Antioxidants attenuate the plasma cytokine response to exercise in humans

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Submitted 8 August 2002; accepted in final form 18 November 2002

Antioxidants attenuate the plasma cytokine response to exercise in humans. J Appl Physiol 94: 1025–1032, 2003. First published November 27, 2002; 10.1152/japplphysiol.00735.2002.—Exercise increases plasma TNF-α, IL-1β, and IL-6, yet the stimuli and sources of TNF-α and IL-1β remain largely unknown. We tested the role of oxidative stress and the potential contribution of monocytes in this cytokine (especially IL-1β) response in previously untrained individuals. Six healthy nonathletes performed two 45-min bicycle exercise sessions at 70% of VO₂ max before and after a combination of antioxidants (vitamins E, A, and C for 60 days; allopurinol for 15 days; and N-acetylcysteine for 3 days). Blood was drawn at baseline, end-exercise, and 30 and 120 min postexercise. Plasma cytokines were determined by ELISA and monocyte intracellular cytokine level by flow cytometry. Before antioxidants, TNF-α increased by 60%, IL-1β by threefold, and IL-6 by sixfold secondary to exercise (P < 0.05). After antioxidants, plasma IL-1β became undetectable, the TNF-α response to exercise was abolished, and the IL-6 response was significantly blunted (P < 0.05). Exercise did not increase the percentage of monocytes producing the cytokines or their mean fluorescence intensity. We conclude that in untrained humans oxidative stress is a major stimulus for exercise-induced cytokine production and that monocytes play no role in this process.

interleukins; flow cytometry; oxidative stress; ergometry; vitamins

THE HUMORAL AND CELLULAR CHANGES occurring after strenuous muscular work resemble in some aspects the acute-phase response to trauma and inflammation (31). Thus long-distance running (23, 27, 31, 44), treadmill running (30), cycle ergometry (37, 48), and even wrestling (26) increase the level of circulating tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6. The stimulus or stimuli for this exercise-induced cytokine production remains largely unknown. Exercise results in increased levels of reactive oxygen species (ROS), both in the blood and within the working muscles. The major sources of intracellular ROS generation during exercise are the mitochondrial electron transport chain, the cytosolic NADH oxidase, xanthine oxidase, and membrane-bound oxidases such as the NADPH oxidase (17, 35). Because ROS are general mediators of signal transduction pathways (10), able to induce cytokine production from various cell types (1, 13, 19), we hypothesized that ROS are stimuli for exercise-induced cytokine production. In fact, we have previously demonstrated (in a model of C2C12 myocytes transformed in culture into myotubes) that muscle cells can produce IL-6 in a ROS-dependent pathway (19). Because the contracting muscle is the major source of the exercise-induced IL-6 secretion (18, 29, 45), the ROS dependence of IL-6 production in our previous in vitro study strongly supports our present hypothesis that ROS are stimuli for cytokine production during exercise.

The source(s) of exercise-induced TNF-α and IL-1β are largely unknown. Blood monocytes comprise a heterogeneous composite population of cells (53), which are a major source of immunoinflammatory mediators. Monocytes had been initially suggested as sources of the exercise-induced plasma cytokines (12), which was later refuted on the basis of results obtained at the cytokine mRNA level (23, 50). Furthermore, recent studies, using the sensitive technique of intracellular detection of cytokines with flow cytometry, have excluded monocytes as sources of exercise-induced IL-6, IL-1α, and TNF-α production (43, 44).

These results (43, 44) were obtained in athletes, in whom monocytes are chronically activated (11), and release increased amounts of cytokines (such as IL-6) compared with sedentary controls (33). In fact, even only 12 wk of moderate endurance training results in increased in vitro IL-1β concentrations (3). However, results of similar whole blood culture systems suggest that monocytes from trained athletes have a decreased cytokine secretory capacity in response to a further stimulus [lipopolysaccharide (LPS) stimulation of the culture system] when compared with sedentary controls (33). Thus monocytes from athletes might be less responsive to an acute exercise stimulus than monocytes from untrained humans.
cytes derived from untrained individuals. In fact, even different training programs can differentially affect monocyte activation (4). Furthermore, the duration and intensity of exercise influence the subpopulations of monocytes being activated secondary to exercise (11). The duration of exercise in the studies conducted in athletes was quite prolonged (2 h or longer; Refs. 23, 29, 43, 44, 50). Thus we investigated whether monocytes in untrained healthy volunteers subjected to a relatively short (45 min) exercise bout respond differently to the stimulus of exercise from what is reported in trained athletes (43, 44).

Furthermore, in these previous conclusive studies in athletes in which cytokines were detected at the protein level by use of flow cytometry (43, 44), IL-1β, which is the secreted isoform of IL-1 (in contrast to IL-1α, which remains mostly intracellular (9)) was not assessed. We therefore investigated the potential contribution of monocytes to exercise-induced IL-1β elevation by using the same sensitive technique (flow cytometry), given that some previous reports at the mRNA (29) and protein (38) level suggest that exercise can occasionally increase IL-1β expression in monocytes.

This study was performed to test the role of ROS (as stimulus) and the potential contribution of monocytes to exercise-induced cytokine (especially IL-1β) production, in normal, healthy, untrained volunteers.

METHODS

Subjects

Six healthy male volunteers with a mean age of 33 ± 5 yr (range 28–44), weight of 79 ± 17 kg, and height of 177 ± 10.5 cm were studied. None of the subjects participated in regular exercise training or competitive sports activities or had febrile illness during the month before testing. Our institutional ethics committee approved the study protocol, and all participants gave informed consent. All subjects refrained from exercising or any other strenuous activity for 24 h before testing. Testing was always performed at the same time of the day (to avoid circadian rhythm variation effects in the antioxidant supplementation regimen presented below). After completing the antioxidant supplementation (2 mo duration), they were subjected to an exercise session of the same duration (45 min) and intensity (70% VO2 max) as the first one. Thus each subject in our study served as self-control to eliminate any biological variability in the response to antioxidant supplementation. This design was chosen instead of a randomized crossover design because of the nature of the antioxidants: vitamins with long-lasting effects after the completion of the supplementation.

Antioxidant Supplementation

Subjects received a combination of antioxidants, including 200 mg vitamin E, 50,000 IU vitamin A, and 1,000 mg vitamin C daily for 60 days before the second exercise session; allopurinol 600 mg/day for 15 days; and N-acetylcysteine 2 g/day for 3 days and 800 mg on the morning before the second exercise session.

Blood samples. Blood samples were collected into sterile syringes and were transferred to precooled sterile EDTA tubes or sodium heparin tubes for flow cytometric evaluation (used within 2 h from venipuncture). Samples in EDTA tubes were immediately spun in a refrigerated centrifuge to separate plasma from cells and thus avoid ex vivo cytokine secretion and were next placed in polystyrene tubes and stored at −70°C until assayed.

Determination of Cytokine Proteins in Plasma

Plasma levels of TNF-α, IL-1β, and IL-6 were measured with commercially available high-sensitivity ELISA kits (Quantikine, R&D Systems, Minneapolis, MN). All assays were performed in duplicate. The intra-assay coefficient of variation was <10% for the three cytokines tested. Samples from the same subject obtained before and after antioxidant supplementation were always analyzed in the same assay.

Measurement of Creatine Kinase

Creatine kinase was measured by the Biochemistry Department of Evangelismos Hospital by use of standard laboratory procedures (automated analysis, Hitachi System 737, Roche Diagnostics, Mannheim, Germany). Plasma concentrations of cytokines and creatine kinase were adjusted for changes in plasma volume during and after the exercise session.

Intracellular Flow Cytometric Detection of Cytokines in Monocytes

Flow cytometric detection of cytokines was performed as previously described (38, 43, 44).

Antibodies and reagents. Anti-TNF-α phycoerythrin (PE) (rat IgG1; clone MP6-XT22) and anti-IL-6 PE (rat IgG1; clone M2-6A3) along with their respective isotype-matched (IgG1 and IgG2a) control monoclonal antibodies (MAbs) were obtained from Becton-Dickinson. The anti-IL-1β PE (rat IgG1; clone AS10) and the isotype-matched IgG1 control MAb were from Becton-Dickinson. CD14 (monocyte-specific cell surface marker) directly conjugated with fluorescent isothiocyanate (FITC) was purchased from Becton-Dickinson. The cell permeabilization kit was obtained from Harlan Sera-Lab.
vive serum albumin (BSA), NaN3, LPS, brefeldin A (BFA), and paraformaldehyde were purchased from Sigma Chemical.

Cell stimulation and staining. Whole blood (1 ml) was stimulated with LPS (1 μg/ml) in the presence of BFA (10 μg/ml) to block the export of newly synthesized cytokines from the Golgi apparatus and was cultured at 37°C for 4 h in a humid 5% CO2 atmosphere. Another aliquot of whole blood was incubated with BFA without LPS stimulation to evaluate spontaneous cytokine production during isotime culture. Incubations were performed in sterile 15-ml polypropylene tubes (Sarstedt) to prevent adherence of cells.

Both unstimulated and in vitro LPS-stimulated cultured cells (50 μl) were incubated with saturating concentrations of anti-CD14-FITC surface stain for 20 min at room temperature in the dark. After incubation, cells were fixed with permeabilization solution A (SERA-LAB, 75 ml) for 15 min, washed with 2 ml of PBSA (containing 0.1% sodium azide and 0.1% BSA). Afterward, 75 μl of permeabilization reagent B (Sera-Lab) was added, as well as saturating concentrations of the appropriate anticytokine MAbs, and the mixture was incubated for 20 min. After a final wash with 2 ml of PBS-BSA-azide, the cells were resuspended in 500 μl of 1% paraformaldehyde and stored at 4°C in the dark until analyzed.

Flow cytometric acquisition and analysis. A flow cytometer (Epics Elite Esp., Coulter Electronics) calibrated for twocolor analysis was used. An electronic acquisition gate was set on CD14-positive cells according to FITC emission and 90° side light scattering. In this gate at least 2,000 CD14 monocyte events were acquired for analysis of cytokine staining. Results were expressed as the percentage of cytokine-producing monocytes, as well as the mean fluorescent intensity (MFI) in unstimulated and stimulated cultures. For negative control, intracellular isotype controls were used.

Statistical Analysis

Normal distribution of the data was tested by the Kolmogorov-Smirnov one sample test. Because data were nonnormally distributed, nonparametric tests were used. Plasma cytokine levels were analyzed along two different ways. First, changes over time were compared by using Friedman’s two-way (repeated-measures) ANOVA by ranks, followed by Wilcoxon’s matched-pairs (signed-ranks) tests for post hoc comparisons. A Bonferroni-type adjustment for multiple comparisons was carried out according to Hochberg and Benjamini (15). Second, both the changes of cytokine values postexercise over the baseline preexercise values and the areas under the curve of the change in plasma cytokine levels over time (30) were compared before and after antioxidant supplementation by using Wilcoxon matched-pairs tests. The percentages of cytokine-positive monocytes or MFI values at different time points within the same exercise session were compared by using Friedman’s ANOVA followed by Wilcoxon’s matched-pairs tests post hoc. Comparisons between the percentage of cytokine-positive monocytes or MFI values at isotime points before and after antioxidant supplementation were carried out by using Wilcoxon’s matched-pairs tests. Correlations were determined by using the nonparametric Spearman-R test. Values reported are means ± SE. A P value <0.05 was considered as statistically significant.

RESULTS

The VO2 max was on the average 3,070 ± 98 ml/min. Accordingly, the mean VO2 was 2,130 ± 86 ml/min during the first trial and 2,195 ± 98 ml/min in the trial after antioxidant supplementation. The heart rate was 182 ± 13 beats/min during the first session and 185 ± 12 beats/min after antioxidant supplementation.

Plasma Cytokine Levels

Before antioxidants. The three cytokines increased in all subjects after the exercise session (Fig. 1). Both IL-1β (Fig. 1B) and IL-6 (Fig. 1C) were increased at the time exercise was terminated and reached their peak value at 30 min postexercise. IL-1β increased by threefold and IL-6 by sixfold. TNF-α also increased, yet with a different time course, exhibiting the peak value immediately postexercise (Fig. 1A).

After antioxidants. After antioxidant supplementation, plasma IL-1β was below the detection limit of the ELISA that we used both before and after the exercise session that we used both before and after the exercise session (Fig. 1B). The TNF-α response to exercise was nearly abolished (Fig. 1A), and the IL-6 response was significantly blunted (Fig. 1C). The changes of the TNF-α and IL-6 values above the baseline (preexercise) values secondary to exercise were significantly lower after antioxidants (Fig. 2, A and C). The areas under the curve of the plasma cytokine response over time were significantly smaller from the corresponding ones before antioxidants (Fig. 2, B and D).

Plasma Creatine Kinase

No change was observed in the plasma creatine kinase level either in the postexercise recovery period (up to 120 min after exercise cessation) or the day after exercise. This response was not altered by antioxidants (data not shown).

Intracellular Cytokine Production by Monocytes

The number of monocytes did not change significantly secondary to exercise. Before antioxidants, monocyte counts were 236 ± 39 cells/dl before exercise, 272 ± 51 cells/dl at the end of exercise, 246 ± 42 cells/dl at 30 min postexercise, and 241 ± 55 cells/dl at 2 h postexercise (P = NS). A similar response in the number of monocytes was observed after antioxidants. Also, exercise did not increase the production of cytokines by monocytes. Neither the percentage of cytokine-positive monocytes nor their mean fluorescence intensity increased after exercise (data not shown). In fact, the only significant change was a decrease in the percentage and the mean fluorescence intensity of monocytes spontaneously producing TNF-α postexercise (Friedman’s ANOVA χ² = 10.31, P = 0.016 for the percentage of TNF-α producing monocytes and Friedman’s ANOVA χ² = 10.53, P = 0.014 for the mean fluorescence intensity).

Correlations

No correlation was observed between the plasma cytokine level and the percentage of cytokine-positive monocytes or the MFI at isotime points. Furthermore, no correlation was observed between the percentage of cytokine-positive monocytes or the MFI at any time point, and the plasma cytokine level at all later time points.
DISCUSSION

The main findings of our study are that, in healthy untrained subjects, 1) antioxidant supplementation drastically affects the plasma TNF-α, IL-1β, and IL-6 induction secondary to exercise, so that plasma IL-1β becomes undetectable, the TNF-α response is abolished, and the IL-6 response is significantly blunted; and 2) exercise does not increase either the percentage of monocytes producing the cytokines or the MFI.

Antioxidant Supplementation and the Cytokine Response to Exercise

Exercise is accompanied by increased levels of ROS within the working myocytes and the extracellular and vascular compartments. ROS have been recognized as mediators of signal transduction pathways (41) able to induce cytokine production from various cell types (1, 13, 19). Our finding that antioxidants significantly blunted the strenuous exercise-induced cytokine response is in concert with the role of ROS as stimuli for the cytokine induction. Our results suggest that ROS act as important mediators of the signal transduction pathways for the exercise-induced cytokine response. It should be noted that cytokines are rapidly upregulated at both the translational (9, 18) and the post-translational level (9), and the kinetics of ROS production is suitable for signaling purposes in such a quick response.

The effect of antioxidants in blunting the cytokine response to exercise is in concert with results obtained in animal models of exercise. Accordingly, Yamashita et al. (52) showed in rats that an acute bout of treadmill exercise increased the TNF-α and IL-1β content of the myocardium, whereas in the presence of the antioxidant (N-2-mercaptoethylglycine), no increase in either cytokine was observed, because of abolishment of ROS production. Antioxidants are effective in attenuating the cytokine response observed in various disease models, such as burn trauma (16), hemorrhagic shock (47), and inflammatory bowel disease (36). Taken together, these results suggest that oxidative stress-mediated cytokine production is a general process observed in health and disease.

The cytokines are usually induced through ROS-mediated activation of the transcription factor NF-κB, although other mechanisms might be operating as well, such as activation of other transcription factors and ROS-mediated shedding of preformed membrane-
bound TNF-α molecules (10). Our previous in vitro results of ROS-dependent IL-6 production from myocytes through activation of NF-κB (19) are in accordance with this proposed mechanism, because muscles are the major IL-6-producing tissues during exercise.

The few studies addressing the role of antioxidants in strenuous exercise-induced cytokine production (27, 32) were not able to show significant effects, although a tendency for IL-6 reduction after vitamin C supplementation has been reported (27). There are many potential explanations for the different findings between our work and those studies. Previous studies have used exercise protocols that included an eccentric component (27, 32), whereas cycling is a form of relatively pure concentric exercise. Eccentric contractions produce direct muscle injury with leukocyte infiltration; the cytokine response that eccentric exercise elicits is different (49) from concentric exercise, which is less injurious for the muscles (7, 31). Our exercise protocol was not damaging to the muscles, as evidenced by the lack of any change in the creatine kinase level after the exercise sessions both before and after antioxidant supplementation. The lack of response observed in trained athletes (27, 32) may be related to regular training, which may increase the natural antioxidant defense system (17, 22, 34), thus attenuating the effect of antioxidants (7). In contrast, we studied healthy volunteers who were untrained nonathletes, to eliminate the possible confounding factor of training on antioxidant defense systems.

In this study, we used a cocktail of ROS scavengers (vitamins A, E, C, and N-acetylcysteine) and inhibitors of ROS-producing enzymes (allopurinol) (2, 7, 14, 42, 46). Vitamin E is the major lipid-soluble antioxidant in cell membranes and is particularly efficient at quenching free radicals originating from the mitochondria and biomembranes. It protects against lipid peroxidation by reacting with a variety of oxygen radicals, including singlet oxygen, lipid peroxidation products, and the superoxide radical, to form a relatively innocuous tocopherol radical (7, 17). Vitamin C is a water-soluble antioxidant in the cytosol and the extracellular fluid that can directly scavenge superoxide, hydroxyl radicals, and singlet oxygen and can also interact with the tocopherol radical to regenerate reduced tocopherol (7, 17). Vitamin A is the most efficient “quencher” of singlet oxygen (7), whereas allopurinol inhibits the ROS-producing enzyme xanthine oxidase (51). N-acetylcysteine improves cysteine availability for the biosynthesis of reduced glutathione (41), which serves multiple functions in protecting tissues from oxidative damage by scavenging hydroxyl radicals and singlet oxygen and by reducing tocopherol radicals, and hydrogen- and organic-peroxides via a reaction catalyzed by glutathione peroxidase (17, 41).

Because multiple sources of ROS exist in the cell, such as the mitochondrial electron transport chain, cytosolic NADH oxidase, xanthine oxidase, and membrane-bound oxidases (17, 35, 51), it is to be expected that a combination of scavengers and inhibitors of
ROS-producing enzymes would be more effective in lowering ROS levels than any single agent. In fact, the defense against oxidative stress depends on an orchestrated synergism between several antioxidants (41). We used doses of antioxidants known to lower the exercise-induced ROS levels in humans. Accordingly, oral N-acetylcysteine (even in lower doses than the ones we used) increased the ROS-scavenging capacity of human plasma secondary to bicycle exercise (42). Vitamin E supplementation (300 mg/day for 4 wk) lessened exercise-induced ROS-mediated lipid peroxidation (46). Vitamin C supplementation (1,000 mg, 2 h before exercise) prevented exercise-induced oxidative stress in healthy humans (2), and allopurinol (300 mg/day for 2 days) prevented exercise-induced blood glutathione oxidation and lipid peroxidation in patients with chronic obstructive pulmonary disease (14).

Thus, although we have not determined any surrogate marker of the level of oxidative stress in our subjects, we are confident that the antioxidant cocktail we administered was effective in reducing the oxidative stress response to exercise. Consequently, the observed attenuation of the cytokine response to exercise was most likely due to reduced ROS production, although pharmacological effects not related to ROS attenuation cannot be excluded.

The antioxidant supplementation blunted but did not completely abolish the cytokine response to exercise. In fact, it is rather likely that the stimuli for cytokine production during exercise are multiple (31). Accordingly, carbohydrates attenuate the IL-6 response to exercise (25), and glycogen depletion greatly augments it (18). Furthermore, adrenergic stimulation could also have a contributing role (30) in IL-6 induction. The stimuli for the IL-1β and TNF-α induction during exercise are less well studied. Our results suggest that oxidative stress is a strong stimulus for the exercise-induced IL-1β and TNF-α response. Antioxidants abolished the exercise-induced elevation of TNF-α (Fig. 1A), whereas IL-1β was below the detection limit of the ELISA we used during the second exercise session, which suggests that antioxidants affected both the constitutive (baseline) and the exercise-induced production of IL-1β (Fig. 1B). Thus the possibility exists that the proinflammatory cytokine induction during exercise is differentially regulated by various stimuli, some of them being common (e.g., ROS), whose relative importance varies with each respective cytokine. Alternatively, oxidative stress may decrease the triggering thresholds of the various stimuli for cytokine induction, in a similar way to its enhancing effect on the antigen receptor-dependent signal cascades (10).

Although attenuation of exercise-induced cytokine production is the most likely cause of the effect of antioxidants in our study, increased cytokine clearance cannot be excluded, because a complex balance between production and clearance determines the concentration of cytokines, and the effect of oxidative stress on the latter is largely unknown.

Monocytes and the Cytokine Response to Exercise

The role of monocytes in exercise-induced cytokine production has been investigated for more than a decade. Early reports, using cultures of monocytes derived from sedentary and exercised subjects and stimulated with LPS or phytohemagglutinin, found increased levels of IL-1 and IL-6 in the culture supernatant (12), which suggested that monocytes were the sources of exercise-induced cytokine elevation. However, studies addressing at the mRNA level the possible cytokine induction in monocytes failed to show any effect of exercise (23, 29, 50). These results could not definitely exclude monocytes in exercise-induced cytokine production because similar cytokine mRNA levels might translate into different levels of secreted protein (9), given the well-described dissociation between transcription and translation for these cytokines (40). In contrast, intracellular flow cytometric detection of cytokines, as used in our study, allows for sensitive determination of cytokine production by the specific cell type, because it measures the amount of protein arrested at the Golgi complex of a specific cell population at a specific time point. Thus it accurately reflects the amount of cytokine secreted. Using flow cytometry, Starkie and colleagues (43, 44) were the first to exclude monocytes as sources of exercise-induced IL-1α, TNF-α, and IL-6 production in endurance-trained men secondary to long-distance running and cycling. The contribution of monocytes to exercise-induced plasma IL-1β elevation cannot be determined from these studies, because the intramonocyte expression of IL-1α and not IL-1β was studied. IL-1α remains mostly intracellular, in contrast to IL-1β, which is the secreted cytokine and thus can account for the plasma IL-1β elevation (9).

Contrary to our hypothesis, untrained individuals did not exhibit any different response from highly trained athletes. Furthermore, our results show that monocytes do not contribute to exercise-induced plasma IL-1β elevation. Thus our results in untrained healthy individuals confirm the findings of Starkie and colleagues (43, 44) obtained in athletes in excluding any role of monocytes in exercise-induced plasma cytokinemia.

Potential Sources of the Exercise-Induced Plasma Cytokines

Because monocytes are not the source of exercise-induced cytokine induction, the question remains which cells and/or tissues are the origins. Although our study does not address this issue, accumulated evidence could provide some answers. IL-6 originates from the exercising muscles (18, 29, 45) although small amounts are secreted from the tendon (20), the brain (28), and the adipose tissue (postexercise; Ref. 21). The cells of origin have not been determined with certainty, yet in vitro results suggest that myocytes are the IL-6-producing cells. Accordingly, we have recently shown (in a model of C2C12 myocytes transformed in culture into myotubes) that muscle cells can produce...
IL-6 in a ROS-dependent pathway (19). Thus the working muscles are the likely sources of the IL-6 induction secondary to exercise.

The origin(s) of IL-1β and TNF-α is less clear. The working muscles are likely candidates because both the mRNA and the proteins of these cytokines have been detected at the tissue level in muscles (6, 39), yet the effect of exercise on the expression of these cytokines has not been consistent (8). The adipose tissue (24), the lung (8), and the heart (52) are other potential sources of exercise-induced TNF-α production. Finally, the liver, a major source of proinflammatory cytokines during endotoxia, could also contribute to exercise-induced cytokine elevation, especially in circumstances of extreme exercise that can be accompanied by endotoxia (5). More studies are certainly needed to elucidate the tissues and cells of cytokine origin during exercise.

In conclusion, we have shown that in healthy untrained humans antioxidants blunt the exercise-induced increase in plasma TNF-α, IL-1β, and IL-6, which suggests that oxidative stress is a strong stimulus for exercise-induced increase in these cytokines. Monocytes do not contribute to exercise-induced cytokininemia in these untrained subjects, a response similar to the one observed in trained athletes.

The authors thank Drs. S. N. A. Hussain and K. Pantopoulos, McGill University, Montreal, Canada and Dr. A. Comtois, McGill University and Department of Exercise Science, Concordia University, Montreal, Canada for helpful suggestions and for carefully reviewing the manuscript.

This work was supported by the THORAX Foundation, Athens, Greece. T. Vassilakopoulos was a recipient of a Fellowship from Proponents Foundation.

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