Effects of age and physical fitness on microcirculatory function

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ABSTRACT
Sedentary aging is associated with endothelial dysfunction and nitric oxide (NO) impairment. The aim of the present study was to assess the effects of regular physical exercise on nitrite/nitrate (NOx) concentrations and microcirculatory function in older men compared with young individuals. We measured NOx plasma concentrations and baseline and stimulated skin blood flow (SBF) by laser Doppler flowmetry in 39 male athletes [range, 22–72 years; maximal oxygen consumption (˙V O2max), 60.0 ± 4.7 ml · min⁻¹ · kg of body weight⁻¹ (mean ± S.D.)] and 45 age- and sex-matched sedentary controls (˙V O2max, 38.0 ± 7.1 ml · min⁻¹ · kg of body weight⁻¹). NOx concentrations were higher in athletes than in controls (50.4 ± 16.3 compared with 39.0 ± 15.4 µmol/l; P < 0.005), whereas baseline SBF was comparable. Hand SBF after heating and ischaemia and foot SBF after heating were higher in athletes (P < 0.0001) than in controls. By comparing the lowest and the highest tertile of age, sedentary young subjects had higher NOx concentrations than sedentary older subjects (43.3 ± 13.4 compared with 31.8 ± 12.2 µmol/l respectively; P < 0.05). Exercise abolished this difference (49.1 ± 9.6 µmol/l for young subjects and 52.1 ± 11.5 µmol/l for older subjects; not significant). Resting SBF was similar in all the subgroups, but stimulated SBFs were lower in both subgroups of untrained compared with trained subjects. NOx concentrations were positively correlated with ˙V O2max (r = 0.46, P < 0.001). Stimulated SBFs were correlated with NOx (r > 0.30, P < 0.05). These findings show that chronic exercise may improve endothelial function in older (and young) men, probably by increasing NO availability.

INTRODUCTION
Aging represents a well-documented cardiovascular risk factor [1]. One of the putative physiopathological mechanisms through which aging may lead to cardiovascular damage is the promotion of changes in arterial structure and function [2].

Endothelium plays an important role in the local regulation of vascular tone and structure. This regulation is mediated mainly by nitric oxide (NO) synthesis and action [3]. A reduction in NO availability and action would lead to endothelial dysfunction, and this derangement has been associated with other cardiovascular risk factors, such as dyslipidaemia, diabetes mellitus, essential hypertension and aging [4–7]. Thus endothelial dysfunction represents a common feature and a crucial event in cardiovascular disease [8].

Regular physical activity has long been considered necessary for the achievement and the maintenance of optimal health. Experimental and clinical studies have provided strong evidence that physical training has beneficial effects on multiple cardiovascular risk factors, such as dyslipidaemia, high blood pressure and glucose tolerance [9,10]. By contrast, sedentary lifestyle has been identified as a risk factor for the development of some cardiovascular risk conditions and for coronary artery

Key words: aging, exercise, endothelial function, microcirculation, nitric oxide (NO).
Abbreviations: BMI, body mass index; NO, nitric oxide; eNOS, endothelial NO synthase; NOx, nitrate + nitrite; pu, perfusion units; SBF, skin blood flow; ˙V O2max, maximal oxygen consumption.
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disease. It has been reported that there is a strong relationship between physical inactivity and cardiovascular mortality [11]. Consequently, daily physical aerobic activity is considered to be an effective component in preventing cardiovascular events [1,9]. Recent evidence, obtained from functional studies, suggests that regular physical activity is associated with an increase in NO availability and action in elderly [12–15] and young people [13,16–18]. Thus it is possible that regular exercise can counterbalance the effects of aging on endothelial dysfunction.

In the present study, our aim was to assess the relationship between age, physical exercise, NO availability and endothelial function in both healthy older and young subjects.

**METHODS**

**Subjects**

Thirty-nine competitive long-distance male runners [age, 42.4 ± 15.3 years (mean ± S.D.; range, 22–72 years) and 45 healthy age- and sex-matched sedentary volunteers (range, 22–75 years) were studied. Athletes had been training for 21 ± 12 years. They were recruited from various running clubs throughout the surrounding regions of Pisa and from the National Veterans Sport Club of Pisa. They performed vigorous endurance exercise more than five times/week and were active in national and international road-running races. The sedentary subjects were recruited through various forms of advertisements.

All subjects were free of cardiovascular disease or other major medical disorders as assessed by clinical history, physical examination, basal and stress electrocardiography, blood chemistry, haematology and urine analysis. Subjects smoking more than five cigarettes/day and/or consuming more than 60 g of ethanol/day (corresponding to half a litre of wine), or receiving any drug treatment within the previous 2 months, were excluded from the study.

Athletes were selected on the basis of maximal oxygen consumption (\(\text{VO}_2\text{max}\)), as assessed by a graded exercise test (cycle ergometer), of greater than 50 ml · min\(^{-1}\) · kg of body weight\(^{-1}\), whereas control subjects had a \(\text{VO}_2\text{max}\) lower than 40 ml · min\(^{-1}\) · kg of body weight\(^{-1}\) [19]. The time period between the last exercise and blood sampling was at least 48 h.

All participants, who fasted for at least 12 h before the start of the study, were requested to adhere to a low-nitrate diet (e.g. exclusion of foodstuffs containing a high concentration of nitrates, such as cured meat, fruits and, in particular, leafy green vegetables) for 72 h before collection of blood samples.

Fully informed consent was obtained from each subject entering the study. The investigation conforms with the Declaration of Helsinki. The protocol was approved by the Local University Hospital Ethics Committee.

**Recording of blood pressure and blood sampling**

All subjects were examined at 09:00 hours in a quiet air-conditioned room with the temperature maintained at 22–24 °C. After introduction of an indwelling cannula (20 gauge; Abbocath, Sling, Ireland) into the left antecubital vein, each subject was allowed to rest in a supine position for at least 15 min before blood pressure was recorded. Venous blood samples were collected in tubes containing EDTA and processed immediately for separation of plasma. Measurement of blood pressure was carried out at least five times by means of a Dinamap automatic recorder (Critikon, Tampa, FL, U.S.A.), with an interval of 3 min between single readings.

**Microcirculatory studies**

The two groups underwent a laser Doppler study of skin blood flow (SBF) by laser Doppler flowmetry (Periflux PF4001, standard probe PF408; Perimed, Jarfalla, Sweden) at upper and lower extremities. The laser Doppler probe was fixed to the skin surface of the third digit of the left hand and of the first digit of the left foot. SBF was measured under basal conditions, after local heating at 44 °C and during reactive hyperaemia following a 3-min brachial artery occlusion. The apparatus and technique have been described in detail previously [20]. The output signal was linearly related to red cell flow as predicted by theoretical [21] and experimental [22] models.

The laser Doppler apparatus was connected to a PC by RS232 interface. The program installed on the computer (Perisoft) allows the storage and analysis of the recordings. The measurement of SBF is expressed in arbitrary units [‘perfusion units’ (pu)]. Coefficient of variation (three measurements) for baseline SBF was less than 5 %.

**Analytical methods**

Serum concentrations of total cholesterol, high-density lipoprotein-cholesterol and triacylglycerols (triglycerides) were determined by standard laboratory methods. The concentration of low-density lipoprotein-cholesterol was calculated using the Friedwald equation, and expressed as mmol/l.

We measured circulating levels of nitrite + nitrate (NOx), which represent the final and stable end-product of the NO metabolic pathway. Venous blood samples were collected in the presence of EDTA and immediately centrifuged at 2500 g for 15 min. Plasma samples were stored at – 80 °C for less than 2 weeks before analysis. At the time of NOx assay, plasma samples were ultrafiltered through 30 kDa cut-off filters and centrifuged at 1000 g for 60 min in order to remove haemoglobin, which is
Table 1  Clinical characteristics of the study populations

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Sedentary controls</th>
<th>Athletes</th>
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<tbody>
<tr>
<td>(n = 45)</td>
<td>(n = 39)</td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>41.4 ± 17.2</td>
<td>42.4 ± 15.3</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>23.8 ± 1.6</td>
<td>23.9 ± 2.4</td>
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<tr>
<td>SBP (mmHg)</td>
<td>121.3 ± 14.5</td>
<td>119.5 ± 13.4</td>
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<tr>
<td>DBP (mmHg)</td>
<td>74.8 ± 14.1</td>
<td>73.7 ± 11.8</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>67.3 ± 4.5</td>
<td>50.9 ± 3.6‡</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.4 ± 0.8</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.4 ± 0.3</td>
<td>1.7 ± 0.2∗</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.3 ± 0.6</td>
<td>2.6 ± 0.3∗</td>
</tr>
<tr>
<td>V̇O₂max (ml·min⁻¹·kg⁻¹)</td>
<td>38 ± 7.1</td>
<td>40 ± 4.7‡</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.4 ± 0.3</td>
<td>1.7 ± 0.2∗</td>
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known to interfere with subsequent spectrophotometric measurements. NOx concentrations in different dilutions of plasma ultrafiltrate were determined by a colorimetric assay kit (Cayman, Ann Arbor, MI, U.S.A.) based on a three-step Griess reaction [23]. The results are expressed as µmol/l. The lower limit of sensitivity for the evaluation of plasma NOx concentration by the Griess colorimetric assay was 1 µmol/l. The assay was found to be linear over the range of NOx concentration 0–35 µmol/l, with correlation coefficients greater than 0.99. Coefficients of variation within or between days were lower than 5.5%.

Statistical analysis

Results are expressed as means ± S.D. ANOVA was used to assess the mean differences between groups, followed by Fisher’s post-hoc test. Linear correlation analysis was used to assess relationships between variables. Differences were considered significant at a P value of < 0.05.

All statistical procedures and curve fitting for statistical analysis were performed by means of StatView version 4.57 (Abacus Concepts Inc., Cary, NC, U.S.A.).

RESULTS

Demographic and clinical characteristics of the athletes and sedentary groups are shown in Table 1. V̇O₂max was significantly higher in athletes than in sedentary subjects (P < 0.0001) as expected. NOx values were significantly higher in athletes compared with sedentary control subjects (50.4 ± 16.3 and 39.0 ± 15.4 µmol/l respectively; P < 0.005). Baseline SBF was similar in athletes and controls (hand SBF, 206 ± 36 compared with 190 ± 29 pu respectively, and foot SBF, 225 ± 41 compared with 179 ± 34 pu respectively; not significant). The hand reflow after ischaemia (374 ± 37 compared with 278 ± 24 pu) and after heating (332 ± 29 compared with 271 ± 27 pu), as well as foot flow after heating (378 ± 44 compared with 270 ± 30 pu), were significantly higher in athletes than in sedentary individuals (P < 0.0001 for all measurements).

A positive statistical interaction between age and V̇O₂max was found (P < 0.05). No age–endurance interaction was observed with respect to NOx concentrations or SBF measurements.

Subgroup analysis

We selected the lowest (n = 35; age, 28 ± 3 years) and the highest (n = 27; age, 61 ± 4 years) tertile of age to assess the relationship between age, fitness, V̇O₂max, NOx and SBF measurements at the extremes of the age distribution (Figures 1 and 2).

The subgroups of older and young individuals, when divided by trained status, were well-matched by age and body mass index (BMI; results not shown). As expected, V̇O₂max was higher in both subgroups of trained subjects when compared with sedentary individuals. Young trained subjects exhibited the highest V̇O₂max (66.6 ± 5.6 ml·min⁻¹·kg of body weight⁻¹). The young sedentary subgroup had half the V̇O₂max observed in trained counterparts (36.8 ± 9.8 ml·min⁻¹·kg of body weight⁻¹; P < 0.0001), as expected. Older trained individuals

Figure 1  V̇O₂max and NOx concentrations in the four subgroups

V̇O₂max (upper panel) and NOx (lower panel) were higher in both subgroups of trained individuals. Results are expressed as means ± S.D. Young sedentary, n = 17; young athletes, n = 18; older sedentary, n = 12; older athletes, n = 15. Upper panel, *P < 0.0001 compared with sedentary subgroups; §§P < 0.02 compared with older counterparts; ††P < 0.0002 compared with young athletes. Lower panel, **P < 0.01 and §§§P < 0.002 compared with older sedentary. ns, not significant.
had a \( \dot{V}O_2 \)max 50\% higher than older controls (44.8 ± 5.7 compared with 30.4 ± 4.9 ml·min\(^{-1}\)·kg of body weight\(^{-1}\); \( P < 0.02 \); Figure 1).

Plasma NOx concentrations were higher in both subgroups of trained subjects, which was independent of age, and were slightly higher in sedentary young people compared with sedentary older subgroup (\( P < 0.05 \); Figure 1). Interestingly, physical exercise was not associated with a significantly higher NOx in young individuals; in contrast, endurance training was associated with significantly higher NOx (\( P < 0.002 \)) in older subjects. Young athletes exhibited higher NOx concentrations than older controls (\( P < 0.01 \)). Training removed the difference in NOx concentrations observed between young and older sedentary individuals (Figure 1).

**DISCUSSION**

By studying a group of sedentary and trained subjects with a large age range (22–75 years), we have tried to assess the relationship between age, exercise, NOx concentrations and endothelial function. Our findings have shown that NOx concentrations were lower in older subjects, and that stimulated peripheral SBFs (e.g., after heating or ischaemia) were lower in sedentary people independent of age. Higher microcirculatory function was observed in both groups of young and older individuals who regularly perform physical exercise. This difference was probably due to an increased action of NO and was related to the higher \( \dot{V}O_2 \)max that characterizes regular physical activity.

There is a large amount of experimental and clinical data indicating that endothelial dysfunction is involved not only in disease states such as atherosclerosis, hypertension, heart failure or diabetes, but also in the normal physiological process of aging. Decreased endothelium-dependent vasodilation of the forearm and coronary

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**Figure 2** Hand basal SBF, hand and foot SBF after heating and hand SBF after ischaemia in the four subgroups

Results are expressed as means ± S.D. Young sedentary, \( n = 11 \); young athletes, \( n = 13 \); older sedentary, \( n = 10 \); older athletes, \( n = 12 \). **\( P < 0.004 \), \( * P < 0.0004 \), \( \S P < 0.001 \) and \( \S S P < 0.05 \) compared with sedentary subgroups.

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**Figure 3** Correlation analysis between \( \dot{V}O_2 \)max and NOx in the whole population

Baseline SBF measured at the hand and foot were similar across the four subgroups (Figure 2). Stimulated SBF (after heating or ischaemia) was significantly higher in athletes than in sedentary people in both subgroups (Figure 2).

Differences between groups were essentially the same even after corrections for weight and BMI.

**Correlation analysis**

In the whole group, \( \dot{V}O_2 \)max was negatively correlated with age (\( r = −0.40 \), \( P < 0.002 \)). In sedentary individuals, this relationship was still significant (\( r = 0.37 \), \( P < 0.03 \)), whereas, in athletes, this relationship was more pronounced (\( r = 0.70 \), \( P < 0.0001 \)).

NOx concentrations were positively correlated with \( \dot{V}O_2 \)max (\( r = 0.46 \), \( P < 0.001 \); Figure 3). A direct relationship between NOx concentrations and stimulated SBF was observed (Figure 4).
Nitric oxide and microcirculatory flow in older athletes

Correlation analysis between NOx and SBF in the whole population

Figure 4

—with cardiovascular disease, levels of urinary NO metabolites increase in direct relationship with the gain in functional capacity, suggesting that increased NO production may be a major adaptive mechanism by which aerobic exercise training benefits the cardiovascular system [30].

The mechanisms by which chronic exercise improves vascular NO availability are not completely known. Exercise induces a complex integrated physiological response that involves activation of circulating hormones and local autacoids, such as catecholamines, adenosine and ATP, which could exert an influence in the long-term regulation of NO biosynthesis. In addition, exercise increases myocardial oxygen consumption and reduces vascular resistance at the coronary level. This effect will elicit endothelial shear stress, which may increase endothelial NO synthase (eNOS) expression and activity [31]. In line with this view, in vitro studies indicate that shear stress increases eNOS expression in endothelial cells [32,33]. Interestingly, shear-stress-responsive elements have been found in the eNOS gene promoter, indicating that shear stress can transcriptionally regulate eNOS expression [34]. Other mechanisms implicated in the increased availability of NO include the activation of Ca2+ -activated K+ channels [29] and the increased expression of eNOS facilitated by exercise-induced expression of antioxidant enzymes [35,36].

In the present study, we observed that older trained subjects have significantly higher NOx plasma concentrations than matched sedentary controls. Similar results have been reported previously by us [12] and other groups [13–15]. In the group of older trained individuals, NOx concentrations were similar to that observed in young people. This effect of exercise on NOx concentrations was not observed in young individuals, suggesting that eNOS in young people is expressed at a higher level of activity independently of the trained status. Accordingly, previous data [12] have shown that endothelium-dependent vasodilation is preserved in a young population and cannot be improved by chronic aerobic exercise training. We have reported previously [12] in elderly subjects that chronic physical activity improves endothelial function (as measured by venous plethysmography) by preventing oxidative stress and restoring NO availability.

The use of the laser Doppler techniques to analyse the microcirculatory SBF has been validated in previous studies [37,38]. In fact, in patients with essential hypertension, SBF measured with laser Doppler flowmetry was increased by acetylcholine infusion in parallel with that observed with strain-gauge venous plethysmography [37]. In patients with Raynaud disease, an NO-generating system increased microcirculatory SBF as assessed by photoplethysmography [38].

In the present study, NOx concentrations were significantly related to post-heating and post-ischaemia SBF,
suggesting that NOx participates in the post-stimulus regulation of blood flow, but the relatively low correlation coefficient (r ~ 35) suggests that other factors are implicated in the improvement of stimulated SBF observed in subjects performing chronic exercise. In this regard, other mediators that are modified by training, such as prostaglandins [39], endothelin [40] and the oxidant/antioxidant status [35], may be implicated.

It is possible that the beneficial effects of regular physical training could be mediated by the increase in NO availability and action, and this could be the basis for the beneficial effects of exercise in some clinical conditions associated with low fitness level, such as aging. It has been reported previously [41] that endothelial NO action could modulate sympathetic activity in humans. In this context, it is possible that regular exercise may improve endothelial function by reducing the chronic suppressive influence exerted by sympathetic-adrenergic tone either directly or by enhancing the sympathoinhibitory effect of NO.

In conclusion, the present study suggests that chronic regular aerobic training is associated with higher NO generation in both young and older subjects. These changes are associated with higher microcirculatory endothelial-dependent function.

REFERENCES


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