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Original Research Communication

Physical Exercise-Induced Expression of Inducible Nitric Oxide Synthase and Heme Oxygenase-1 in Human Leukocytes: Effects of RRR-α-Tocopherol Supplementation

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ABSTRACT

This study evaluated the effects of RRR-α-tocopherol (500 IU/day, 8 days) on *in vivo* cytokine response and cytoplasmic expression of inducible nitric oxide synthase (iNOS) and the antioxidant stress protein heme oxygenase-1 (HO-1) in human leukocytes after exhaustive exercise. Thirteen men were investigated in a double-blind, placebo-controlled, cross-over study with a wash-out period of 28 days. The exercise procedure consisted of an incremental treadmill test followed by a continuous run until exhaustion at 110% of the individual anaerobic threshold (total duration 28.5 ± 0.8 min). HO-1 and iNOS protein were assessed in mono- (M), lympho-, and granulocytes (G) using flow cytometry. Plasma interleukin-6 (IL-6) and IL-8 were measured by ELISA. IL-6 rose significantly whereas IL-8 did not exhibit significant changes after exercise. Changes of IL-6 were not affected by RRR-α-tocopherol. Exercise induced an increase of iNOS protein primarily in M and G. A small, but significant, increase of HO-1 protein was measured in M and G. RRR-α-Tocopherol did not show any significant effects on cytoplasmic expression of iNOS and HO-1 at rest and after exercise. In conclusion, exhaustive exercise induces expression of iNOS and HO-1 in human leukocytes by a mechanism that is not sensitive to RRR-α-tocopherol supplementation. Antiox. Redox Signal. 2, 113–126.

INTRODUCTION

INFLAMMATORY RESPONSE to strenuous physical exercise includes an increased release of cytokines (Lewicki *et al.*, 1988; Northoff *et al.*, 1994), reactive oxygen species (ROS) (Thiebauld and Mannes, 1997), and an augmented expression of stress proteins (Fehrenbach and Niess, 1999). Previous investigations revealed a stimulating effect of vigorous physical exercise on expression of inducible nitric oxide synthase (iNOS) in human leukocytes (Niess *et al.*, 1999a). Furthermore, increased leukocyte expression of heat shock proteins (Fehrenbach *et al.*, 2000) and the antioxidant stress protein heme oxygenase-1 (HO-1) (Niess *et al.*, 1999b) have been observed in response to intensive endurance running. The regulatory pathways leading to an exercise-induced expression of iNOS and stress proteins in immunocompetent cells remain unclear.

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Up-regulation of iNOS and stress protein synthesis in leukocytes depends in part on activation of redox-sensitive transcription factors, such as nuclear factor-κB (NF-κB) (Goldring *et al.*, 1995; Kurata *et al.*, 1996; Grimble, 1998; Powell *et al.*, 1998). Furthermore, cytokine-induced expression of iNOS (Okuda *et al.*, 1997) and HO-1 (Terry *et al.*, 1999) is mediated by protein kinase C (PKC)-dependent pathways. It has been shown that physical exercise leads to increased generation of ROS. In experiments with rats, Davies *et al.* (1982) and Jackson *et al.* (1985) found direct indications of augmented free-radical generation in muscle due to exercise. More recently, this has been documented by Asthon *et al.* (1998) in human serum after exhaustive exercise. Exercise-induced oxidative stress results in part from generation of ROS in immunocompetent cells. Because of the pivotal role of ROS in orchestrating redox-sensitive gene transcription, an influence of ROS on exercise-associated modulation of immune functions can be assumed. Antioxidants suppress *in vitro* stimulability for cytokine release (Payette *et al.*, 1990; Powell *et al.*, 1998), expression of iNOS (Hecker *et al.*, 1996), and HO-1 (Okinaga *et al.*, 1996). Investigation of these suppressive effects under *in vivo* conditions may help gain more insight into the pathways leading to exercise-induced changes of immune function. Therefore, we questioned whether short-term supplementation of the antioxidant RRR-α-tocopherol affects post-exercise expression of iNOS and antioxidant stress protein HO-1 in human leukocytes *in vivo*. Using an exhaustive exercise protocol on the treadmill, we evaluated cytoplasmic expression of iNOS and HO-1 by flow cytometry (Bachelet *et al.*, 1998) separately in lympho-, mono-, and granulocytes with and without short-term supplementation of RRR-α-tocopherol in a placebo-controlled, double-blind cross-over design.

MATERIALS AND METHODS

Subjects

Thirteen male subjects (26.5 ± 0.9 years, 180.7 ± 1.3 cm, 74.1 ± 3.0 kg) were enrolled in the study. They did not perform any kind of specific sports conditioning and devoted less than 3 h · week^{-1} to recreational and occupational physical activities. The subjects were nonsmokers with normal dietary habits and did not take any medication or vitamin supplements. Further recommendations were given to avoid dietary imbalance, and subjects were advised to record food intake during the study period, using a standardized protocol. The study was approved by our institute's Ethics Committee and conformed to the 1975 Declaration of Helsinki for research on human subjects. All subjects gave informed consent to participate in the investigation.

We used a placebo-controlled, double-blind, cross-over design with a wash-out period of 28 days between parts A and B. Supplementation started on day 0 and lasted 8 days. On day 8, the subjects performed two exhaustive treadmill runs. In part A, the subjects randomly received 500 IU RRR-α-tocopherol (Optovit® fortissimum 500 capsules prepared by HERMES Arzneimittel GmbH, Munich, Germany) per day or placebo. In part B, the intake of RRR-α-tocopherol or placebo was reversed for the same subjects.

Exercise procedure

The exercise protocol started with a graded exercise test (IET) on the treadmill (Saturn, HP COSMOS, Traunstein, Germany) that was performed until exhaustion. The initial speed was 6 km · h^{-1} with an increment of 2 km · h^{-1} every 3 min. The incline of the treadmill was kept constant at 1%. Capillary blood for lactate measurement was obtained from the earlobe after every stage. Running speed at the individual anaerobic threshold (IAT) was assessed according to Dickhuth *et al.* (1991). After a resting period of 15 min, the subjects performed a continuous run (CR) on the treadmill until exhaustion at a running velocity of 110% of the IAT. During part B, the subjects were instructed to reproduce the identical running times in IET and CR as performed in part A. Also, running speed during CR was the same in parts A and B. Total running time (IET + CR) was 28.5 ± 0.8 min (RRR-α-tocopherol) and 28.6 ± 0.9 min (placebo), respectively (Table 1).

TABLE 1. EXERCISE CHARACTERISTICS

Characteristics[a]	*RRR-α-Tocopherol*	*Placebo*
V_{max} IET (km · h^{-1})	16.0 ± 0.4	16.1 ± 0.4
LA_{max} IET (mmol l^{-1})	10.7 ± 0.9	10.2 ± 0.8
T_{max} CR (min)	10.5 ± 1.0	10.6 ± 1.0
LA_{max} CR (mmol l^{-1})	9.1 ± 0.6	9.2 ± 0.5
Total running time (min)	28.5 ± 0.8	28.6 ± 0.9

Values are means ± SEM

[a] V_{max} IET, maximal running velocity in the incremental exercise test; LA_{max} IET, maximal blood lactate in the incremental exercise test; T_{max} CR, running time in the continuous run; LA_{max} CR, maximal blood lactate after the continuous run.

Blood sampling

Using EDTA as an anticoagulant, venous blood samples were taken in a sitting position at rest, 0, 0.25, 1, 3, 24, and 48 hr after the end of CR. Whole-blood aliquots for flow cytometry and complete blood cell counts were kept at room temperature, and the procedures of analysis were started within 1 hr after collection. Whole blood (30 ml) was centrifuged (4°C, 1,000 × *g*, 15 min) immediately after sampling, and plasma aliquots were stored at −80°C until further analysis. Capillary blood for lactate measurements was obtained from the earlobe before, 0, and 5 min after the runs.

Analytical procedures

Complete blood cell counts including hemoglobin, hematocrit, and differential leukocyte counts were performed by an automated Coulter Counter (Cell Dyn 3500, Abbott, Germany). Lactate concentrations of hemolyzed capillary blood were measured electrochemically using a lactate analyzer (EBIO, Eppendorf, Germany). Plasma creatine kinase activity (CK) was determined in our clinical laboratory routine (Hitachi 717, Boehringer, Ingelheim, Germany). Furthermore, plasma samples were analyzed for concentrations of interleukin-6 (IL-6) and IL-8 by an enzyme-linked immunoassay method (Genzyme, Duoset, Cambridge, MA). The measurements of thiobarbituric reactive substances (TBARS) in plasma were performed as recently described (Jentzsch *et al.*, 1996) using the difference in absorption at 535 nm and 572 nm (Unicam UV 2 spectrometer, UK). Butylated hydroxytoluene and 2-thiobarbituric acid were of highest grade commercially available (Sigma, Deisenhofen, Germany). For assessment of cholesterol concentrations in plasma, we used a colorimetric test (Chol MPR 1, Boehringer Mannheim, Germany). Post-exercise values of plasma parameters were corrected for changes in plasma volume according to Dill and Costill (1974).

Determinations of α-tocopherol concentrations in plasma

Plasma concentrations of α-tocopherol were analyzed using a modification of the high-performance liquid chromatography (HPLC) method as previously described (Biesalski *et al.*, 1986). After precipitation of proteins with 200 μl ethanol, 200 μl of plasma was extracted twice with 500 μl of hexane. Hexane phases were separated, dried under continuous nitrogen stream, and redissolved in 200 μl of hexane. α-Tocopherol was separated on a 250 × 4 mm Merck LiChrosphere CN column (Merck, Darmstadt, Germany) using hexane as an eluent. Detection procedures were performed by UV detection (293 nm, UV/VIS Detector 432, Kontron, Germany). Plasma concentrations of α-tocopherol were adjusted for plasma cholesterol levels.

Assessment of cytoplasmic expression of iNOS and HO-1 in leukocytes

Cytoplasmic expression of iNOS and HO-1 in lympho-, mono-, and granulocytes was measured by flow cytometry as described before (Bachelet *et al.*, 1998; Niess *et al.*, 1999b). A total of 5 ml of EDTA-blood was layered above

5 ml of Lymphoflot (Biotest, Dreieich, Germany) and settled for 60 min by gravity without centrifugation. The overlay was removed and the cell concentration adjusted with phosphate-buffered saline (PBS) (Biochrom KG, Berlin, Germany) to 1×10^7 cells $\cdot$ ml^{-1}. A total of 100 μl of the suspension was used for the flow-cytometric analysis.

The leukocytes were analyzed by indirect immunofluorescence using the iNOS- and HO-1-specific antibodies (iNOS, rabbit polyclonal antiserum and OSA-100, rabbit polyclonal antiserum, StressGen Biotechnologies, Canada). A total of 1×10^6 cells were first fixed at room temperature in a solution containing formaldehyde, then permeabilized according to the manufacturer's instructions (Fix & Perm kit, An der Grub, Vienna, Austria), and, at the same time, incubated with the primary iNOS- or HO-1-specific antibody for 15 min. After washing the labeled cells twice and incubating in the presence of the second fluorescein-conjugated goat anti-rabbit $F(ab')_2$ IgG antibody (Dianova, Hamburg, Germany) for 20 min, the cells were analyzed using the flow cytometer EPICS-XL-MLC (Coulter, Krefeld, Germany).

Negative controls were performed using normal rabbit serum (DAKO, Glostrup, Denmark) and the second antibody (Dianova, Hamburg, Germany). The lympho-, mono-, and granulocyte populations were differentiated by size and granularity in the scattergram and gated. Fluorescence histograms (see Fig. 6) were area-corrected to 10,000 cells. The values were corrected for background fluorescence with the negative controls. Data are presented as per cent positive cells (%) and mean fluorescence intensity (mean fluorescence channel, mfc).

Statistics

Statistical analyses and graphics were computed by the statistical software package JMP (SAS Institute, Inc., Cary, NC) and KaleidaGraph (Synergy Software, Abelbeck) for Macintosh computer. Data are expressed as means ± standard error (SEM). Differences between post-exercise measurements (0, 3, 24, and 48 hr after exercise) and the pre-exercise values were evaluated using the *t*-test for paired values. After Bonferroni adjustment, statistical significance was set at $p \leq 0.0125$.

Intraindividual differences between measurements with RRR-α-tocopherol and placebo were calculated for every time of measurement and tested for significance by the *t*-test for paired values. A *p* value of <0.01 was regarded as significant.

RESULTS

RRR-α-Tocopherol supplementation induced a marked increase of α-tocopherol in plasma that was still apparent 48 hr after exercise (Fig. 1). Blood lactate values rose after IET and CR but did not show any differences between verum and placebo group (Table 1). Plasma levels of CK peaked 24 hr after exercise, but no effects of RRR-α-tocopherol on CK release from muscle were observed (Table 2). Table 2 depicts the changes in leukocyte counts. Neutrophils peaked 3 hr after the end of CR, whereas lymphocyte and monocyte counts exhibited an increase only directly after exercise. RRR-α-Tocopherol showed no effects on exercise-induced changes in leukocyte differential counts. Plasma TBARS concentrations showed only minor changes after exercise and were not influenced by RRR-α-tocopherol (Fig. 2). Plasma IL-6 exhibited a slight but significant increase

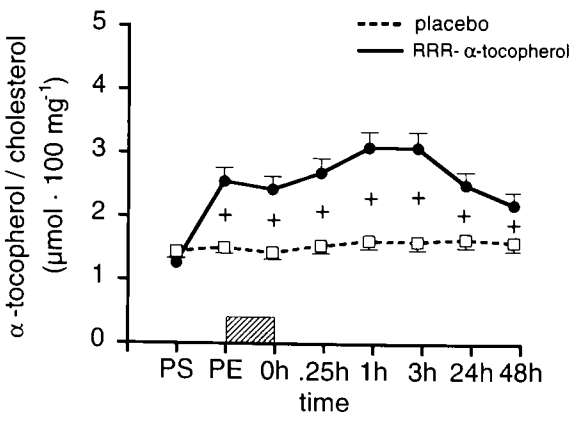

FIG. 1. Cholesterol-adjusted α-tocopherol concentrations in plasma presupplementation (PS), in pre-exercise (PE), and at several time points after exercise with RRR-α-tocopherol or placebo (mean ± SEM). (+) Significant differences between RRR-α-tocopherol and placebo ($p < 0.01$).

TABLE 2. PLASMA CREATINE KINASE AND LEUKOCYTE COUNTS

				Post-exercise			
		Presupplementation	*Pre-exercise*	*+ 0 hr*	*+3 hr*	*+ 24 hr*	*+ 48 hr*
Creatine kinase (U l^{-1})	RRR-α-tocopherol	53 ± 5	51 ± 4	56 ± 5	71 ± 8	108 ± 20[a]	76 ± 10
	Placebo	56 ± 7	47 ± 4	54 ± 4	93 ± 16	163 ± 41[a]	105 ± 22[a]
Neutrophils ($10^9 \cdot l^{-1}$)	RRR-α-tocopherol	3.3 ± 0.4	3.3 ± 0.3	5.4 ± 0.6[a]	8.5 ± 0.6[a]	3.5 ± 0.5	3.4 ± 0.3
	Placebo	3.2 ± 0.3	3.7 ± 0.4	5.2 ± 0.4[a]	9.2 ± 0.5[a]	3.7 ± 0.3	3.5 ± 0.3
Lymphocytes ($10^9 \cdot l^{-1}$)	RRR-α-tocopherol	1.8 ± 0.1	2.0 ± 0.1	3.8 ± 0.3[a]	2.0 ± 0.1	2.0 ± 0.2	2.0 ± 0.1
	Placebo	1.7 ± 0.1	2.0 ± 0.1	3.9 ± 0.3 [a]	1.9 ± 0.1	2.3 ± 0.1	2.1 ± 0.1
Monocytes ($10^9 \cdot l^{-1}$)	RRR-α-tocopherol	0.39 ± 0.03	0.37 ± 0.03	0.60 ± 0.05[a]	0.51 ± 0.05	0.38 ± 0.04	0.36 ± 0.03
	Placebo	0.35 ± 0.02	0.36 ± 0.03	0.56 ± 0.04[a]	0.51 ± 0.04[a]	0.45 ± 0.05	0.40 ± 0.06

Plasma creatine kinase and leukocyte counts (means ± SEM) presupplementation, pre-exercise, and at several time points after exercise.
[a]Denotes significant changes compared to pre-exercise values ($p < 0.0125$). No significant differences between RRR-α-tocopherol and placebo were found.

directly after CR, whereas changes of IL-8 were not induced by exercise. RRR-α-Tocopherol did not affect changes in IL-6 and IL-8 (Fig. 3).

Analysis by flow cytometry revealed a significant increase of iNOS-positive lympho- and granulocytes after exercise (Fig. 4), but a concomitant rise in fluorescence intensity was only observed in granulocytes (Table 3). Most of the monocytes had already been detected as positive for iNOS protein at rest (RRR-α-tocopherol: 88.9 ± 4.7%, placebo: 92.6 ± 2.5%) and did not exhibit significant changes after exercise. Monocyte fluorescence intensity for iNOS, as reflected by mfc values, showed an increase in both groups 24 hr post-exercise (Fig. 5).

A high percentage of HO-1-positive monocytes was found at rest (RRR-α-tocopherol: 92.7 ± 3.1%, placebo: 96.3 ± 1.1%) without significant differences compared to the other sampling times. Monocyte fluorescence intensity for HO-1 exhibited a delayed rise 48 hr after exercise (Fig. 5). Exhibiting lower values at rest, granulocytes of the RRR-α-tocopherol group showed a significant increase of HO-1-positive cells when measured 3 and 24 hr post-exercise (Fig. 7). Fluorescence intensity of granulocytes increased 3 and 48 hr post-exercise in both groups (Table 3).

Directly after exercise, 7 out of 13 subjects exhibited a lower percentage of HO-1-positive granulocytes and mean fluorescence intensity for HO-1 when reveiving RRR-α-tocopherol (Fig. 8). But significant effects of RRR-α-tocopherol on HO-1 as well as iNOS protein were not detectable (Figs. 4,5,7, Table 3).

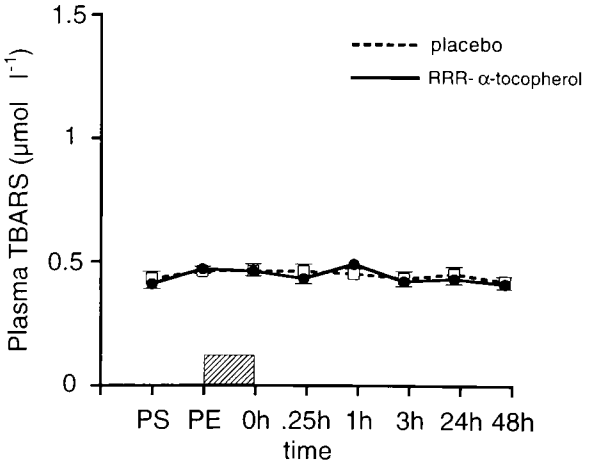

FIG. 2. Plasma concentrations of thiobarbituric reactive substances (TBARS) presupplementation (PS), pre-exercise (PE), and at several time points after exercise with RRR-α-tocopherol or placebo (mean ± SEM). No significant exercise-related changes or differences between RRR-α-tocopherol and placebo were found.

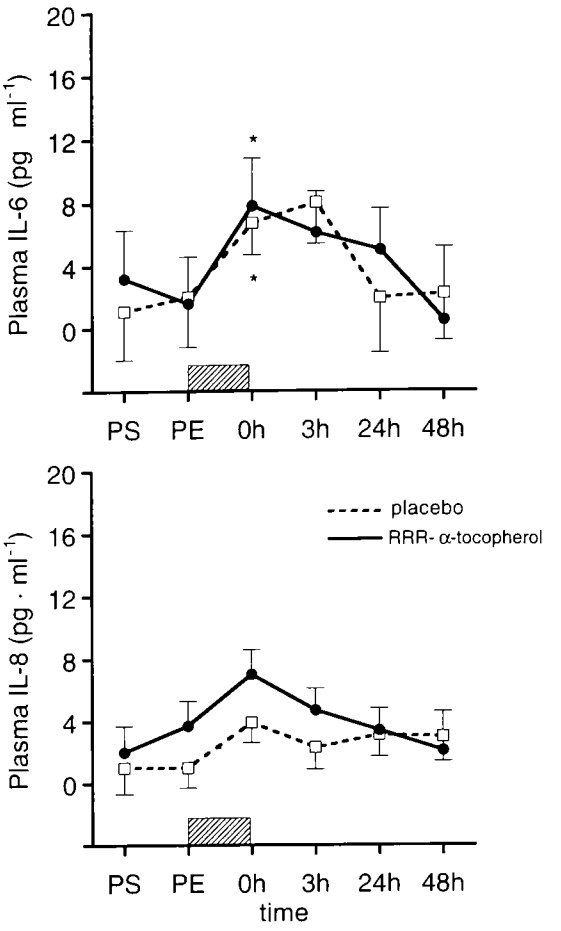

FIG. 3. Plasma concentrations of interleukin-6 (IL-6, upper panel) and IL-8 (lower panel) presupplementation (PS), pre-exercise (PE), and and at several time points after exercise with RRR-α-tocopherol or placebo (mean ± SEM). (*) Significant changes compared to pre-exercise values ($p < 0.0125$). No significant differences were found between RRR-α-tocopherol and placebo.

DISCUSSION

Effects of exercise

The present investigation revealed an increased *in vivo* expression of iNOS in leukocytes of untrained subjects in response to exhaustive treadmill running. This finding corresponds with our recent results (Niess *et al.*, 1999a), which showed an augmented ex-

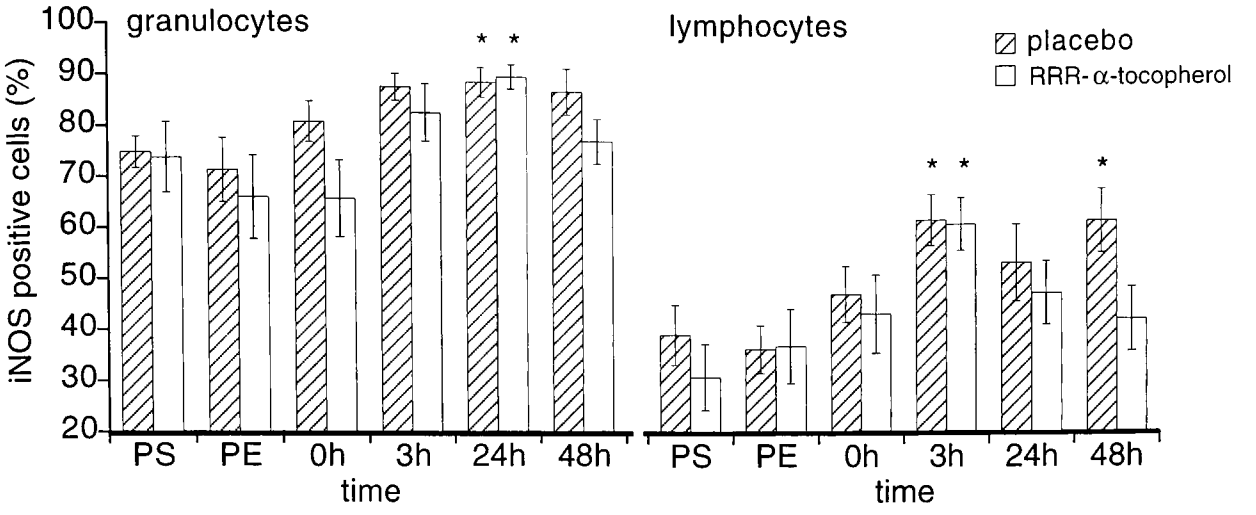

FIG. 4. Expression of iNOS in granulo-(left) and lymphocytes (right) measured by indirect immunofluorescence (flow cytometry) presupplementation (PS), pre-exercise (PE), and at several time points after exercise in the RRR-α-tocopherol or placebo group. Results (mean ± SEM) are presented in percent iNOS-positive cells. (*) Significant changes compared to pre-exercise values ($p < 0.0125$). No significant differences were found between RRR-α-tocopherol and placebo.

pression of iNOS mRNA and protein after vigorous exercise. Our flow cytometric results exhibited differences in the protein expression of iNOS between lympho-, mono-, and granulocytes both at rest and after exercise. A more pronounced baseline expression was observed in monocytes and granulocytes. Exhaustive treadmill running caused an increase of iNOS protein content in these cell populations that was reflected by an augmented mean fluorescence intensity. The percentage of iNOS-positive lymphocytes also increased after exercise, but no relevant rise was observed in the amount of iNOS protein expressed per cell. Within the human immune system, iNOS can be expressed in monocytes/macrophages, neutrophils, eosinophils, T lymphocytes, and mesangial cells in response to inflammatory stimuli (Barnes and Liew, 1993; Weinberg *et al.*, 1995; Lincoln *et al.*, 1997; Wallerath *et al.*, 1997). Cytokine release and thermal stress act as potent stimulators of iNOS in immunocompetent cells (Kroencke *et al.*, 1995; Lincoln *et al.*, 1997), and also seem to be of causal importance in the context of exercise. Evidence exists that induction of iNOS is modulated by the redox-sensitive NF-κB (Goldring *et al.*, 1995). This may explain the more pronounced expression of iNOS in cell populations such as granulo and monocytes, which are more susceptible to oxidative stress.

Although our investigations did not include a parallel assessment of leukocyte NO· release, the increased expression of iNOS in immunocompetent cells found after exhaustive exercise may contribute to a rise in endogenous NO· production. With regard to the various functions of NO·, the biological significance of an exercise-induced generation of NO· by immunocompetent cells must be discussed in conjunction with other features of the immune response to exercise. Changes such as depressed proliferation of lymphocytes (Eskola *et al.*, 1978; Nieman *et al.*, 1995), partial suppression of the oxidative burst in neutrophils (Gabriel and Kindermann, 1997; Kokot *et al.*, 1988), and DNA strand breaks in leukocytes (Hartmann and Niess, 2000) have been described to occur as effects of physical exercise and are assumed to be induced in part by oxidative stress (Niess *et al.*, 1999a).

In contrast to our earlier finding of a pronounced increase of HO-1 expression in lympho-, mono-, and granulocytes after a half-marathon run (Niess *et al.*, 1999b), the results of the present study revealed lower exercise-associated increases of this stress protein. Percentage of HO-1-positive cells rose 3 hr post-

TABLE 3. EXPRESSION OF iNOS AND HO-1 PROTEIN (FLUORESCENCE INTENSITY) IN LYMPHO- AND GRANULOCYTES

			Post-exercise				
		Presupplementation	*Pre-exercise*	*+ 0 hr*	*+ 3 hr*	*+ 24 hr*	*+ 48 hr*
iNOS in lymphocytes (mfc)	RRR-α-tocopherol	1.25 ± 0.03	1.30 ± 0.04	1.28 ± 0.03	1.40 ± 0.07	1.32 ± 0.03	1.28 ± 0.03
	Placebo	1.25 ± 0.02	1.26 ± 0.02	1.29 ± 0.03	1.40 ± 0.06	1.33 ± 0.04	1.38 ± 0.04
iNOS in granulocytes (mfc)	RRR-α-tocopherol	1.56 ± 0.08	1.37 ± 0.03	1.36 ± 0.04	1.55 ± 0.07[a]	1.53 ± 0.06	1.48 ± 0.06
	Placebo	1.49 ± 0.04	1.37 ± 0.03	1.43 ± 0.03	1.66 ± 0.11[a]	1.55 ± 0.07[a]	1.66 ± 0.08[a]
HO-1 in lymphocytes (mfc)	RRR-α-tocopherol	1.36 ± 0.03	1.34 ± 0.04	1.38 ± 0.06	1.44 ± 0.05	1.40 ± 0.05	1.45 ± 0.04
	Placebo	1.36 ± 0.03	1.39 ± 0.05	1.42 ± 0.04	1.50 ± 0.07	1.40 ± 0.05	1.46 ± 0.04
HO-1 in granulocytes (mfc)	RRR-α-tocopherol	1.72 ± 0.08	1.51 ± 0.07	1.58 ± 0.06	1.75 ± 0.07[a]	1.78 ± 0.09	1.91 ± 0.10[a]
	Placebo	1.68 ± 0.08	1.61 ± 0.08	1.77 ± 0.08	1.85 ± 0.09[a]	1.80 ± 0.14	1.97 ± 0.13[a]

Expression of iNOS and HO-1 protein in lympho- and granulocytes (means ± SEM) as measured by indirect immunofluorescence and expressed in fluorescence intensity (mfc = mean fluorescence channel).

[a]Denotes significant changes compared to pre-exercise values ($p < 0.0125$). No significant differences between RRR-α-tocopherol and placebo were found.

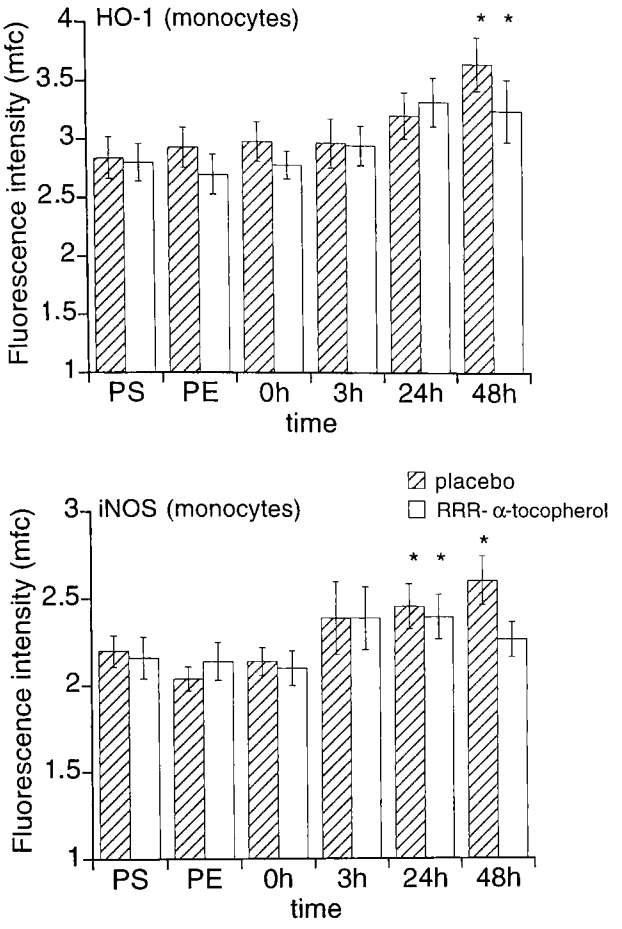

FIG. 5. Expression of HO-1 and iNOS in monocytes measured by indirect immunofluorescence (flow cytometry) presupplementation (PS), pre-exercise (PE), and at several time points after exercise in the RRR-α-tocopherol or placebo group. Results (mean ± SEM) reflect fluorescence intensity (mfc) of the cells. (*) Significant changes compared to pre-exercise values ($p < 0.0125$). No significant differences were found between RRR-α-tocopherol and placebo.

exercise in lymphocytes, but significant effects on fluorescence intensity reflecting an augmented amount of protein expressed per cell were only detectable in granulo- and monocytes. Compared to the half-marathon run, the present exhaustive exercise protocol induced a more pronounced lactacidosis, but the total duration of running was shorter. The shorter duration of exercise seems to result in lower increases in neutrophil counts, plasma CK, and the absence of post-exercise lymphopenia or increase of IL-8 as compared to the half-marathon run. This indicates that the present exercise protocol induced a lower level of overall stress. This suggestion was further confirmed when we found only a moderate increase of IL-6 and unchanged plasma concentrations of TBARS and ascorbate radical (data not shown, in preparation), which were assessed simultaneously. Therefore, one main finding of our investigation was that severe and prolonged physical stress is necessary to induce a marked up-regulation of HO-1 protein in human leukocytes in response to exercise. Recent studies have suggested protective roles of HO-1 in various inflammatory conditions (Willis *et al.*, 1996). In this context, HO-1 provides special protection against oxidative damage (Camhi *et al.*, 1995). Reactions of HO-1 lead to degradation of heme to bilirubin (Abraham *et al.*, 1988). Another way in which HO-1 protects from oxidative stress is through coinduction of ferritin, diminishing the intracellular pool of free iron (Vile and Tyrell, 1993). As shown recently, human HO-1 deficiency is paralleled by enhanced endothelial cell injury caused by oxidative stress (Yachie *et al.*, 1999). *In vitro* studies of Marini *et al.* (1996) revealed the important role of HO-1 in the maintenance of proliferative capacity as well as the survival of lymphocytes,

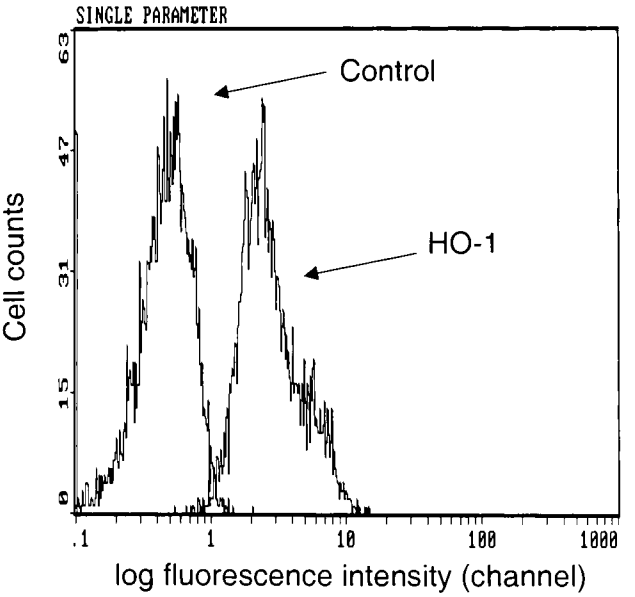

FIG. 6. Exemplary flow cytometric histogram (intracellular indirect immunofluorescence) of HO-1 in granulocytes. The histogram depicts the peaks of HO-1 protein (OSA-100 rabbit polyclonal HO-1 antibody + fluorescein-conjugated anti-rabbit antibody) and of the negative control (normal rabbit serum + fluorescein-conjugated anti-rabbit antibody).

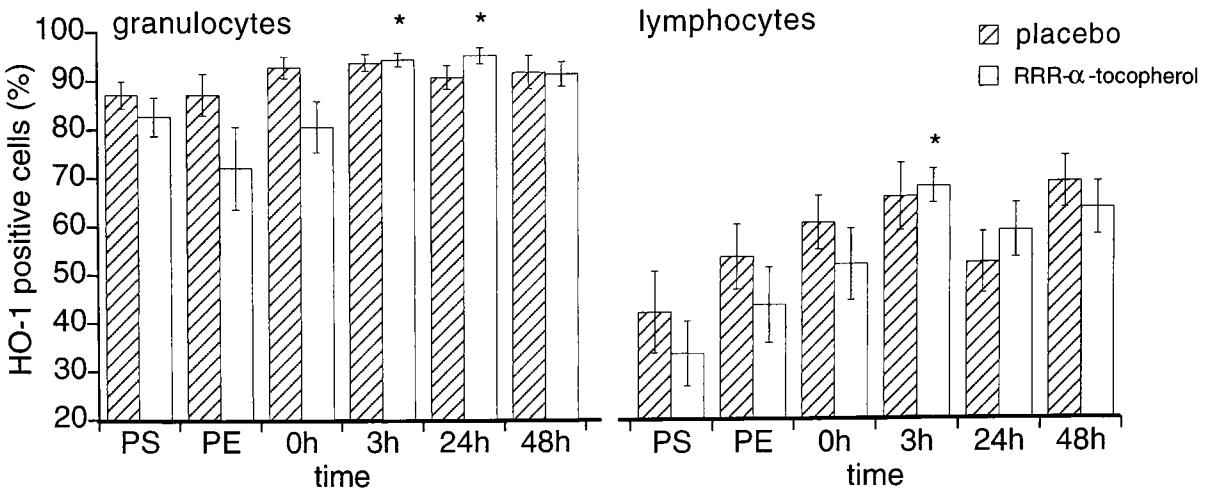

FIG. 7. Expression of HO-1 in granulo-(left) and lymphocytes (right) measured by indirect immunofluorescence (flow cytometry) presupplementation (PS), pre-exercise (PE), and at several time points after exercise in the RRR-α-tocopherol or placebo group. Results (mean ± SEM) are presented in percent HO-1-positive cells. (*) Significant changes compared to pre-exercise values ($p < 0.0125$). No significant differences were found between RRR-α-tocopherol and placebo.

which are stressed by ROS. This mechanism may also be of importance during and after strenuous exercise.

Effects of RRR-α-tocopherol supplementation

α-Tocopherol is the primary lipid-soluble antioxidant (Meydani, 1995) and influences cellular responses to ROS, including modulation of signal transduction pathways (Azzi *et al.*, 1992). In the present investigation, ingestion of 500 IU RRR-α-tocopherol daily over a period of 8 days was sufficient to induce a 2.3-fold increase in plasma lipid-standardized α-tocopherol. This coincides with reports that chronic administration of 440 mg of α-tocopherol resulted in steady-state plasma levels occurring after 4–5 days of supplementation (Dimitrov *et al.*, 1991).

Few studies are available regarding the impact of nutritional antioxidants on immune response to strenuous exercise. Under adminis-

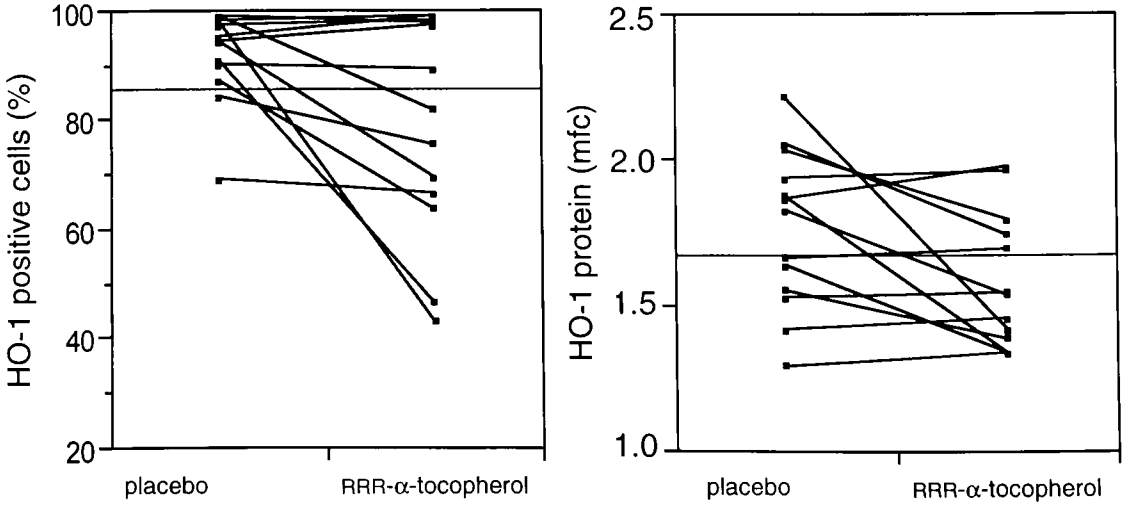

FIG. 8. Individual differences (RRR-α-tocopherol versus placebo) in percent HO-1-positive cells (left panel) and mean fluorescence intensity (mfc) for HO-1 protein (right panel) in granulocytes directly after exercise. No significant differences were found between RRR-α-tocopherol and placebo.

tration of α-tocopherol, Cannon *et al.* (1991) observed suppression of IL-6 release and endotoxin-stimulated generation of IL-1β in humans after downhill running. In another investigation, supplementation with *N*-acetylcysteine abolished neutrophil priming for oxidant release after exercise in rats (Huupponen *et al.*, 1995). In contrast, Nieman *et al.* (1997) found that ascorbate supplementation did not influence natural killer cell activity, lymphocyte proliferation, granulocyte/monocyte phagocytosis, or neutrophil oxidative burst after prolonged endurance exercise in marathon runners.

Several *in vitro* studies revealed a suppressive impact of antioxidants on expression of iNOS (Hattori *et al.*, 1995; Pahan *et al.*, 1998) and HO-1 (Ossola *et al.*, 1997; Bornman *et al.*, 1999) as well as on cytokine release (Payette *et al.*, 1990; Powell *et al.*, 1998). This seems to be due to inhibitory effects on the redox-sensitive transcription factor NF-κB (Blackwell and Christman, 1997). Inhibition of iNOS and stress protein synthesis by antioxidants may also be the result of an inactivation of cellular PKC, which has been shown to be sensitive to α-tocopherol (Kanno *et al.*, 1996; Azzi *et al.*, 1997). As shown recently, α-tocopherol exerts a suppressive effect on PKC activity in human monocytes and thereby decreases superoxide anion generation (Cachia *et al.*, 1998).

In the present investigation, slight rises of plasma IL-6 after exercise did not show any changes under administration of RRR-α-tocopherol. With respect to stress protein synthesis, half of the individuals exhibited a lower expression of HO-1 in granulocytes directly after exercise. But this finding did not reach significance. In neutrophils, antioxidant effects of vitamins seem to reduce autooxidative activity upon activation (Baehner *et al.*, 1977). This mechanism may lower generation of ROS and the pro-oxidant state of these cells when activated by exercise and affect redox-sensitive signaling transduction. However, clear effects of RRR-α-tocopherol on expression of HO-1 protein were not apparent in our investigation. Beyond the influence of ROS, a direct stimulating effect of cytokines on the expression of HO-1 has been shown for TNFα in liver cells (Cantoni *et al.*, 1991). TNFα release is known to be augmented due to strenuous exercise (Northoff *et al.*, 1994), which may affect HO-1 synthesis. Whether or not hyperthermia can enhance human HO-1 expression via a direct pathway is still a controversial discussion (Okinaga *et al.*, 1996; Bornman *et al.*, 1999).

Complex mechanisms also regulate iNOS (Lincoln *et al.*, 1997), and it has been shown that different kinds of antioxidants differentially affect iNOS expression at both the transcriptional and the post-transcriptional level (Hecker *et al.*, 1996). Additional influence of various cytokines via a PKC-dependent pathway, thermal stress (Kroencke *et al.*, 1995), and low concentrations of NO· itself (Sheffler *et al.*, 1995) are known to exert stimulating effects on the expression of iNOS. But similar to HO-1, exercise-induced expression of iNOS protein was not suppressed by supplementation with RRR-α-tocopherol. Therefore, it is interesting to note that in our experimental arrangement exercise-induced *in vivo* expression of HO-1 and iNOS protein in human leukocytes seems to be mediated by mechansims that are not sensitive to RRR-α-tocopherol supplementation. From a methodological viewpoint, our results let us speculate if a more vigorous exercise protocol, such as prolonged intensive endurance running with concomitant more pronounced induction of cytokine release and expression of iNOS and HO-1, would be better suited to work out influences of antioxidants on immune response in the human system.

In conclusion, exhaustive running exercise of approximately 30 min duration performed by untrained subjects is capable of inducing *in vivo* expression of iNOS protein in leukocytes, which seems to be more pronounced in mono- and granulocytes. An increased expression of iNOS in immunocompetent cells may contribute to an exercise-induced rise of endogenous NO· production and reflects an inflammatory response to exercise. This type of exercise also enhances expression of leukocyte HO-1, although compared to prolonged endurance running, the effects observed are rather marginal. Our study failed to detect clear effects of α-tocopherol on leukocyte iNOS and HO-1. This finding suggests that post-exercise expression of these proteins in human leukocytes is mediated by mecha-

nisms that are not sensitive to α-tocopherol supplementation.

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ABBREVIATIONS

CK, creatine kinase; CR, continuous run; EDTA, ethylenediamine tetra-acetate; ELISA, enzyme-linked immunosorbent assay; HO-1, heme oxygenase-1; HSP, heat shock protein; HPLC, high-performance liquid chromatography; IAT, individual anaerobic threshold; IET, incremental exercise test; IL-6, interleukin-6; IL-8, interleukin-8; INOS, inducible NO-synthase; LAmax CR, maximal blood lactate after the continuous run; LAmax IET, maximal blood lactate after the incremental exercise test; mfc, mean fluorescence channel; NF-κB, nuclear factor-κB; NO, nitric oxide; PBS, phosphate-buffered saline; PKC, protein kinase C; ROS, reactive oxygen species; TBARS, thiobarbituric-reactive substances; Tmax CR, running time in the continuous run; TNFα, tumor necrosis factor-α; VmaxIET, maximal running velocity in the incremental exercise test.

REFERENCES

ABRAHAM, N.G., LIN, H.-C., and SCHWARTZMAN, M.L. (1988). The physiological significance of heme oxygenase. Int. J. Biochem. **20,** 543–558.

ASHTON, T., ROWLANDS, C.C., JONES, E., YOUNG, I.S., JACKSON, S.K., DAVIES, B., and PETERS, J.R. (1998). Electron spin resonance spectrometric detection of oxygen-centred radicals in human serum following exhaustive exercise. Eur. J. Appl. Physiol. **77,** 498–502.

AZZI, A., BOSCOBONIC, D., and HENSEY, C. (1992). The protein kinase family. Eur. J. Biochem. **208,** 547–557.

AZZI, A., BOSCOBOINIK, D., CLEMENT, S., MARILLEY, D., OZER, N.K., RICCIARELLI, R., and TASINATO, A. (1997). α-Tocopherol as a modulator of smoth muscle cell proliferation. Prostaglandins Leukot. Essent. Fatty Acids **57,** 507–514.

BACHELET, M., MARIETHOZ, E., BANZET, N., SOUIL, E., PINOT, F., POLLA, C.Z., DURAND, P., BOUCHAERT, P., and POLLA, B.S. (1998). Flow cytometry is a rapid and reliable method for evaluating heat shock protein 70 expression in human monocytes. Cell Stress Chaperones **3,** 168–176.

BAEHNER, R.L., BOXER, L.A., ALLEN, J.M., and DAVIS, J. (1977). Autooxidation as a basis for altered function by polymorphonuclear leucocytes. Blood **50,** 327–335.

BARNES, P.J., and LIEW, F.Y. (1993). Nitric oxide and asthmatic inflammation. Immunol. Today **16,** 128–130.

BIESALSKI, H.K., GREIFF, H., BRODDA, K., HAFNER, G., and BAESSLER, K.H. (1986). Rapid determination of vitamin A (retinol) and vitamin E (alpha-tocopherol) in human serum by isocratic adsorptions HPLC. Int. J. Vitam. Nutr. Res. **56,** 319–327.

BLACKWELL, T.S., and CHRISTMAN, J.W. (1997). The role of nuclear factor-kappa B in cytokine gene regulation. Am. J. Respir. Cell Mol. Biol. **17,** 3–9.

BORNMAN, L., BALADI, S., RICHARD, M.J., TYRELL, R.M., and POLLA, B.S. (1999). Differential regulation of stress proteins and ferritin in human monocytes. J. Cell Physiol. **178,** 1–8.

CACHIA, O., EL BENNA, J., PEDRUZZI, E., DESCOMPS, B., GOURGEROT-POCIDALO, M.-A., and LEGER, C.-L. (1998). Alpha-tocopherol inhibits the respiratory burst un human monocytes. J. Biol. Chem. **273,** 32801–32805.

CAMHI, S.L., LEE, P., and CHOI, A.M.K. (1995). The oxidative stress response. New Horizons **3,** 170–182.

CANNON, J.G., MEYDANI, S.N., FIELDING, R.A., FIATARONE, M.A., MEYDANI, M., FARHANGMEHR, ORENCOLE, S.F., BLUMBERG, J.B., and EVANS, W.J. (1991). Acute phase response in exercise: association between vitamin E, cytokines, and muscle proteolysis. Am. J. Physiol. **260,** R1235–R1240.

CANTONI, L., ROSSI, C., RIZZARDINI, M., GADINA, M., and GHEZZI, P. (1991). Interleukin-1 and tumor necrosis factor induce hepatic haem oxygenase. Feedback regulation by glucocorticoids. Biochem. J. **279,** 891–894.

DAVIES, K.J.A., QUINTANILHA, A.T., BROOKS, G.A., and PACKER, L. (1982). Free radicals and tissue damage produced by exercise. Biochem. Biophys. Res. Commun. **107,** 1198–1205.

DICKHUTH, H.H., HUONKER, M., MÜNZEL, T., DREXLER, H., BERG, A., and KEUL, J. (1991). Individual anaerobic threshold for evaluation of competitive athletes and patients with left ventricular dysfunction. In: *Advances in Ergometry.* N. Bachl, T. Graham, and H. Löllgen, eds. (Springer Verlag, Berlin, Heidelberg, New York) pp. 173–179.

DILL, D.B., and COSTILL, D.L. (1974). Calculation of percentage changes in volumes of blood plasma and red cells in dehydration. J. Appl. Physiol. **37,** 247–248.

DIMITROV, N.V., MEYER, C., GILLILAND, D., RUPPENTHAL, M., CHENOWETH, W., and MALONE, W. (1991). Plasma tocopherol concentrations in response to supplemental vitamin E. Am. J. Clin. Nutr. **53,** 723–729.

ESKOLA, J., RUUSKANEN, O., SOPPI, E., VILJANEN, M.K., JÄRVINEN, M., TOIVONEN, H., and KOU-

VALAINEN, K. (1978). Effect of sport stress on lymphocyte transformation and antibody formation. Clin. Exp. Immunol. **31,** 339–345.

FEHRENBACH, E., and NIESS, A.M. (1999). The role of heat shock proteins in the exercise response. Exerc. Immunol. Rev. **5,** 57–77.

FEHRENBACH, E., PASSEK, F., NIESS, A.M., POHLA, H., WEINSTOCK, C., DICKHUTH, H.-H., and NORTHOFF, H. (2000). HSP expression in human leucocytes is modulated by endurance exercise. Med. Sci. Sports Exerc. **32,** (in press).

GABRIEL, H., and KINDERMANN, W. (1997). The acute immune response to exercise: What does it mean? Int. J. Sports Med. **18,** S28–S45.

GOLDRING, C.E.P., NARAYANAN, R., LAGADEC, P., and JEANNIN, J.-F. (1995). Transcriptional inhibition of the inducible nitric oxide synthase gene by competitive binding of NF-kB/rel proteins. Biochem. Biophys. Res. Commun. **209,** 73–79.

GRIMBLE, R.F. (1998). Nutritional modulation of cytokine biology. Nutrition **14,** 634–640.

HARTMANN, A., and NIESS, A.M. (2000). DNA damage in exercise. In: *Handbook of Oxidants and Antioxidants in Exercise.* C. K. Sen, L. Packer, and O. Hanninen, eds. (Elsevier B.V., Amsterdam). Part III, Chapter 9, pp. 195–217.

HATTORI, S., HATTORI, Y., BANBA, N., KASAI, K., and SHIMODA, S. (1995). Pentamethyl-hydroxychromane, vitamin E derivate, inhibits induction of nitric oxide synthase by bacterial lipopolysaccharide. Biochem. Mol. Biol. Int. **35,** 177–183.

HECKER, M., PREISS, C., KLEMM, P., and BUSSE, R. (1996). Inhibition by antioxidants of nitric oxide synthase expression in murine macrophages: role of nuclear factor kappa B and interferon regulatory factor 1. Br. J. Pharmacol. **118,** 2178–2184.

HECKER, M., PREISS, C., SCHINIKERTH, V.B., and BUSSE, R. (1996). Antioxidants differentially affect nuclear factor kappa B-mediated nitric oxide synthase expression in vascular smooth muscle cells. FEBS Lett. **380,** 224–228.

HUUPPONEN, M.R.H., MAKINEN, L.H., SEN, C.K., RANKINEN, T., VAISANEN, S., and RAURAMAO, R. (1995). The effect of N-acetylcysteine on exercise induced priming of human neutrophils. Int. J. Sports Med. **16,** 399–403.

JACKSON, M.J., EDWARDS, R.H.T., and SYMONS, M.C.R. (1985). Electron spin resonance of intact mammalian skeletal muscle. Biochim. Biophys. Acta **847,** 185–190.

JENTZSCH, A.M., BACHMANN, H., FÜRST, P., and BIESALSKI, H.K. (1996). Improved analysis of malondialdehyde in human body fluids. Free Rad. Biol. Med. **20,** 251–256.

KANNO, T., UTSUMI, T., TAKEHARA, Y., IDE, A., AKIYAMA, J., YOSHIOKA, T., HORTON, A.A., and UTSUMI, K. (1996). Inhibition of neutrophil-superoxide generation by α-tocopherol and coenzyme Q. Free Radic. Res. **24,** 281–289.

KOKOT, K., SCHAEFER, R.M., TESCHNER, M., GILGE, U., PLASS, R., and HEIDLAND, A. (1988). Activation of leucocytes during prolonged exercise. Adv. Exp. Med. Biol. **240,** 57–63.

KROENCKE, K.-D., FEHSEL, K., and KOLB-BACHOFEN, V. (1995). Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. Biol. Chem. Hoppe-Seyler **376,** 327–343.

KURATA, S., MATSUMO, M., and YAMASHITA, U. (1996). Concomitant transcriptional activation of nitric oxide synthase and heme oxygtenase genes during nitric oxide-mediated macrophage cytostasis. J. Biochem. Tokyo **120,** 49–52.

LEWICKI, R., TCHORZEWSKI, H., MAJEWSKI, E., NOWAK, Z., and BAJ, Z. (1988). Effect of maximal physical exercise on T-lymphocyte sub-populations and interleukin 1 (IL-1) and interleukin 2 (IL-2) production in vitro. Int. J. Sports Med. **9,** 114–117.

LINCOLN, J., HOYLE, C.H.V., and BURNSTOCK, G. (1997). *Nitric Oxide in Health and Disease.* (Cambridge University Press, Cambridge).

MARINI, M., FRABETTI, F., MUSIANI, D., and FRANCESCHI, C. (1996). Oxygen radicals induce stress proteins and tolerance to oxidative stress in human lymphocytes. Int. J. Radiat. Biol. **70,** 337–350.

MEYDANI, M. (1995). Vitamin E. Lancet **345,** 170–175.

NIEMAN, D.C., SIMANDLE, S., HENSON, D.A., WARREN, B.J., SUTTLES, J., DAVIS, J.M., BUCKLEY, K.S., AHLE, J.C., BUTTERWORTH, D.E., FAGOAGA, O.R., and NEHLSEN-CANNARELLA, S.L. (1995). Lymphocyte proliferative response to 2.5 hours of running. Int. J. Sports Med. **16,** 404–408.

NIEMAN, D.C., NEHLSEN-CANNARELLA, S.L., HENSON, D.A., BUTTERWORTH, D.E., FAGOAGA, O.R., WARREN, B.J., and RAINWATER, M.K. (1997). Carbohydrate, ascorbic acid, and the immune response to running. Int. J. Sports Med. **18,** S112.

NIESS, A.M., DICKHUTH, H.-H., NORTHOFF, H., and FEHRENBACH, E. (1999a). Free radicals and oxidative stress in exercise—immunological aspects. Exerc. Immunol. Rev. **5,** 22–56.

NIESS, A.M., PASSEK, F., LORENZ, I., SCHNEIDER, E.M., DICKHUTH, H.-H., NORTHOFF, H., and FEHRENBACH, E. (1999b). Expression of the antioxidant stress protein heme oxygenase-1 (HO-1) in human leukocytes—Acute and adaptional responses to endurance exercise. Free Rad. Biol. Med. **26,** 184–192.

NORTHOFF, H., WEINSTOCK, C., and BERG, A. (1994). The cytokine response to strenous exercise. Int. J. Sports Med. **15,** S167–S171.

OKINAGA, S., TAKAHASHI, K., TAKEDA, K., YOSHIZAWA, M., FUJITA, H., SASAKI, H., and SHIBAHARA, S. (1996). Regulation of human heme oxygenase-1 gene expression under thermal stress. Blood **87,** 5074–5084.

OKUDA, S., KANDA, F., KAWAHARA, Y., and CHIHARA, K. (1997). Regulation of inducible nitric oxide synthase expression in L6 rat skeletal muscle cells. Am. J. Physiol. **272,** C35–C40.

OSSOLA, J.O., GROPPA, M.D., and TOMARO, M.L. (1997). Relationship between oxidative stress and heme

oxygenase induction by copper sulfate. Arch. Biochem. Biophys. **337,** 332–337.

PAHAN, K., SHEIKH, F.G., NAMBOODIRI, A.M., and SINGH, I. (1998). *N*-Acetyl cysteine inhibits induction of NO production by endotoxin or cytokine stimulated rat macrophages, C6 glia cells and astrocytes. Free Rad. Biol. Med. **24,** 39–48.

PAYETTE, H., ROLA-PLESZCZYNSKI, M., and GHADIRIAN, P. (1990). Nutrition factors in relation to cellular regulatory immune variables in a free-living elderly population. Am. J. Clin. Nutr. **52,** 927–932.

POWELL, J.R., BARRAT, M.E.J., HILLEBRAND, A., HURST, T.L., and NEBE VON CARON, G. (1998). The effect of dietary antioxidants upon human blood monocyte immune functions in vitro. Pathophysiol. **5** (Suppl. No. 5), 72.

SHEFFLER, L.A., WINK, D.A., MELILLO, G., and COX, G.W. (1995). Exogenous nitric oxide regulates IFN-gamma plus lipopolysaccharide-induced nitric oxide synthase expression in mouse macrophages. J. Immunol. **155,** 886–894.

TERRY, C.M., CLIKEMAN, J.A., HOIDAL, J.R., and CALLAHAN, K.S. (1999). TNF-α and IL-1α induce heme oxygenase -1 via protein kinase C, Ca^{2+}, and phospholipase A_2 in endothelial cells. Am. J. Physiol. **276,** H1493–H1501.

THIEBAULD, C.M., and MANNES, R. (1997). Free radicals and inflammatory reaction. Int. J. Sports Med. **18,** S115.

VILE, G.F., and TYRELL, R.M. (1993). Oxidative stress resulting from ultraviolet A irridation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. J. Biol. Chem. **268,** 14678–14681.

WALLERATH, T., GATH, I., AULITZKY, W.E., POLLOCK, J.S., KLEINERT, H., and FORSTERMANN, U. (1997). Identification of the NO synthase isoforms expressed in human neutrophil granulocytes, megakaryocytes and plateletes. Thromb. Haemost. **77,** 163–167.

WEINBERG, J.B., MISOKONIS, M.A., SHAMI, P.J., MASON, S.N., SAULS, D.L., DITTMAN, W.A., WOOD, E.R., SMITH, G.K., MCDONALD, B., BACHUS, K.E., HANEY, A.F., and GRANGER, D.L. (1995). Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin and nitric oxide production by blood monocytes and peritoneal macrophages. Blood **86,** 1184–1195.

WILLIS, D., MOORE, A.R., FREDERICK, R., and WILLOUGHBY, D.A. (1996). Heme oxygenase: a novel target for the modulation of the inflammatory response. Nature Med. **2,** 87–90.

YACHIE, A., NIIDA, Y., WADA, T., IGARASHI, N., KANEDA, H., TOMA, T., OHTA, K., KASAHARA, Y., and KOIZUMI, S. (1999). Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 defiency. J. Clin. Invest. **103,** 129–135.

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2. E IORI, E PAGNIN, A GALLO, L CALO, E MURPHY, F OSTUNI, G FADINI, A AVOGARO. 2007. Heme oxygenase-1 is an important modulator in limiting glucose-induced apoptosis in human umbilical vein endothelial cells. *Life Sciences* . [CrossRef]
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5. M. Valeria Catani, Isabella Savini, Guglielmo Duranti, Daniela Caporossi, Roberta Ceci, Stefania Sabatini, Luciana Avigliano. 2004. Nuclear factor #B and activating protein 1 are involved in differentiation-related resistance to oxidative stress in skeletal muscle cells. *Free Radical Biology and Medicine* **37**:7, 1024-1036. [CrossRef]
6. M Urso. 2003. Oxidative stress, exercise, and antioxidant supplementation. *Toxicology* **189**:1-2, 41-54. [CrossRef]
7. Jennifer M Sacheck, Paul E Milbury, Joseph G Cannon, Ronenn Roubenoff, Jeffrey B Blumberg. 2003. Effect of vitamin E and eccentric exercise on selected biomarkers of oxidative stress in young and elderly men. *Free Radical Biology and Medicine* **34**:12, 1575-1588. [CrossRef]
8. Henning Bay Nielsen. 2003. Lymphocyte Responses to Maximal Exercise. *Sports Medicine* **33**:11, 853-867. [CrossRef]
9. J Sacheck. 2001. Role of vitamin E and oxidative stress in exercise. *Nutrition* **17**:10, 809-814. [CrossRef]
10. Chandan K. Sen. 2001. Antioxidants in Exercise Nutrition. *Sports Medicine* **31**:13, 891-908. [CrossRef]