Exercise-induced oxidative stress in older adults as measured by antipyrine oxidation

Exercise-Induced Oxidative Stress in Older Adults as Measured by Antipyrine Oxidation

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Aging is associated with increased susceptibility to free radical-mediated tissue damage. Measuring exercise-induced oxidative stress, however, is a major problem in free radical research. We used an exogenous marker (antipyrine) to measure oxidative stress in older adults during submaximal exercise. Antipyrine pharmacokinetics is independent of blood flow to the liver. Furthermore, antipyrine reacts quickly with hydroxyl radicals (10^{10}-10^{11} $L \cdot mol^{-1} \cdot s^{-1}$) to form para- and ortho-hydroxyantipyrine (o-APOH). o-APOH is not formed in man through the mono-oxygenase pathway of cytochrome P450. Thirty-four subjects (62 ± 1 years) orally ingested 10 mg antipyrine/kg body mass. One hour after ingestion subjects cycled 45 minutes at 50% maximal power output. Exercise significantly increased the ratio of para-hydroxyantipyrine (p-APOH) to native antipyrine in plasma (.0014 ± .0001 *v* .0021 ± .0002; *P* < .0001). Also, the ratio of o-APOH was significantly increased after exercise (.0014 ± .0001 *v* .0019 ± .0002; *P* < .0001). Exercise significantly increased plasma levels of plasma malondialdehyde MDA) (.55 ± .07 *v* .92 ± .21 μmol/L; *P* < .01). In conclusion, in older adults, oxidative stress occurs during cycling at submaximal intensity as measured with free radical reaction products of antipyrine.

PHYSICAL EXERCISE markedly increases oxygen uptake particularly in skeletal muscle.[1] Increased oxygen consumption further increases the leaking of reactive oxygen species (ROS), such as superoxide, hydroxyl radical ($OH^{\cdot}$), and singlet oxygen, as products of oxidative phosphorylation, from the mitochondrial electron transport chain.[2] The imbalance, in favor of the free radicals, between the increased ROS generation and the scavenging capacity is termed oxidative stress.

Aging is associated with an increased susceptibility to free radical-mediated tissue damage.[3-5] Monitoring the amount of oxidative stress in vivo, however, is a major problem in free radical research.[6] Most human studies investigating exercise-induced oxidative stress have relied on indirect endogenous indices of free radical damage, such as plasma malondialdehyde (MDA) levels,[7] conjugated dienes,[8] and serum lipid peroxides.[9] Although these studies showed an increase in postexercise lipid peroxidation, the major disadvantage of using endogenous markers in vivo is the possibility that they are not only formed by free radicals, but also by other pathways, for example, MDA could be formed by the enzymatic breakdown of prostaglandin endoperoxides.[6] A suitable approach to measure oxidative stress directly in vivo is aromatic hydroxylation by salicylic acid[10,11] or phenylalanine.[12,13] These methods are based on the ability of $OH^{\cdot}$ to attack the benzene rings of aromatic molecules and to produce hydroxylated compounds that can be measured directly. Salicylic acid, however, has a high biologic half-life of about 15 to 20 minutes, and the metabolic breakdown depends on blood flow to the liver, which are disadvantages during endurance exercise experiments.[14]

This study used antipyrine (2,3-dimethyl-1-phenyl-3-pyrazoline-5-one) as an aromatic probe for assessing oxidative stress. The properties of antipyrine make it a very suitable marker. Following oral ingestion, antipyrine is uniformly distributed in the total body water after approximately 1 hour.[15] Antipyrine breakdown is independent of blood flow to the liver, which is important during exercise where blood flow is altered. Due to the reactive phenyl group, the reaction rate constant with $OH^{\cdot}$ is in the order of 10^{10}-10^{11} $L \cdot mol^{-1} \cdot s^{-1}$.[16] Exposure of an antipyrine solution in water to ^{60}Co γ-radiation leads to the formation of 3 phenolic antipyrine derivatives: para-hydroxyantipyrine (p-APOH), ortho-hydroxyantipyrine (o-APOH), and meta-hydroxyantipyrine (m-APOH). The last 2 metabolites are not endogenously formed.[17] The ratio of the hydroxylated products of antipyrine and the plasma concentration of antipyrine reflects the oxidative stress in vivo.

Although older people may be more susceptible to oxidative stress, so far studies have only measured exercise-induced oxidative stress in older adults by using endogenous markers. Therefore, the purpose of this study was to examine the effect of submaximal exercise on oxidative stress in older adults by using free radical reaction products of antipyrine.

MATERIALS AND METHODS

Design

In this study, 34 older subjects (62 ± 1 years) subjects performed a maximal exercise test and at least 2 hours thereafter a 45-minute cycling test at submaximal intensity. Exercise-induced oxidative stress was measured before and after the 45-minute cycling test by using free radical reaction products of antipyrine.

Subjects

The subjects were recruited from advertisements in the local media. Selection criteria were age over 55 years, no health problems, and no participation in regular sports activities or exercise programs during the previous year. Physical characteristics of the subjects are shown in Table 1. Detailed information concerning the purpose and methods used in the study was provided, and written consent was obtained. The local Ethical Committee approved the study.

Protocol

After an overnight stay in the laboratory, subjects first performed a maximal exercise test (7:30 AM) of about 10 to 15 minutes. Maximal

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Submitted February 9, 2001; accepted May 21, 2001.

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0026-0495/01/5012-0008$35.00/0
doi:10.1053/meta.2001.28086

Table 1. Subject Characteristics (means ± SEM)

No. (men/women)	21/13
Age (yr)	62 ± 1
Body mass (kg)	75.1 ± 2.4
Height (m)	1.71 ± 0.2
BMI ($kg \cdot m^{-2}$)	26 ± 1
Fat mass (%)	32 ± 2
VO_{2max}, ($L \cdot min^{-1}$)	1.98 ± 0.09
W_{max} (W)	130 ± 7
50% W_{max} (W)	65 ± 3

NOTE. Percent fat mass was determined by using deuterium dilution combined with a measurement of underwater weighing.

Abbreviation: BMI, body mass index.

power output (W_{max}) and maximal O_2 consumption ($\dot{V}O_{2max}$) were determined on an electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands), as described previously.[18] During the test, O_2 uptake was measured continuously using a computerized open system (SensorMedics 2900 analyzer, Anaheim, CA). Additionally, heart rate was measured continuously (Polar Sport Tester, Kempele, Finland). At least 1 hour after the exercise test, a Teflon catheter (Baxter Quick Cath II, Swinford, Ireland) was inserted into an antecubital vein, and a resting blood sample (10 mL) was drawn. After that (9:00 AM), subjects orally ingested 10 mg antipyrine (Janssen, Geel, Belgium) per kilogram body mass. One hour after ingestion (10:00 AM), they started to cycle for 45 minutes at 50% W_{max}. Blood samples were drawn before the exercise protocol, 20 minutes during the exercise protocol, and immediately after exercise. Blood was collected into EDTA (1.34 mmol/L) and glutathione (GSH, 0.65 mmol/L)-containing tubes and was centrifuged immediately (3,000 rpm) 10 minutes at 4°C. Aliquots of plasma were frozen immediately in liquid nitrogen and stored at −20°C until further analysis of MDA and of antipyrine and its phenolic derivatives.

Oxidative Stress

Antipyrine and MDA were used as markers for oxidative stress. Antipyrine and its hydroxylated metabolites were measured in plasma by reversed-phase high-performance liquid chromatography–mass spectrometry (HPLC-MS) as described previously.[19] Briefly, a reversed-phase Supersphere RP18 Endcapped column (LC-Packings, Amsterdam, The Netherlands), 150 × 1 mm ID, d_p = 4 μm was attached to a LC system consisting of a LC-10AT pump (Shimadzu, 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 Ionspray ionization was used as interface between HPLC and MS. Sample pretreatment consisted of C18 solid phase extraction (Sep-Pak C18; Cartridges, Waters, MA) to wash out salts and proteins. Cartridges were conditioned with methanol and H_2O. After that, 450 μL plasma was inserted in the cartridge, followed by 2 mL ammonium-acetate buffer (10 mmol/L, pH 5). The cartridge was flushed with 1.5 mL methanol to elute the target components. The samples were evaporated to dryness under nitrogen pressure and dissolved with 450 μL H_2O after which they stayed 30 minutes in a waterbath (30°C). Afterwards, treated samples were filtered by using Spartan 13/20 filters (Schleicher & Schuell, Dassel, Germany).

Because a competitive effect exists between antipyrine and other biomolecules for reaction with $OH^{\cdot}$, the formation of the free radical reaction products of antipyrine is dependent on the available concentration of antipyrine. Therefore, ratios of phenolic derivatives to native antipyrine are used, similar to the salicyclic acid method.[20]

MDA was 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^2 H_2O and 2.5 mL 1 mol/L HCl. A total of 111 μL plasma and 1,000 μL TBA solution was mixed and vortexed in an Eppendorf cup, after which it was placed in a waterbath (95°C) for 1 hour. Samples were cooled down to room temperature, and the absorption was measured spectrophotometrically at 532 nm (Spectronic 1001, Meyvis, Bergen op Zoom, The Netherlands).

Statistics

All data are means ± SEM. Statistical analysis of the data was performed using a nonparametric Wilcoxon signed-ranked test, because the data was not normally distributed and to minimize for big interindividual differences. Statistical significance was set at $P < .05$. The StatView5.0 program (SAS Institute, Cary, NC) was used as the statistical package.

RESULTS

Forty-five minutes of cycling at a submaximal intensity significantly increased the ratio of p-APOH to native antipyrine when compared with the ratio before exercise (.0014 ± .0001 *v* .0021 ± .0002; $P < .0001$; Fig 1A). Additionally, the ratio of o-APOH to native antipyrine was significantly increased after exercise when compared with the ratio before exercise (.0014 ± .0001 *v* .0019 ± .0002; $P < .0001$; Fig 1B). The 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 (158.5 ± 9.8 μmol/L and 155.6 ± 9.4 μmol/L before and after exercise, respectively). MDA levels in plasma were significantly increased immediately after exercise (.55 ± .07 *v* .92 ± .21 μmol/L; $P < .01$; Fig 2). Also after 20 minutes of cycling, MDA levels were significantly increased when compared with pre-exercise levels (.55 ± .07 *v* .71 ± .13 μmol/L; $P < .05$).

DISCUSSION

So far, studies in older adults that examined exercise-induced oxidative stress have relied on endogenous markers. This study investigated exercise-induced oxidative stress in older adults by using antipyrine as an exogenous marker. Results showed that the ratios of phenolic derivatives to native antipyrine were significantly increased after a 45-minute exercise at a submaximal intensity. Thus, in older adults submaximal exercise increased oxidative stress, which is in accordance with previous studies that have relied on endogenous markers.[21,22] Meydani et al[22] observed a steady increase in the urinary TBA level after a 45-minute running down on an inclined treadmill in 55 to 74-year-old subjects. Leaf et al[21] observed a significant increase in the postexercise levels of expired ethane and pentane in 44 to 77-year-old cardiac patients. However, endogenous markers have several methodologic disadvantages.[6]

An appropriate approach to measure oxidative stress directly in vivo is aromatic hydroxylation by using salicylic acid[10,11] or phenylalanine.[12,13] A disadvantage of salicylic acid, however, is that the metabolic breakdown is dependent on blood flow to the liver, which makes it less suitable during exercise experiments where liver blood flow will be altered. Additionally, the biological half-life is about 15 to 20 minutes, resulting in a high metabolic breakdown.[12] A serious disadvantage of phenylala-

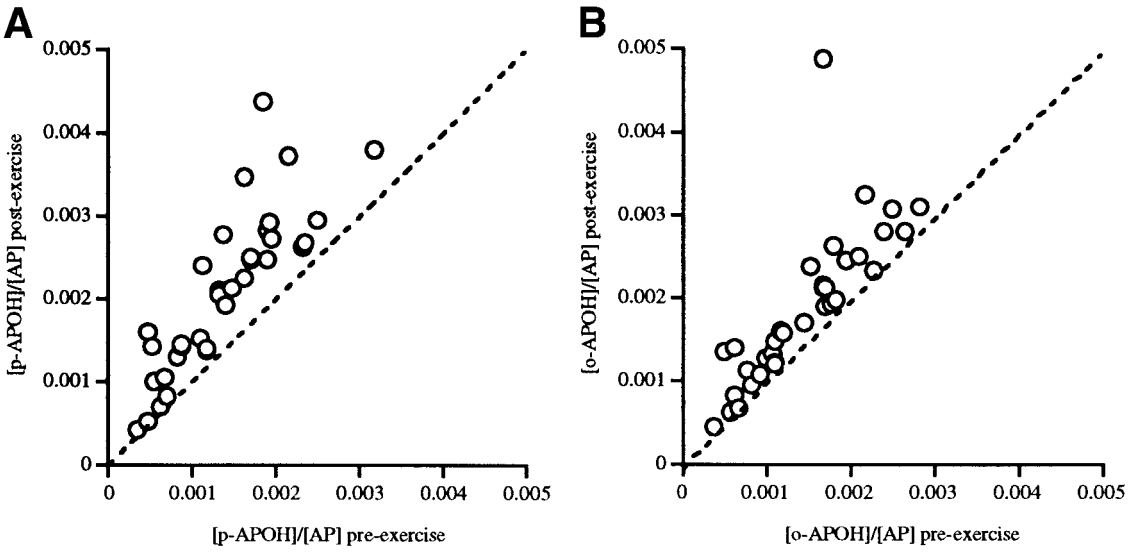

Fig 1. (A) Ratio of p-APOH to native antipyrine (AP) in plasma of all subjects before (x-axis) and immediately after exercise (y-axis). If there was no difference between the ratio before and after exercise, the line of identity (dotted line) would have been found ($P < .0001$). (B) Ratio of o-APOH to native AP in plasma of all subjects before (x-axis) and immediately after exercise (y-axis). If there was no difference between the ratio before and after exercise, the line of identity (dotted line) would have been found ($P < .0001$).

nine is its relatively low rate constant for reaction with $OH^{\cdot}$, at 1.9^9 L · mol^{-1} · s^{-1}.[12]

The novel antipyrine method used in this study has some advantages over the existing methods. First, the reaction rate constant with $OH^{\cdot}$ is in the order of 10^{10}-10^{11} L · mol^{-1} · s^{-1}, which is higher than the reaction rate of salicylic acid and phenylalanine with $OH^{\cdot}$.[16] Reaction with $OH^{\cdot}$ results in the formation of 2 in plasma measurable hydroxylated metabolites, of which at least 1 (o-APOH) is not known to be formed by the human mono-oxygenase pathway of cytochrome P450.[17] Second, metabolic breakdown of antipyrine is independent on blood flow to the liver, which is important during exercise where blood flow is altered.[15] The biological half-life of antipyrine is about 12 hours, and antipyrine can be ingested in a relatively high dose. After oral ingestion, antipyrine is uninformly distributed over the total body water and not only the extracellular water compartment like salicylic acid.[15] Coolen et al[23] furthermore showed that the sensitivity of the HPLC to separate antipyrine and its phenolic derivatives increased by a factor 100 to 1,000 when MS was used as a detector instead of the commonly used ultraviolet (UV)-spectrophotometer. The recently developed method[19] used in this study is highly selective in measuring the expected low concentrations of antipyrine and its phenolic derivatives in plasma. Therefore, this method should have been sensitive and selective to measure the potentially small changes in plasma concentrations of antipyrine and its free radical reaction products in exercising older humans.

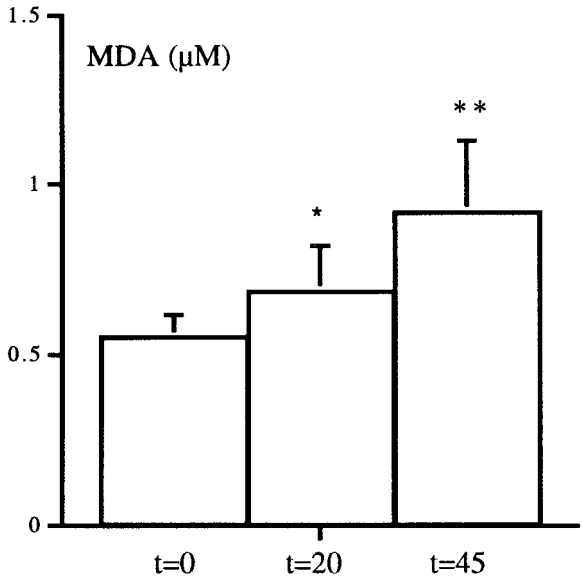

Fig 2. Plasma concentration of MDA ± SEM (μmol/L), before (t = 0), 20 minutes (t = 20), and immediately after exercise at 50% W_{max} (t = 45). Statistical difference when compared with t = 0, *$P < .05$; **$P < .01$.

Interestingly, Coolen[24] observed no significant increase in the free radical products of antipyrine in young adults after exercise. Those 19 young male adults (23 ± 1 years; 22 ± 1 kg · m^{-2}) cycled for 2 hours at the same relative submaximal intensity (50% W_{max}; 154 ± 7 W) as the elderly subjects in this study. The exercise-induced oxidative stress of the young adults was compared with a 2-hour resting condition. No significant changes in the ratios of p-APOH and o-APOH to antipyrine could be observed. Additionally, no significant difference in the level of MDA before and after 2 hours of exercise was observed. Coolen[24] proposed that the training status of the subjects (VO_{2max}: 4.1 ± 0.1 L · min^{-1}) was too high to observe an effect on exercise-induced oxidative stress at such a moderate intensity, which is similar to a previous finding.[8] It has to be mentioned, however, that food intake was not

measured in both younger and older adults. Therefore, we cannot exclude that part of the differences found can be attributed to differences in food intake. In the present study, the level of MDA significantly increased during and after exercise (Fig 2). Although the older subjects in this study were relatively unfit (Table 1), which makes comparison of the data with young adults rather difficult, it could be argued that the increased levels of MDA and free radical reaction products of antipyrine might indicate a clear exercise-induced oxidative stress. In Fig 1, it is clearly shown that exercise results in an increase in the ratio of the hydroxylated metabolites to the plasma antipyrine concentration in almost every subject.

It could be argued, however, that subjects performed the maximal exercise test and the submaximal exercise trial on 1 day, which could have influenced the measured exercise-induced oxidative stress. To minimize the effect of the maximal exercise test, the time between the 2 cycling test was at least 2 hours. Recently, Leaf et al[25] observed in 7 healthy men and women that the expired ethane and pentane levels (markers of lipid peroxidation) were already declining 5 minutes after performing a maximal exercise test. This suggests that the residual effect of the time frame used on the measured oxidative stress is probably negligible. Additionally, all the subjects were able to perform the 45-minute cycling test without physical complaints.

It has to be mentioned, however, that the lack of a resting control group is a limitation of this study. It could be argued that the increase in p-APOH and o-APOH was due to the fact that under normal resting conditions 2% to 5% of the total electron flux leaks to form primary short-lived ROS, such as superoxide, hydrogen peroxide and $OH^{\cdot}$.[26] This explanation seems unlikely, because an increase in the ratios of p-APOH and o-APOH to native antipyrine was observed between the pre- and postexercise condition. In addition, the increase in these ratios was highly significant ($P < .0001$). Furthermore, the plasma MDA levels were increased almost 70% after exercise (Fig 2). Although the TBA assay lacks specificity when applied to human plasma,[2] it seems that in older adults, submaximal exercise results in an increased oxidative stress.

In view of the age-related increase in the susceptibility to oxidative stress, one might speculate if untrained older adults would benefit from following an exercise training program. Leeuwenburgh et al[27] showed that 10 weeks endurance training increased GSH peroxidase and superoxide dismutase content in the deep vastus lateralis muscle of young rats, but had no effect on antioxidant enzyme content in old rats. They suggested that aged skeletal muscle had a limited capacity to further increase its antioxidant potential. So far, no studies have been conducted in healthy older adults in which oxidative stress was measured before and after a training period. It would be informative to know whether training is effective in the induction of the antioxidant system, and whether the elderly are able to cope with an increase in oxidative stress following a training program.

In conclusion, the present study demonstrated that in older adults, oxidative stress occurred during a single bout of submaximal endurance exercise as measured with free radical reaction products of antipyrine.

ACKNOWLEDGMENT

The authors wish to thank Joan Senden for placement of the Teflon catheters and assistance during the MDA analyses. We would also like to thank Joost van Dongen from the Laboratory for Macromolecular and Organic Chemistry for assistance during the HPLC-MS experiments.

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