expression [12] and by activating redox-sensitive transcription factors, take on the role of intracellular messengers. Nuclear factor-κB (NF-κB) is a typical example of transcription factor which is activated by intracellular reactive oxygen species, such as H_2O_2, superoxide anion or hydroxyl radicals [13]. However, the role played by ROS activation of NF-κB in physical exercise remains only poorly understood [14] and no data on the effects of utilizing anaerobic exercise protocols are available.

This study was aimed to investigate changes in blood markers of oxidative damage induced by short-term supramaximal anaerobic exercise and to determine whether oxidative stress was associated to activation of the transcription factor NF-κB. The Wingate test (WAnT) was chosen because it strongly activates lactic acid production [15,16], and causes a major increase in plasma catecholamine levels [8]. In order to identify potential cumulative effects of exercise, both single WAnT and series of four tests separated by 60 min rest intervals were carried out.

Materials and methods

Subjects and procedures

Eight voluntary professional cyclists participated in this study. Subjects were informed of the purpose of the investigation and the possible risks involved before giving their written consent to participate. The experimental protocol was approved by the local ethics committee according to the principles set forth in the Declaration of Helsinki of the World Medical Association.

The cyclists visited the laboratory on two different days, D1 and D2, separated by 10–15 days. On D1, the experiment started at about 9 a.m., 2 h after a standardized breakfast. On arrival, each subject was asked to lie down and a catheter was inserted into a braquiocephalic vein. The subject sat on a cycloergometer (Monark® 816E) and the first blood sample was drawn after 15 min, to determinate rest values. A 10-min warm-up followed at a submaximal power of about 50% VO_{2max}. The WAnT was then performed as previously described [17]. During this exercise the subject was asked to cycle for 30 seg as rapidly as possible with verbal encouragement from the researchers. Performance during the test was judged using the usual parameters of peak power (W_{peak}) and mean power (W_{mean}). The fatigue index was expressed as a percentage: (peak power − final power/peak power) × 100 [18].

The second part of the study took place on D2 and was aimed to identify the effects of consecutive series of short-term anaerobic exercise. The same steps that D1 were followed. The single difference was the exercise protocol that consisted of a series of four WAnTs, with rest intervals of 60 min between them.

Blood sample preparation

Venous blood samples were taken using EDTA as an anticoagulant. On D1, blood samples were obtained, using a catheter closed by stylet (Vasoran and Mandrin, B. Braun), from the braquiocephalic vein at rest, immediately after the Wingate exercise and at 15, 60, 120 min and 24 h after cessation of exercise. On D2, blood samples were obtained at rest, immediately before each Wingate test and 24 h after cessation of exercise.

Immediately after extraction, blood samples (0.5 ml) were treated, at 4°C, either with 0.5 ml ice-cold perchloric acid (PCA) (12%), containing 40 mM NEM and 2 mM bathophenanthrolinedisulfonic acid for oxidised glutathione (GSSG) assay or 0.5 ml ice-cold trichloroacetic acid (TCA) (30%), containing 2 mM EDTA for reduced glutathione (GSH) assay, and mixed thoroughly. Samples were centrifuged at 15,000*g* for 5 min at 4°C and the acidic supernatants were used for derivatization or spectrophtometric determination of GSH [19].

A measure of 2 ml of whole blood were centrifuged immediately after sampling (1500*g*, 10 min, 4°C) and plasma aliquots were stored at −80°C until further determination of TBARS.

Leukocytes were separated from 10 ml of the whole blood by centrifugation with 3 volume of buffer containing Tris 10 mM and EDTA 10 mM (1500*g*, 10 min, 4°C). The pellets were centrifuged again with 3 volume of buffer Tris 10 mM and EDTA 1 mM. The pellet of leukocytes obtained was stored at −80°C until further analysis.

Peripheral blood mononuclear cells (PBMC) were separated from 12 ml of the whole blood by density-gradient centrifugation on Ficoll separating solution (Biochrom AG). For each sample, two 15-ml centrifuge tubes were used to layer 6 ml of blood onto 4 ml of Ficoll. The suspension was centrifuged for 30 min at 450*g* and 20°C. The mononuclear cell layer was removed with manual pipetteing, washed one time in Hank's solution and centrifuged for 10 min at 20°C and 275*g* after the wash. Washed cells were resuspended in 1 ml of PBS. Analyses were performed on frozen cells.

Assessment of the glutathione status in blood

Reduced glutathione determination was performed by a modification of the glutathione S-transferase (GST) assay described by Brigelius et al. [20] The following reaction mixture was added into a cuvette: 825 μl of 0.5 M potassium phosphate buffer, pH 7, containing 1 mM EDTA, 25 μl of the acidic supernatant of

the sample and 10 μl of chlorodinitrobenzene solution (2 mg/ml of ethanol) recording the absorbance at 340 nm as a baseline. The glutathione S-transferase solution was prepared by dissolving 500 U/ml of phosphate buffer. Then, 10 μl of dialyzed glutathione S-transferase were added and absorbance was recorded at 340 nm until the end point of the reaction ($E = 9.6/\text{mM/cm}$).

For oxidised glutathione analysis, blood samples were derivatized by the following procedure: 50 μl an internal standard solution (1 mM γ-glutamylglutamate prepared in 0.3% PCA) was added to 500 μl of acidic supernatant. Ten microliters of a pH indicator solution (1 mM m-cresol purple) was also added and samples were neutralized up to pH 8.0–8.5 with 2 M potassium hydroxide containing 0.3 M 3-(*N*-morpholino) propanesulfonic acid (MOPS) to prevent excessive alkalinization. Then, samples were centrifuged at 15,000*g* for 5 min and 50 μl of 1% 1-fluoro-2,4-dinitrobenzene, dissolved in ethanol, were added to an aliquot of 25 μl of each supernatant. After derivatization, samples were stored in darkness at −20°C until injection.

Samples processed were dissolved in 50 μl of 80% methanol and, 25 μl was injected into high-performance liquid chromatography (HPLC) system. A Spherisorb-NH_2 column (Waters, 5 μm, 0.46 × 25 cm) was used. The flow rate was 1.0 ml/min during the gradient. The mobile phase and the gradient were the same as those described previously by Viña et al. [19]. Solvent A was 80% methanol, and solvent B was 0.5 M sodium acetate in 64% methanol. After injection of the derivatized sample, the mobile phase was held at 80% A, 20% B for 5 min followed by a 10 min linear gradient up to 1% A, 99% B. Then, the mobile phase was held at 99% B until GSSG eluted.

Assessment of the thiobarbituric reactive species (TBARS) in plasma

Thiobarbituric acid (TBA) reactants were measured according to a modification of the high pressure liquid chromatography method of Richard et al. [21]. Ten microliters of 2% (w/v) solution of BHT in 100% ethanol were added to each tube in order to inhibit the progression of lipid peroxidation. Then, into each test tube, 100 μl of plasma was vortex-mixed with 750 μl of kit working solution (TBA-PCA (2:1, v/v), prepared fresh daily). The tubes were tightly capped and placed in a 95°C water bath for 60 min. They were then chilled in an ice-water bath. The tubes were centrifuged and maintained at 4°C until HPLC analyses. The MDA-TBA adduct is unstable at neutral pH, and so each sample was separately neutralized within 10 min of injection. About 20 μl of 5 M potassium hydroxide was added to 300 μl of sample to bring the pH of the reaction mixture to 6.0. After neutralizing, the samples were immediately centrifuged at 3000*g* for 3 min and then analysed. A measure of 50 μl of samples were injected into HPLC system equipped with a Prodigy analytical stainless-steel column (Phenomenex, 5 μm, 0.46 × 25 cm). Isocratic separation was performed at 1.0 ml/min flow-rate. Mobile phase consisted in 50 mM phosphate buffer (pH 6.0): methanol (58:42, v/v). The absorbance of each sample was recorded at the column outlet at 532 nm.

Assay of 8-hydroxy-2-deoxyguanosine (8-OHdG)

Isolation of cell DNA was performed using a method by Loft and Poulsen [22]. Briefly, leukocytes were resuspended in 2 ml of 10 mM Tris–HCl buffer (pH 7.5) containing 320 mM sucrose, 5 mM $MgCl_2$, 0.1 mM deferrioxamine and 1% Triton X. After centrifugation at 1500*g* for 10 min, the pellet was resuspended in 600 μl of 10 mM Tris–HCl buffer (pH 8.0) containing 5 mM EDTA, 0.15 mM deferrioxamine, and 10% sodium dodecyl sulfate and then was incubated at 50°C for 15 min with RNAse A (1 mg/ml) and T1 (1 U/μl). Leukocytes then were incubated at 37°C for 1 h with proteinase K (20 μg/μl). After incubation, the mixture was extracted with isopropanol in the presence of sodium iodure (1.2:2 vol/vol), and DNA was precipitated from the aqueous phase. DNA was solubilized in 200 μl of water. We then added P1 nuclease (1.5 U/μl) and incubated the product at 37°C for 60 min. Finally, the mixture was digested for 30 min at 37°C with alkaline phosphatase (0.1 U/μl) in the presence of 20 μl of 0.4 M Tris–HCl buffer (pH 8.8). From the hydrolysed mixture, 50 μl were injected into the high-performance liquid chromatography apparatus. The nucleosides were separate by C18 reversed-phase column (Phenomenex, 5 μm, ID 0.46 × 25 cm). The eluting solution was 100 mM sodium acetate (pH 5.2) containing 4.5% methanol and 4.25% acetonitrile at 1.0 ml/min flow-rate. The 8-OHdG and dG were detected using an ESA Coulochem II electrochemical detector in line with an ultraviolet detector as reported previously [22]. The 8-OHdG levels were expressed as the ratio of 8-OHdG/10^5 dG.

Electrophoretic mobility shift assays (EMSAs)

Binding activity of NF-κB was determined in nuclear extracts of PBMC by means of EMSA as described Hofmann et al. [23]. Nuclear extracts of PBMC were harvested by the method of Andrews and Faller [24] as reported: PBMC were lysed in 800 μl of cold buffer A (10 mM HEPES-KOH, pH 7.9, 0.1 mM EDTA-Na, 0.1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) and incubated for 15 min on ice. Cells were centrifuged for 3 min at 16,000*g*, and the supernatant

was discarded. The pellet was resuspended in 60 μl of cold buffer C (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 0.4 mM NaCl, 1 mM EDTA-Na, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF), incubated for 15 min on ice, and centrifuged for 4 min at 16,000*g*. The supernatant containing nuclear proteins was quick-frozen at −80°C. Protein concentration was determinated according to the Lowry method [25]. Oligonucleotides were end labeled with [γ-^{32}P]ATP to a specific activity $>5 \times 10^7$ cpm/μg DNA: NF-κB consensus: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Binding of NF-κB was performed in 50 mM Tris–HCl, pH 7.5, containing 5 mM EDTA-Na, 200 mM NaCl, 20% glycerol, 5 mM β-mercaptoethanol and 0.1 μg/μl poly (dI/dC) in a total of 25 μl as described [26]. Nuclear extract (26 μg) was incubated for 20 min at room temperature in binding buffer in the presence of ~1 ng labeled oligonucleotide [~250 μCi (Amersham Redivue)]. For competition studies, 3.5 pmol of unlabeled NF-κB oligonucleotide (competitor) or 3.5 pmol of labelled NF-κB oligonucleotide mutate (noncompetitor) were mixed 15 min before the incubation with the labelled oligonucleotide.

Protein–DNA complexes were separated from the free DNA probe by electrophoresis through 6% native polyacrylamide gels containing 10% ammonium persulfate and 0.5x Tris-borate-EDTA buffer. Gels were dried under vacuum on Whatmann DE-81 paper and exposed for 48–72 h to Amersham Hyperfilms at −80°C.

IκB-alpha protein degradation

For Western blot analysis of IκB-alpha protein degradation, PBMC cells were homogenised with 150 μl of 0.25 mM sucrose, 1 mM EDTA, 10 mM Tris and a protease inhibitor cocktail [27]. Protein concentration was determinated according to the Lowry method [25]. Samples containing 50 μg of protein were separated by SDS-polyacrylamide gel electrophoresis (9% acrylamide) and transferred to PVDF membranes. Non-specific binding was blocked by preincubation of the PVDF membrane in PBS containing 5% bovine serum albumin for 1 h. The membrane was then incubated overnight at 4°C with polyclonal anti-IκB-alpha antibodies (Santa Cruz Biotechnology). Bound primary antibody was detected using a peroxidase conjugated secondary antibody (DAKO) by chemiluminiscence using the ECL kit (Amersham). The density of the specific IκB-alpha (36 kDa) bands were quantitated with an imaging densitometer. The membrane was stripped in 6.25 mM Tris, pH 6.7, 2% SDS and 100 mM mercaptoethanol at 50°C for 15 min and probed again for anti-beta-actin antibodies (Sigma) to verify equal protein loading in each lane.

Expression of the results and statistical analysis

Brief and intense exercise such as the Wingate test has been demonstrated to induce non-negligible plasma volume changes, which necessarily modify all plasma concentrations measured [28–30]. Therefore, the blood chemical values (glutathione and TBARS) measured in this study were corrected taking into account plasma volume changes using the equation suggested by Dill and Costill [31].

Data were expressed as mean ± standard error of means (S.E.M). The results for NFκB and IκB are presented as percentages from resting values. Comparisons between rest and the other sampling points were performed by one-way analysis of variance (ANOVA) with repeated measures. *Post hoc* comparisons were made with the Newman-Keuls test. A value of $p < 0.05$ was regarded as significant. A SPSS + vrs. 12.0 statistical software (Chicago, IL) was used.

Results

Following a single WAnT, the mean value of W_{peak} was 11.84 W/kg and the mean W_{mean} was 10.05 W/kg (Table I). This values were within the range previously described for competitive cyclists [32] and confirmed the good anaerobical performance of subjects. The fatigue index by a single WAnT was 37%. When the exercise protocol consisted of a series of four WAnTs with rest intervals of 60 min between them, there was no significant decrease in W_{peak} and W_{mean} and the fatigue index did not significantly change throughout time (Table I).

Plasma TBARS concentration was not significantly modified following a single WAnT, although values tended to increase at 60 and 120 min (Table II). When a series of four tests were performed, values were not significantly modified at any of the testing periods (Table III).

Table II also reports blood concentrations of GSH and GSSG and the GSSG/GSH ratio after a single WAnT. A significant decrease in GSH levels was observed immediately after and at 15, 60 and 120 min of the test (−14, −23, −29 and −17%, respectively), followed by a return to basal value after 24 h. This decrease was parallel to a significant increase of

Table I. Effects of a single Wingate test (WAnT) and four consecutive WAnTs on anaerobic power and fatigue index.

	W_{peak} (W/kg)	W_{mean} (W/kg)	Fatigue index (%)
Single WAnT	11.8 ± 0.2	10.1 ± 0.2	37.1 ± 2.7
1st WAnT	12.0 ± 0.2	9.9 ± 0.3	38.6 ± 4.3
2nd WAnT	11.8 ± 0.4	10.0 ± 0.4	34.4 ± 5.0
3rd WanT	12.3 ± 0.4	10.6 ± 0.3	32.0 ± 4.4
4th WAnT	11.3 ± 0.4	10.1 ± 0.4	37.5 ± 9.0

Results are expressed as means ± SEM. Number of subjects: $n = 8$.

Table II. Time course of blood reduced glutathione (GSH), oxidized glutathione (GSSG), ratio GSSG/GSH × 10^3 and plasma thiobarbituric acid reactive substances (TBARS) at rest, immediately after a single Wingate test and following 15 min, 60 min, 120 min and 24 h of recovery.

	GSH (μM)	GSSG (μM)	GSSG/GSH × 10^3	TBARS (μM)
Rest	590 ± 28	26.0 ± 4.1	44.0 ± 3.0	0.088 ± 0.014
0 min	507 ± 9*	27.8 ± 4.5	55.8 ± 3.7*	0.097 ± 0.018
15 min	452 ± 11*	24.7 ± 4.1	55.8 ± 1.6*	0.089 ± 0.018
60 min	416 ± 10*	23.7 ± 1.6	59.7 ± 2.0*	0.113 ± 0.021
120 min	487 ± 10*	28.4 ± 3.8	57.7 ± 1.4*	0.103 ± 0.010
24 h	629 ± 35	26.3 ± 4.0	43.8 ± 7.2	0.094 ± 0.024

Results are expressed as means ± SEM. *Significant changes compared to resting values ($p < 0.05$). Number of subjects: $n = 8$.

the GSSG/GSH ratio (+27, +27, +26 and +31%, respectively). Concentration of oxidized glutathione in blood was not significantly affected by the WAnT (Table II).

As shown in Table III, blood GSH decreased significantly for the first three of the WAnTs (−21, −16 and −15%, respectively vs rest) and normalised thereafter. The GSSG/GSH was also increased after the first three tests (+43, +47 and +35%) and did not differ significantly from pre-exercise values thereafter.

Leukocyte 8-OHdG levels were measured before and 24 h after the WAnTs. Following a single test values were still increased by 29% (5.04 ± 1.12 vs 3.92 ± 0.89 8-OHdG/10^5 dG; $p < 0.05$), while a 35% increase was detected following the series of four tests (5.09 ± 1.22 vs 3.78 ± 0.58 8-OHdG/10^5 dG; $p < 0.05$).

A single WAnT caused a significant increase in NF-κB binding activity to NF-κB consensus sequence in all individuals tested (Figure 1). Densitometric analysis confirmed that binding activity reached a maximum (+91%) at 60 min post-test and returned to baseline levels within 24 h. Figure 2 shows the effects of the series of four WAnTs on the activation of the transcription factor NF-κB. The signal intensity obtained by EMSA demonstrated a significant activation of NF-κB that persisted for the first three tests (+43, +49 and +34%, respectively), returning to resting values thereafter.

A significant decrease in IκB protein levels was observed after a single WAnT. This decrease was more pronounced at 60 and 120 min post-exercise (−44 and −46%, respectively), returning to basal values at 24 h (Figure 3). Figure 4 shows the IκB protein levels following the series of four WAnTs. Values decreased progressively from the first to the forth test (from −37 to −54%) and still remained reduced at 24 h.

Discussion

Based on the available evidence it appears that anaerobic exercise, whether it involves isometric, eccentric, isotonic, or sprint training, can induce oxidative damage [33]. The results of sprint protocols in animals argue in favour of this assumption. Thus, Alessio et al. [34] have shown that lipid peroxidation levels in the skeletal muscle of rats increase after sprint exercise at a speed of 45 m/min for 1 min and muscle TBARS have been reported to increase acutely in mice performing 15 sprints at 35 m/min for 30 s. However, very scarce and contradictory data are available in humans and only a few studies utilizing sprint protocols have been undertaken [4,30].

When plasma TBARS were measured as a marker of oxidative stress, no change was detected following a single WAnT. However, although oxidative stress during exercise has most frequently been assessed by measuring the malondialdehyde levels using the TBARS assay, this method has been often criticized for its lack of sensitivity and specificity [35]. In addition, results of previous studies by Leaf et al. [36] and Groussard et al. [30] have lead to the assumption that high intensity exercise results in MDA removal from plasma during recovery and that TBARS is, therefore, not a suitable marker of oxidative stress for this type of exercise [30,36].

TABLE III. Time course of blood reduced glutathione (GSH), oxidized glutathione (GSSG), ratio GSSG/GSH × 10^3 and plasma thiobarbituric acid reactive substances (TBARS) at rest, immediately after each of a series of 4 Wingate tests and following 24 h of recovery.

	GSH (μM)	GSSG (μM)	GSSG/GSH × 10^3	TBARS (μM)
Rest	579 ± 21	21.5 ± 3.5	40.9 ± 2.9	0.102 ± 0.016
1st WAnT	456 ± 10*	25.0 ± 2.3	58.4 ± 3.7*	0.099 ± 0.010
2nd WAnT	484 ± 37*	24.1 ± 1.9	60.1 ± 2.6*	0.096 ± 0.009
3rd WAnT	490 ± 18*	24.4 ± 1.7	55.4 ± 1.4*	0.103 ± 0.023
4th WAnT	578 ± 13	26.5 ± 2.7	45.5 ± 2.5	0.087 ± 0.022
24 h	609 ± 31	22.7 ± 2.3	39.3 ± 2.0	0.087 ± 0.010

Results are expressed as means ± SEM. *Significant changes compared to resting values ($p < 0.05$). Number of subjects: $n = 8$.

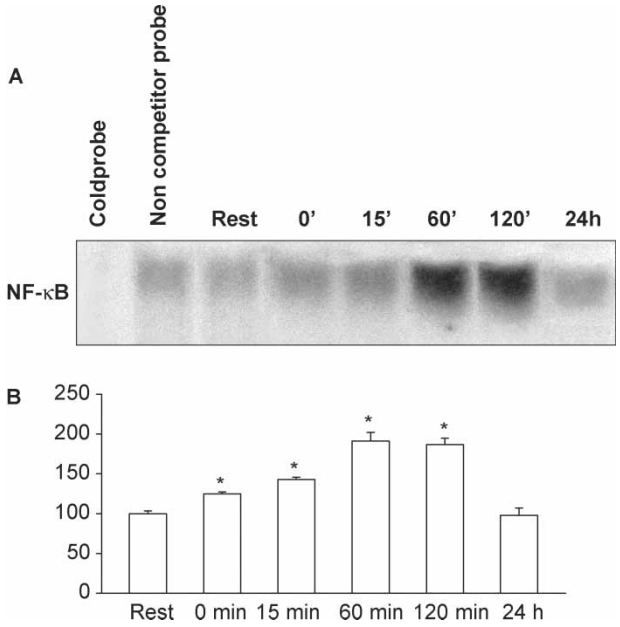

Figure 1. Nuclear factor κB activation in PBMC at rest, immediately after a single Wingate test and following 15, 60, 120 min and 24 h of recovery. A—shows representative EMSA; B—presents results expressed as percentage of resting values (means ± SEM). *Significant changes compared to resting values ($p < 0.05$). Number of subjects: $n = 8$.

Researchers have routinely studied glutathione status as a marker of oxidative stress within biological systems, as this seems to be one of the most reliable indices of exercise-induced oxidant production [37]. Following interaction of ROS with reduced glutathione, oxidized

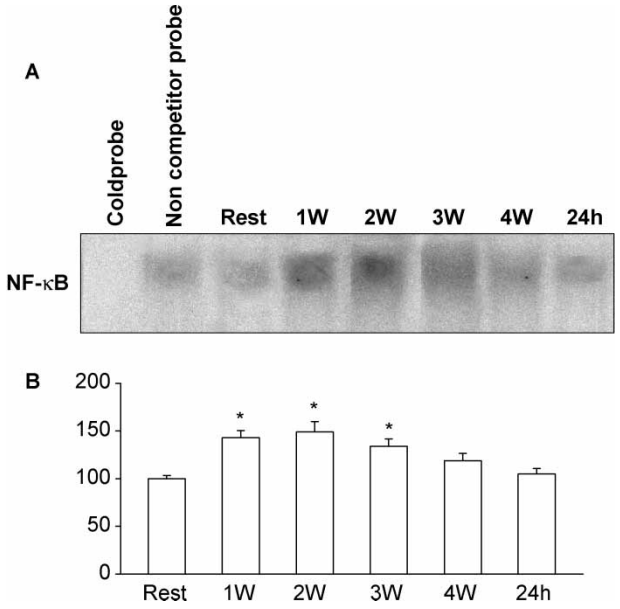

Figure 2. Nuclear factor κB activation in PBMC at rest, immediately after each of a series of 4 Wingate tests and following 24 h of recovery. A—shows representative EMSA. B—presents results expressed as percentage of resting values (means ± SEM). *Significant changes compared to resting values ($p < 0.05$). Number of subjects: $n = 8$.

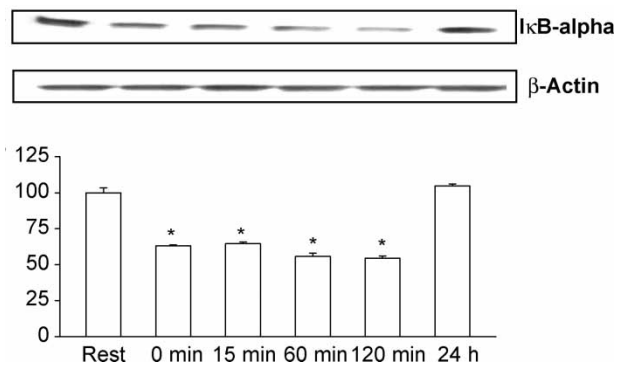

Figure 3. Western blot analysis of IκB-alpha in PBMC at rest, immediately after a single Wingate test and following 15, 60, 120 min and 24 h of recovery. A—shows representative western blot photographs. B—presents results expressed as percentage of resting values (means ± SEM). *Significant changes compared to resting values ($p < 0.05$). Number of subjects: $n = 8$.

glutathione disulfide is produced, and increased GSSG/GSH ratio is a characteristic biological response to oxidative stress. Inal and colleagues [38] noted a decrease in blood GSH following a 100-m swim sprint, leading them to suggest an increased oxidative stress imposed on the glutathione system. Most recently, Groussard et al. [30] found a decrease in erythrocyte glutathione after a short-term supramaximal anaerobic exercise. Our data confirm both results together with a significant increase in the GSSG/GSH ratio, a fact that has been previously reported only in individuals performing aerobic submaximal exercise [39].

Marzatico et al. [4] studied sprint athletes following the performance of six sprints and noted elevated plasma MDA at 6–48 h post exercise, and plasma conjugated dienes at 6 h post exercise. Similarly, Thompson et al. [40] observed trained athletes after a 90 min shuttle run of intermittent walking, jogging and sprinting, and reported increased levels of plasma MDA. In contrast to these results, we found no significant change of plasma TBARS induced by a set

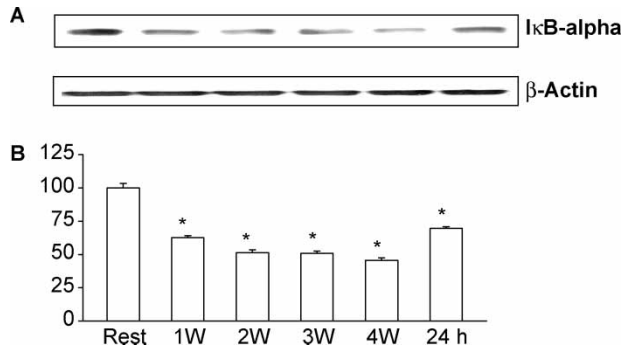

Figure 4. Western blot analysis of IκB-alpha in PBMC at rest, immediately after each of a series of 4 Wingate tests and following 24 h of recovery. A—shows representative western blot photographs. B—presents results expressed as percentage of resting values (means ± SEM). *Significant changes compared to resting values ($p < 0.05$). Number of subjects: $n = 8$.

of four WAnTs separated by 60 min rest intervals, and values even tended to decrease after the fourth test and 24 h later. In addition to factors previously mentioned, this could be explained by an exercise-induced adaptation process that upregulates anti-oxidant defense mechanisms and appears to function both for aerobic and anaerobic exercise [6]. The observed maximal decreases of GSH and increases of the GSSG/GSH ratio after the first and second WAnT, indicating no evidence of persistent or cumulative exercise effects, and the lack of significant changes both in the peak power and mean power along the series of tests would be in line with this hypothesis.

Specific to DNA oxidation, ROS associated damage may involve both strands breaks as well as single base modifications, potentially leading to mutagenesis [41]. Although several studies have tested the effects of aerobic exercise on oxidative damage to DNA, it should be noted that only two studies have focused on DNA oxidation in response to anaerobic exercise [42,43]. In the one involving sprint exercise [43] the number of micronuclei in 3000 binucleated blood lymphocytes was assessed as a marker of DNA damage and was noted to be increased comparing to resting levels at both the 24 and 48 h post exercise time points. The fact that in our study leukocyte 8-OHdG levels were still increased 24 h after either a single or a series of WAnTs, confirms that anaerobic exercise can induce oxidative damage to DNA and increase the formation of methylated bases.

NF-κB is a redox-sensitive transcription factor which is activated by intracellular ROS [44]. This hypothesis is supported by direct addition of H_2O_2 to culture medium activates NF-κB in various cell lines [45] and by the inhibitory effect of antioxidants [46]. In fact, a substantial body of evidence links NF-κB activity to cellular oxidative status, although the mechanism by which NF-κB is activated by ROS is unknown. It is though, however, that oxidizing conditions in the cytoplasm favor translocation of NF-κB to the nucleus, but that reducing conditions are required within the nucleus for NF-κB DNA binding [47].

Data in the literature demonstrate that an intensive physical exercise gives rise to a considerable activation of the transcription factor NF-κB both in laboratory animals [48,49] and in humans [50–52]. Moreover, the exercise-induced activation of NF-κB has been reported to be accompanied by a decrease of the ratio between intracellular reduced and oxidized glutathione [51]. Electrophoretic mobility shift assay from nuclear extracts of peripheral blood mononuclear cells revealed in our study an activation of NF-κB which reached a maximum at 60 min post exercise. This pattern was similar to that of the GSSG/GSH ratio, suggesting that generation of ROS during short-time supramaximal exercise is associated to an activation of transcription factors that could trigger the expression of a wide variety of target genes. In fact, the redox-sensitive activation of NF-kB may be the overture to elevated expression of genes such as those encoding for manganese superoxide dismutase, which exerts an important antioxidant function [49], or for the inducible isoform of the nitric oxide synthase, which participates in the inflammatory responses [53].

The pattern of change in the series of WAnTs was also similar to those found for blood GSH concentration and GSSG/GSH ratio, with a maximal activation after the second test. This result further supports the non-cumulative nature of ROS-induced damage. The gradual decrease of markers of stress could be associated to an adaptation of antioxidant defenses and the disappearance of the stimuli that cause oxidative stress could give rise to a reduced activation of NF-κB.

NF-κB exits in a latent form in the cytoplasm of unstimulated cells, comprising a transcriptionally active dimmer bound to an inhibitor protein IκB [54]. This form of NF-κB is unable to bind to DNA. However, IκBα is rapidly degraded by the ubiquitin-proteasome pathway in response to various inducers that include reactive oxygen intermediates, leading to the release of free NF-κB which translocates to the nucleus where it binds to DNA [55]. Radak et al. [56] very recently investigated the combined effects of aging and regular physical exercise in rats and showed that the content of IκB was inversely related to NF-κB activation. Results in our study indicate that an anaerobic exercise bout such as the WAnT strongly induces IκB degradation and the subsequent activation of NF-κB.

In summary, we conclude that short-term supra-maximal anaerobic exercise induces oxidative stress, as evidenced by the damage to macromolecules and by changes in the glutathione status. These alterations are not accumulated when the test is repeated four times with rest intervals of 60 min between them. Moreover, our data indicate that high intensity anaerobic work gives rise to an activation of the transcription factor NF-κB accompanied by a degradation of IκB. Further studies are necessary to better identify the mechanisms involved in anaerobic exercise-induced oxidative stress and its relation to NF-κB activation.

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