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Exercise Training and Oxidative Stress in the Elderly as Measured by Antipyrine Hydroxylation Products

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Effects of 12 wk exercise training on oxidative stress were examined in elderly humans. We measured oxidative stress during a 45 min cycling test by using antipyrine hydroxylation products. Antipyrine breakdown is independent of blood flow to the liver, which is important during exercise. Furthermore, antipyrine reacts quickly with hydroxyl radicals to form *para*- and *ortho*-hydroxyantipyrine. *Ortho*-hydroxyantipyrine is not formed in man through the mono-oxygenase pathway of cytochrome P450. Twenty subjects (9 women; 60 ± 3 y) participated in the training program. Thirteen subjects (5 women; 64 ± 7 y) served as inactive controls. Subjects trained, twice a week for 1 h, at a fitness center. After 12 wk, maximal oxygen uptake ($p < .005$) and workload capacity ($p < .001$) were only significantly elevated in the training group. After 12 wk, both groups observed no change in the ratios of antipyrine hydroxylates, *para*- and *ortho*-hydroxyantipyrine, to native antipyrine. Furthermore, no differences were observed within or between groups in the exercise-induced increase in the plasma level of thiobarbituric acid reactive species. In conclusion, 12-wk training had no effect on exercise-induced oxidative stress in elderly humans as measured by free radical reaction products of antipyrine. Despite

the fact that training in elderly humans improves functional capacity, it appears not to compromise antioxidant defense mechanisms.

Keywords: Antipyrine, hydroxyl radicals, aromatic hydroxylation, aging

INTRODUCTION

Recent animal studies support the contention that exercise training may result in up-regulation of the antioxidant capacity to cope with increased oxidative stress.^[1–3] Leeuwenburgh *et al.*^[4] however, showed that 10 wk training increased glutathione (GSH) peroxidase and superoxide dismutase (SOD) content in the deep vastus lateralis muscle of young rats, but had no effect in old rats. They suggested that aged skeletal muscle has a limited capacity to further increase its antioxidant potential. This finding was confirmed

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by Oh-Ishi *et al.*^[5] who showed that 6 wk swimming training improved the antioxidant enzyme system only in young mouse diaphragm and not in that of old mice. In humans, data on the effect of training on oxidative stress is scanty and controversial.^[6-9] So far, no studies have been conducted in healthy elderly humans. Therefore, it would be informative to know whether elderly can cope better with exercise-induced oxidative stress after a training program.

Studies in humans have relied on endogenous markers for measuring exercise-induced oxidative stress. We used antipyrine (2,3-dimethyl-1-phenyl-3-pyrazoline-5-one), an exogenous

marker, for assessing oxidative stress. The metabolic pathway of antipyrine has been extensively documented.^[10-13] Antipyrine is metabolized in the hepatic smooth endoplasmic reticulum by at least three reactions (two hydroxylations and a *N*-demethylation), resulting in the formation of four primary metabolites: 4-hydroxyantipyrine, norantipyrine, 3-hydroxymethyl-antipyrine and 3-carboxy-antipyrine (Figure 1). After oral ingestion about 90% is excreted in the urine as unchanged antipyrine (3%), 4-hydroxyantipyrine (29%), norantipyrine (17%), 3-hydroxymethyl-antipyrine (35%), and as 3-carboxyantipyrine (3%).^[14] The properties of antipyrine make it

FIGURE 1 Metabolic pathway of antipyrine and its free radical reaction products.

a very suitable marker for measuring oxidative stress. After oral ingestion, antipyrine is completely absorbed and uniformly distributed in the total body water after approximately 1 h.^[11-13] Antipyrine breakdown is independent of blood flow to the liver, which is important during exercise where blood flow is altered. The half-life time of antipyrine is about 12 h.^[11,12] Furthermore, the reaction constant of antipyrine with hydroxyl radicals is in the order of $10^{10} \text{ l mol}^{-1} \text{ s}^{-1}$.^[15] Exposure of an antipyrine solution in water to ^{60}Co γ -radiation leads to the formation of three phenolic antipyrine derivatives: *para*-hydroxyantipyrine (*p*-APOH), *ortho*-hydroxyantipyrine (*o*-APOH) and *meta*-hydroxyantipyrine (*m*-APOH). The last two metabolites are not endogenously formed (Figure 1).^[16] Moreover, the free radical products of antipyrine could be detected in human plasma that was spiked with a 1 mM radiated antipyrine solution.^[17] A recently performed study in 16 patients with claudication intermittens, an early manifestation of atherosclerosis in the leg which is characterized by a low-grade repetitive ischemia-reperfusion during physical exercise, showed that 5 min of walking exercise ($3 \text{ km} \cdot \text{h}^{-1}$, 8° inclination) resulted in a significantly increased plasma concentration of *p*-APOH and *o*-APOH (Figure 2).^[18] The ratio of

o-APOH to native antipyrine increased significantly from $1.26 \times 10^{-3} \pm 0.14 \times 10^{-3}$ before exercise to $1.47 \times 10^{-3} \pm 0.18 \times 10^{-3}$ 5 min after exercise. Thus, the ratio of the hydroxylated products of antipyrine and the plasma concentration of antipyrine reflects the oxidative stress *in vivo*.

This study was designed to determine whether 12 wk exercise training would affect oxidative stress in exercising elderly. Antipyrine was used as an exogenous marker for measuring oxidative stress. Ratios of hydroxylates to native antipyrine were used to adjust for interindividual differences in biological availability, distribution and metabolism of antipyrine, similar to the salicylic acid method.^[19]

MATERIALS AND METHODS

Subjects

Thirty-three subjects were recruited with advertisements in the local media. Selection criteria were age over 55 y, no health problems and no participation in sports during the previous year. After detailed information concerning the purpose and methods used in the study was provided and written consent was obtained, 20 subjects (9 women) participated in the exercise-trained group (EXER), and 13 subjects (5 women) served as non-trained controls (CONT). Physical characteristics are shown in Table I. The local Ethical Committee approved the study.

FIGURE 2 Typical chromatogram of the formation of *para*- (*p*-APOH) and *ortho*-hydroxyantipyrine (*o*-APOH) in plasma immediately after 5-min exercise in a patient with claudication intermittens (AP: antipyrine).

TABLE I Characteristics of the exercise training (EXER) and control group (CONT). There were no differences between the two groups. Data are expressed as mean $\pm$ SEM

	EXER	CONT
Number (women, men)	20 (9, 11)	13 (5, 8)
Age (y)	60 (1)	64 (2)
Height (cm)	171 (2)	171 (3)
Body mass (kg)	78.6 (3.2)	69.5 (3.2)
BMI ($\text{kg} \cdot \text{m}^{-2}$)	27 (1)	24 (1)
$\text{VO}_{2\text{max}}$ ($\text{l} \cdot \text{min}^{-2}$)	2.02 (0.13)	1.92 (0.14)
W_{max} (W)	134 (9)	124 (10)

Study Design

Exercise-induced increase in oxidative stress was measured in elderly humans, before and after a 12 wk training program, with a 45-min cycling test at submaximal intensity. Oxidative stress was measured by free radical products of antipyrine.

Study Protocol

After an overnight stay at the laboratory, maximal workload capacity ($W_{\max}$) and maximal O_2 consumption ($VO_{2\max}$) were determined, at baseline (T0) and after 12 wk (T12). Subjects exercised incrementally on an electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands), as described recently.^[20] O_2 uptake during the test was measured continuously, using a computerized open system (SensorMedics 2900 analyzer, Anaheim CA, USA). During the incremental exercise test heart rate was measured continuously (Polar Sport Tester, Kempele, Finland).

One hour thereafter a Teflon catheter (Quick Cath[®] II, Baxter Healthcare S.A., Swinford, Ireland) was placed into an antecubital vein and a resting blood sample (10 ml) was drawn. Subjects then orally ingested 10 mg antipyrine (Janssen Chimica, Geel, Belgium) per kg body mass. One hour after ingestion subjects cycled for 45 min at 50% $W_{\max}$. Blood samples were drawn before exercise and immediately after exercise. Blood was collected into EDTA (1.34 mM) and GSH (0.65 mM) containing tubes and was centrifuged immediately (3000 rpm, 10 min at 4°C). Aliquots of plasma were frozen in liquid nitrogen and stored at -20°C until further analysis of antipyrine and its hydroxylates.

Training Program

Subjects trained twice a week, for 1 h, on non-consecutive days for 12 wk. All training sessions were performed at a fitness center and

supervised by a fitness instructor. The sessions consisted of 10 exercises using weight stack machines (Sportesse, Essen, Germany) and one dynamic exercise (120 sec cycling). All exercises were performed in cyclic order. The resistance exercises were performed for about 45 sec (10–15 repetitions) and consisted of the following exercises: chest press, triceps press, crunch, biceps curl, abduction, adduction, leg extension, leg curl, seated row and lat-pull. After each exercise subjects could rest for about 85 sec, to standardize the training protocol as much as possible, before the next exercise started. Each training session included a 15-min warming-up and a 10-min cooling-down. The warming-up consisted of aerobic exercises and the cooling-down consisted of stretch- and relaxing movements. The intensity of the training program was approximately 50% of heart rate reserve (HRR).

Sample Analysis

Antipyrine and its hydroxylates were measured with reversed phase HPLC combined with mass spectrometry (HPLC-MS).^[21] A reversed-phase Supersphere RP18 Endcapped column (LC-Packings, Amsterdam, The Netherlands), 150 × 1 mm I.D., $d_p = 4 \mu\text{m}$ was attached to a LC system consisting of a LC-10AT pump (Shimadzu Ltd., Kyoto, Japan), and a Triathlon autosampler (Spark Holland, Emmen, The Netherlands). The HPLC was connected to an API-300 LC/MS/MS (Perkin Elmer Sciex Instruments, Thornhill, Canada) which operated in the multiple reaction mode and Turbo Ion spray ionization was used as interface. Final data processing was done using an integration program (TurboQuan 1.0, Perkin Elmer Sciex Instruments, Thornhill, Canada). Sample pre-treatment consisted of C18 solid phase extraction (Sep-Pak[®] C18 Cartridges, Waters, USA) in order to wash out salts and proteins. Cartridges were conditioned with 5 ml methanol and 5 ml H_2O . After that 450 μl plasma was inserted in the cartridge,

followed by 2ml ammonium-acetate buffer (10mM, pH adjusted with acetic acid to 5). The cartridge was flushed with 1.5ml methanol to elute the target components. Samples were evaporated to dryness under nitrogen pressure (QBT4 Grant Instruments Ltd., Cambridge, UK), dissolved in 450 μ l H₂O, and placed in a waterbath (30°C) for 30 min. Afterwards, treated samples were filtered by using Spartan 13/20 filters (Schleicher & Schuell, Dassel, Germany).

Thiobarbituric acid-reactive substances (TBARS) were measured in plasma using a fluorescent thiobarbituric acid (TBA) assay. TBA, 0.375 g, (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was dissolved in 250 ml demineralized H₂O and 2.5 ml 1M HCl. 111 μ l plasma and 1000 μ l TBA solution were mixed and vortexed in an Eppendorf cup after which it was placed in a waterbath (95°C) for 1 h. Samples were cooled down to room temperature and the absorption was measured spectrophotometrically at 532 nm (Spectronic 1001, Meyvis, Bergen op Zoom, The Netherlands). Results were expressed as micromoles of MDA.

Statistics

All data are presented as means $\pm$ SEM. To compare differences within the two groups the non-parametric Wilcoxon signed-ranked test was used. To compare differences between the two groups the non-parametric Mann-Whitney U-test was used. Statistical significance was set at $p < .05$. The StatView5.0 program (SAS Institute Inc., Cary, NC) was used as the statistical package.

RESULTS

Twelve weeks of exercise training significantly elevated $\text{VO}_{2\text{max}}$ in EXER (25.8 ± 6.4 vs 28.1 ± 5.4 ml·kg⁻¹·min⁻¹; $p < .01$), whereas $\text{VO}_{2\text{max}}$ in CONT remained unchanged (27.5 ± 4.6 vs 27.6 ± 5.6 ml·kg⁻¹·min⁻¹). Training significantly elevated W_{max} with approximately 8% (134 ± 9 vs

FIGURE 3 Box-and-whisker plots (median with quartiles and range) of the exercise-induced increase in the ratio of *para*-hydroxyantipyrine (A) and *ortho*-hydroxyantipyrine (B) to native antipyrine during 45 min cycling at baseline and after 12 wk in the exercise and control group. No statistical differences were observed between or within the groups.

145 ± 8 W; $p < .001$), no change in W_{max} was observed in CONT (124 ± 10 vs 125 ± 11 W). Therefore, at T12 EXER cycled at a significantly higher absolute intensity of 50% W_{max} when compared with T0, whereas CONT cycled at the same absolute intensity of 50% W_{max} at both T0 and T12.

In EXER, the exercise-induced increase in the ratio of *p*-APOH to native antipyrine was not significantly different after 12 wk training (T0 and T12: $5.7 \times 10^{-4} \pm 0.9 \times 10^{-4}$ and $7.1 \times 10^{-4} \pm 1.0 \times 10^{-4}$; Figure 3A). Additionally, the exercise-induced increase in the ratio of *o*-APOH to antipyrine was not significantly different after 12 wk training (T0 and T12: $2.8 \times 10^{-4} \pm 0.8 \times 10^{-4}$ and $3.9 \times 10^{-4} \pm 0.9 \times 10^{-4}$; Figure 3B).

Additionally, in CONT no differences in the ratios of hydroxylates to antipyrine could be observed. At T0 and T12 the ratios of *p*-APOH were $8.7 \times 10^{-4} \pm 1.5 \times 10^{-4}$ and $6.7 \times 10^{-4} \pm 1.5 \times 10^{-4}$, respectively (Figure 3A). At T0 and T12 the ratios of *o*-APOH were $5.5 \times 10^{-4} \pm 1.5 \times 10^{-4}$ and $4.0 \times 10^{-4} \pm 0.9 \times 10^{-4}$, respectively (Figure 3B). Furthermore, no differences were observed within or between groups in the exercise-induced increase in the plasma level of TBARS. Differences between the level of TBARS pre- and post-exercise at T0 and T12 were $0.33 \pm 0.32 \mu\text{M}$ and $0.14 \pm 0.13 \mu\text{M}$ ($p = .31$) in EXER, and $0.14 \pm 0.26 \mu\text{M}$ and $0.13 \pm 0.06 \mu\text{M}$ ($p = .47$) in CONT, respectively.

DISCUSSION

This study was undertaken to examine the effect of a 12 wk training program on exercise-induced oxidative stress in the elderly. Antipyrine was used as an exogenous marker for measuring oxidative stress. Results showed that in elderly humans training had no effect on the exercise-induced increase in the ratios of free radical reaction products of antipyrine and the concentration of antipyrine in plasma during 45 min cycling.

This study used a newly developed technique to assess oxidative stress. The metabolic pathway of antipyrine has been extensively documented.^[10–13] Recently, it was shown *in vitro* that exposure of an antipyrine solution in water to ^{60}Co γ -radiation leads to the formation of three phenolic antipyrine derivatives: *para*-hydroxyantipyrine, *ortho*-hydroxyantipyrine and *meta*-hydroxyantipyrine (Figure 1).^[16] In case that an antioxidant is added to an antipyrine solution in water, the conversion of antipyrine after exposure to ^{60}Co γ -radiation can be used as indicator for the free radical scavenging capacity of a potential antioxidant.^[18] For example, ascorbic acid significantly reduced the conversion of antipyrine, depending on the added concentration of ascorbic acid. The results of the antipyrine

method were linearly correlated ($r^2 = .99$) with the results of the commonly used deoxyribose assay.

However, the properties of antipyrine make it also a suitable marker for measuring oxidative stress *in vivo*. Antipyrine can be safely ingested in a relatively high dose, usually 10 mg per kg body mass, with a maximum of $4 \text{ g} \cdot \text{day}^{-1}$. After oral ingestion, antipyrine is completely absorbed and uniformly distributed in the total body water after approximately 1 h.^[11–13] The half-life time of antipyrine is about 12 h.^[11,12] Furthermore, antipyrine breakdown is independent of blood flow to the liver, which is important in clinical studies where the blood flow is altered e.g. exercise experiments.^[12] Moreover, antipyrine reacts quickly with hydroxyl radicals (reaction rate constant $10^{10} \text{ l mol}^{-1} \text{ s}^{-1}$) to form two in plasma measurable hydroxylated metabolites, of which at least one (*o*-APOH) is not known to be formed by the human mono-oxygenase pathway of cytochrome P450 (Figure 1).^[16] Thus, the ratio of the hydroxylated products of antipyrine to plasma concentration of antipyrine can be used as a marker for oxidative stress.

Due to its high reactivity, the hydroxyl radical has a very short half-life and is therefore present only transiently in extremely low concentration.^[22] It is therefore necessary that the aromatic probe produce concentrations in body fluids sufficient to scavenge hydroxyl radicals in order to compete with other scavenger molecules. Consequently, the concentration of the hydroxylated products in plasma is extremely low. Recently, Coolen *et al.*^[17] showed that the sensitivity of HPLC to separate antipyrine and its free radical reaction products increased by a factor 100–1000 when MS was used as a detector instead of the commonly used UV-spectrophotometer. In this study, the free radical products of antipyrine could be detected in human plasma that was spiked with a 1 mM radiated antipyrine solution.^[17] Coolen *et al.*^[21] furthermore, demonstrated that HPLC-MS with Turbo Ion spray as interface improved the signal to noise ratio

when compared with electrospray ionization or atmospheric pressure chemical ionization as interfaces. The detection limit for antipyrine with Turbo Ionspray as interface, since no standards of the phenolic derivatives are available, was 1.3 pg. in 20 μ l water, which makes this technique highly sensitive and selective for determination of the phenolic derivatives of antipyrine in plasma. Therefore, the method used in this study should have been sensitive and selective to measure the potentially small changes in plasma concentrations of antipyrine and its hydroxylates in exercising elderly humans.

Recently, a study in 16 patients with claudication intermittens (51–74 y) showed that 5-min walking exercise resulted in a significantly increase in the plasma concentration of the free radical reaction products of antipyrine (Figure 2).^[18] Claudication intermittens is caused by narrowing or obstruction of arteries in the aorto-iliacal region or in peripheral arteries, and it is characterized by a chronic ischemia-reperfusion injury during physical exercise, which results in an increased oxidative stress. The claudicants, seven patients were claudicants in one leg and nine in both legs, orally ingested 15-mg antipyrine per kg body mass. One hour after ingestion, similar to the protocol used in the present study, the claudicants started with the 5-min walking exercise (3 km·h⁻¹, 8° inclination). The exercise resulted in a significant increase ($p = .002$) in the serum lactate concentration (1.59 ± 0.21 and 2.99 ± 0.6 mmol·l⁻¹ before and after exercise, respectively). Interestingly, the exercise-induced increase in the ratio *o*-APOH to antipyrine was similar in claudicants performing 5-min walking exercise ($\Delta 2.1 \times 10^{-4}$) compared to elderly cycling for 45 min at submaximal intensity ($\Delta 2.8 \times 10^{-4}$), which confirms that the increased hydroxyl radical formation in the exercising elderly subjects could be detected by the antipyrine method.

Because the plasma level of *p*-APOH and *o*-APOH depends on that of antipyrine, and because the latter could vary among individuals

ingesting the same dose of antipyrine, ratios of the hydroxylated products of antipyrine and the plasma concentration of antipyrine were used similar to the salicylic acid method.^[19] Additionally, there was no difference in concentration of antipyrine between pre- and post-exercise conditions (158.5 ± 9.8 μ M and 155.6 ± 9.4 μ M pre- and post-exercise, respectively). Therefore, the exercise-induced increase in both ratios was due to an increased concentration of *p*-APOH and *o*-APOH after exercise and not to changes in antipyrine concentration. This study clearly showed no differences in the exercise-induced increase in the ratios of the hydroxylated products of antipyrine and the plasma concentration of antipyrine after 12 wk training (Figure 3). The fact that no differences were observed within or between groups in the exercise-induced increase in the level of TBARS in plasma furthermore suggests that training had no effect on exercise-induced oxidative stress in elderly humans.

The implications of this study are in accordance with findings of Leaf *et al.*^[23] who showed that after 12 wk training in elderly cardiac patients the expired levels of ethane and pentane were unchanged. However, some limitations have to be considered when ethane and pentane, end products of lipid peroxidation, are used as indirect markers of oxidative stress.^[22,24] Firstly, blood flow could influence the level of expired ethane and pentane. During exercise, blood flow increases in muscle tissue, thus liberating alkanes that are stored in membranes. Therefore, increase of pentane exhalation does not necessarily reflect an increase in oxidative stress. Secondly, one must realize that those alkanes, once formed *in vivo*, are susceptible to metabolism. Thirdly, expired alkanes can arise from other sources, e.g. inhaled air or gut bacteria. Furthermore, Leaf *et al.*^[23] showed that the serum level of MDA was significantly decreased after training, which is in contrast with findings of the present study. It has to be mentioned, however, that the TBA assay used to measure MDA lacks specificity when applied to human plasma.^[22,24]

Therefore, the results of Leaf *et al.*^[23] should be interpreted with some caution.

Recently, Leeuwenburgh *et al.*^[14] showed that exercise training significantly increased GSH peroxidase and SOD content in the deep vastus lateralis muscle in young, but not in old rats. Additionally, Oh-Ishi *et al.*^[5] showed that training only improved the antioxidant enzyme system in young mouse diaphragm. Both studies suggested that aged skeletal muscle had a limited capacity to increase its antioxidant potential. Although in the present study antioxidant capacity or enzyme activity was not determined, the fact that aging muscle has a limited capacity to increase its antioxidant enzyme system might explain why we were unable to observe any effect of training on oxidative stress in exercising elderly humans.

Additionally, it could be argued that the intensity of the training program (~50% HRR), was too low to pose a challenge for adaptive responses to the antioxidant defense systems. Powers *et al.*^[25] showed in rats that high intensity training was superior to low-intensity exercise training in the up-regulation of skeletal muscle SOD activity. Nevertheless, low-intensity exercise training resulted in a significant up-regulation of rat skeletal muscle SOD activity. In addition, $\text{VO}_{2\text{max}}$ was significantly increased by approximately 9% in EXER, indicating that the intensity of the training program should have been high enough to induce changes in the antioxidant enzyme system. However, it may be possible that greater elevations of $\text{VO}_{2\text{max}}$ or a longer training period may be necessary before any significant changes in oxidative stress during exercise would occur.

Powers *et al.*^[25] used an entirely aerobic training program, whereas in the present study the training program consisted of a combination of strength and endurance training. Powers *et al.*^[25] also showed that the training-induced up-regulation in the antioxidant enzyme system was limited to highly oxidative muscles (type I and IIa fibers). Furthermore, in a cross-sectional

study, Jenkins *et al.*^[26] reported higher antioxidant enzyme activities in muscle of males with a high $\text{VO}_{2\text{max}}$ than in males with a low $\text{VO}_{2\text{max}}$. In contrast to this finding, Hellsten *et al.*^[27] showed that anaerobic training improved the level of antioxidant protection in human muscle. Additionally, Ørtenblad *et al.*^[28] reported that the activities of antioxidant enzymes were markedly elevated in highly trained jumpers when compared with untrained subjects. Although the previous mentioned studies all used different types of training and different types of markers for assessing oxidative stress, which makes comparison extremely difficult, it could be argued that the type of training used in this study could have had the potential to up-regulate the antioxidant enzyme system, resulting in a lowered oxidative stress during exercise.

In conclusion, 12 wk of exercise training had no effect on exercise-induced increase in oxidative stress in elderly humans as measured by free radical products of antipyrine. Despite the fact that exercise training in elderly humans improves functional capacity, it appears not to compromise antioxidant defense mechanisms.

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