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ORIGINAL ARTICLE

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Enhanced endothelium-dependent vasodilatation in human skin vasculature induced by physical conditioning

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Abstract Functional alterations to the endothelial cells of the vascular system may contribute to the improved circulatory performance induced by physical conditioning. We evaluated microvascular reactivity to iontophoretic application of acetylcholine (ACh) and sodium nitroprusside (SNP) through the skin and blood perfusion measurements in the same area using laser Doppler flowmetry. Whereas ACh acts on smooth muscle cells of the vascular system via the production of vasodilator substances from the endothelium, SNP is an endothelium-independent vasodilator acting on vascular smooth muscle cells directly. The study was performed using two groups of subjects with different levels of aerobic endurance, long distance runners competing at national level ($n = 9$) and controls ($n = 9$). The subjects were tested for 40 min on a treadmill before and after an exercise test at 80% of their maximal oxygen uptake. During stimulation by ACh cutaneous perfusion increased to a higher level in the athletes than in the controls (overall $P < 0.05$), whereas an acute period of exercise abolished this difference (overall $P > 0.6$). There was no significant difference between the athletes and the controls with respect to the SNP-induced increase in cutaneous perfusion either before ($P > 0.9$) or after ($P > 0.9$) exercise. The higher cutaneous perfusion responses to stimulation with ACh in the athletes than in the controls may support the hypothesis that regular exercise modifies the responsiveness of the cutaneous endothelium. The difference in ACh-induced perfusion and in unstimulated forearm perfusion between the two groups was present only at rest. This finding indicated that mechanisms were introduced during exercise, which compensated for the lower endothelial sensitivity to stimulation in the controls at rest.

Key words Acetylcholine · Cutaneous blood flow · Endothelium-dependent vasodilatation · Physical exercise · Laser Doppler flowmetry

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Introduction

Research on the mechanisms behind the improved circulatory performance induced by physical conditioning in humans has mainly focused on cardiac function, pulmonary capacity and structural alterations of the microvasculature (Blomquist and Saltin 1983; Johnson 1989). However, it is also possible that control of the vascular system can be modified by physical exercise. One potential mechanism would be that repetitive vasodilatations induced by exercise sessions may produce adaptive changes in the intrinsic responsiveness of the vascular endothelium.

The properties of the vasodilatation of the endothelial system can be assessed by stimulation with acetylcholine (ACh). This method is based upon the novel discovery of

Furchgott and Zawadzki (1980) that the rabbit aorta dilates in response to the application of ACh only in the presence of an intact endothelium. Their experiments have suggested the existence of a mediator diffusing from endothelial cells to vascular smooth muscle cells and causing vasodilatation. Several years later it was discovered that this "endothelial-derived relaxing factor" was identical to nitric oxide (NO; Ignarro et al. 1987; Palmer et al. 1987). This ACh stimulation has been used to demonstrate impaired endothelium-mediated vasodilatation in diabetes mellitus, essential hypertension, hypercholesterolaemia, heart failure, and atherosclerosis (Drexler 1997; Andreassen et al. 1998).

Recent studies have demonstrated enhanced vascular responsiveness to endothelium-dependent vasodilators in skeletal muscle in exercise-trained subjects (Delp 1995; Kingwell et al. 1996), whereas the cutaneous responsiveness to endothelium-dependent vasodilators in response to exercise has not been tested. Increased flow and the correspondingly increased shear stress to the vessel wall have been found to be stimuli which elicit vasodilatation in the resistance vessels of skeletal muscle (Miller and Vanhoutte 1988; Koller and Kaley 1991). From this discovery and with the knowledge that increased core temperature during exercise results in an increased thermoregulatory contribution to the perfusion, we anticipated that adaptive changes induced by physical conditioning could also be detected in the cutaneous vasculature.

The aim of the present study was to test the hypothesis that physical conditioning enhances endothelial responsiveness to vasodilators in human cutaneous vasculature. The responsiveness to the endothelium-dependent substance ACh delivered iontophoretically through the forearm skin was compared with the endothelium-independent vasodilator, sodium nitroprusside (SNP), in two groups of healthy, male subjects of different levels of aerobic conditioning. Blood perfusion was recorded in the same area using the *non invasive* technique of laser Doppler flowmetry (LDF).

Methods

Subjects

The subjects used in the experiment were nine male long-distance runners (athletes) who had competed in national events for more than 5 years. They were tested in June while undergoing intense training [median 11.0 (range 8.5–17.0) hours a week; median 8.5 (range 5.0–11.0) times a week] and competing in at least one event each week. Their training program during this period included intense interval training. The subjects acting as controls comprised nine healthy, less well-trained soldiers (controls) who performed some physical activity [median 1.5 (range 0.5–7) hours a week; median 1.5 (range 0.5–3.5) times a week]. The subjects had not taken any medication during the week prior to the study. None of the subjects were smokers. Exclusion criteria were a history of cardiovascular disease or other illness. After being informed of the design of the study they gave their written consent. The study was approved by the local Ethics Committee.

Aerobic endurance level

The maximal oxygen uptake ($\dot{V}O_{2\,max}$) was measured during running on a motor driven treadmill at 3° uphill gradient (Hermansen 1973) using the Sensor Medics oxygen analyser (MMC Horizon System, USA). The oxygen uptake ($\dot{V}O_2$) was measured at 4–5 different submaximal levels, each level being measured for 4 min. Heart rate was recorded continuously using an electro-cardiograph (Sirekust 341, Siemens, Germany).

Laboratory procedure

All the subjects were asked to refrain from strenuous exercise for 24 h before the study to avoid the short-term effects of exercise. The $\dot{V}O_{2max}$ test procedure was therefore performed at least 24 h prior to the exercise test. Food intake before the exercise test was restricted to a light meal 2 h prior to the exercise test. The LDF measurements were carried out in a room with the temperature maintained constant at 22 (21–23)°C with the subjects in a supine position. At least 20 min were allowed for the subjects to become accustomed to the room temperature and the situation before the LDF measurements were made on the skin of the left forearm. Skin perfusion was measured immediately before and from 15 min after the exercise test. Skin temperature was measured using a digital skin thermometer (Fluke 2190, John Fluke, USA). The exercise test was performed on a treadmill at 3° uphill gradient for 40 min at 80% of the subjects' individual $\dot{V}O_{2max}$ after warming up for 10 min at 50% of their $\dot{V}O_{2max}$. The entire procedure is given in Table 1.

Plasma lactate

Before starting the exercise test and immediately after its finish blood from antecubital veins was drawn with minimal stasis by repetitive venipuncture using a 20-G needle and placed in pre-cooled plastic tubes containing 3.8% sodium citrate in a 9:1 blood:anticoagulant ratio. The blood was stored on ice (maximum 20 min) before centrifugation (20 min at 22°C and 1500 *g*) and the plasma samples were stored in aliquots at –70°C until further analysis.

Determination of packed cell volume, haemoglobin concentration and mean cell volume (MCV in ethylenediaminetetra-acetic acid EDTA) anticoagulated blood were made on an automatic analysing device (Coulter counter model S plus STKR, Coulter Electronics, Inc., Fl., USA) in EDTA blood. The plasma volume changes were determined according to an equation by which short-term changes in plasma volume can be calculated from changes in the PCV provided that no changes are observed in MCV (Van Beaumont 1972). Plasma lactate concentrations after the exercise test were adjusted for the changes in plasma volume observed after exercise.

Table 1 The laboratory procedure. $\dot{V}O_{2\,max}$ Maximal oxygen uptake, *LDF* laser Doppler flowmetry, *ACh-b* iontophoresis with acetylcholine (ACh) before running, *SNP-b* iontophoresis with sodium nitroprussid (SNP) before running, *ACh-a* iontophoresis with ACh after running, *SNP-a* iontophoresis with SNP after running, $\dot{V}O_{2,50\%}$, $\dot{V}O_{2,80\%}$ 50% and 80% of $\dot{V}O_{2\,max}$

Day 1:
$\dot{V}O_{2\,max}$-test
Day 3 or later:
22-min resting LDF
22-min ACh-b
22-min SNP-b
3-min blood sampling
10-min warming up at $\dot{V}O_{2,50\%}$
40-min exercise test at $\dot{V}O_{2,80\%}$
3-min blood sampling
22-min ACh-a
22-min SNP-a

Laser Doppler flowmetry

The principles of LDF have been thoroughly described elsewhere (Nilsson et al. 1980): LDF gives a semiquantitative assessment of microvascular blood perfusion, which is expressed in arbitrary units (a.u.). The LDF measurements from the skin reflect blood flow in capillaries, arterioles, venules and dermal vascular plexa; they have also been shown to reflect a minor nutritive and a major thermoregulatory part of the perfusion (Bollinger et al. 1991). A commercially available monitor was used for LDF (MBF 3D, Moor Instruments, Axminster, Devon, UK). A sampling frequency of 40 Hz and a time constant of 0.1 s were selected. The LDF measurements were obtained with two fibers, using an optic probe (P10A, Moor Instruments, England), and the data were stored on a personal computer.

Iontophoresis

The technique of iontophoresis allows polar drugs to cross the skin using a small, direct current. It has been shown to be possible to assess reactivity of the microvascular system when blood perfusion is being measured simultaneously in the same area (Müller et al. 1987; Westermann et al. 1988; Andreassen et al. 1998).

A combined probeholder, for iontophoresis and perfusion measurement, was fixed with double-sided adhesive tape on the volar side of the right forearm (Fig. 1) after the skin had been cleaned with isopropyl alcohol and left to dry in the air. The perspex probeholder had a small chamber for deposition of the test solutions which was then in direct proximity to the laser Doppler probe. A battery powered constant current stimulator (MIC 1, Moor Instruments Ltd, England) was used to provide a direct current for the drug iontophoresis. The active electrode was made of platinum, and charged according to the active ions of the drug. Quantities of 1% solutions of ACh (E. Merck, Germany) and SNP (E. Merck, Germany) were used. For ACh anodal current was used to transfer the cation (ACh^+) during iontophoresis, and for SNP cathodal current was applied to transfer the anion as has been described by Müller et al. (1987) and Westermann et al. (1988). A reference electrode was attached to the wrist of the right arm of the subjects.

The doses of drugs delivered were directly proportional to the total charge in millicoulombs which migrates through the skin surface, determined from the product of the constant current measured in milliampers and the duration of current flow in seconds. By applying small currents of brief duration, a transfer of vasoactive drugs into the epidermis beneath the chamber was accomplished. Drug doses were altered by varying the amount or the time of the current. To avoid current-induced stimulation of local sensory nerves, currents higher than 200 mA or total charges greater than 8 mC were avoided (Westermann et al. 1988). Based on pilot studies and earlier recommendations (Westermann et al. 1988) we made dose-response curves for both ACh and SNP, using charges of 0.75 mC (75 mA for 10 s), 1.5 mC (150 mA for 10 s), 3.0 mC (150 mA for 20 s) and 6.0 mC (200 mA for 30 s) with the response being measured for 300 s after each charge of the iontophoresis (Fig. 2a, b). These charges produced a stepwise increase in laser Doppler perfusion, reaching a saturation level at 6.0 mC (Fig. 2a, b). We subtracted the value of the perfusion during the unstimulated state from the response values obtained during iontophoresis of different doses.

For each subject five curves were recorded. One curve was obtained during 22 min of rest, and four curves were obtained during iontophoresis. Calibration of the laser Doppler equipment was checked before measurements on each of the test subjects. The different doses of the same substance (ACh or SNP) were applied at the same location. The test positions for ACh and SNP were separated by at least 5 cm. The chamber used in all the experiments allowed a skin area of 0.64 cm^2 to be treated.

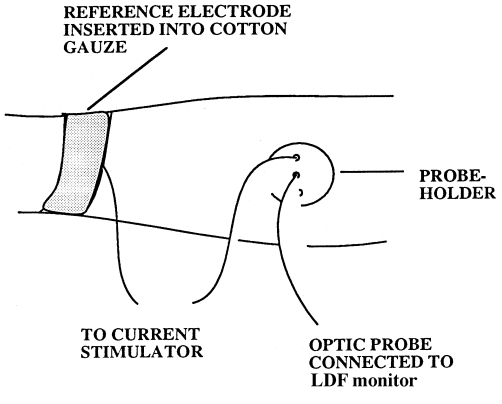

Fig. 1 Iontophoresis procedure on human forearm: arrangement of laser Doppler flowmeter probe holder and electrode for evoking iontophoretic vasodilatation responses

Statistical analysis

Data are illustrated either as group median with range, or as box plots. The five horizontal lines at the boxes are the 10, 25, 50, 75, and the 90th percentiles. Values above or below the 10th and 90th percentile are represented as data points. A two-way analysis of variance (ANOVA; repeated measure design) was used to compare the skin perfusion data between the athletes and the controls. The repeated measures were performed on the data after transformation to obtain normal distribution and equal variance in the two groups. When differences were obtained, post-hoc analyses were performed using the Mann-Whitney test for non-parametric comparisons between the two groups at the different doses. The Mann-Whitney test for comparison of independent samples was also used to evaluate differences between the athletes and the controls in

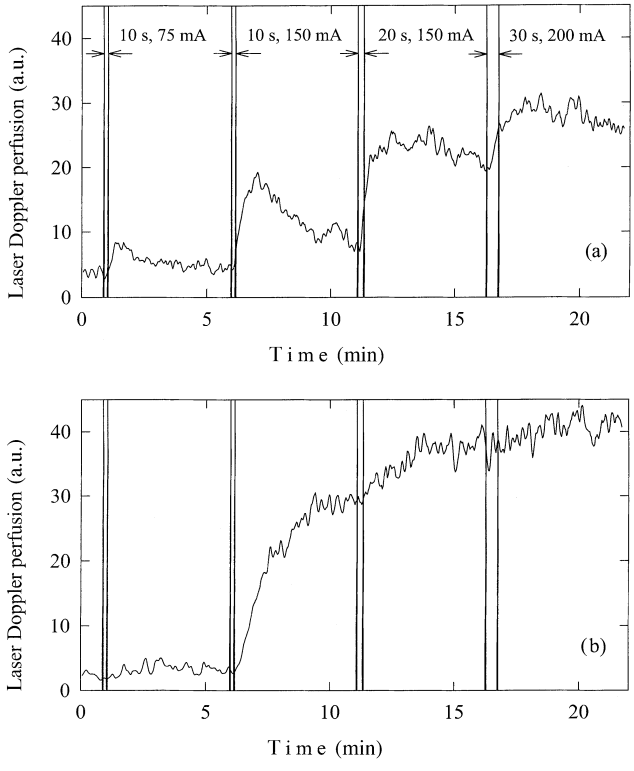

Fig. 2 Laser Doppler perfusion of the response to increasing concentrations of acetylcholine (**a**) and sodium nitroprussid (**b**). The dose-response curves were made by using the charges of 0.75 mC, 1.5 mC, 3.0 mC, and 6.0 mC. The response measuring period for each dose was 300 s

anthropometric and performance data. Statistically significant differences were defined as $P < 0.05$.

Results

Anthropometric and performance data

Anthropometric and performance data of both groups are summarized in Table 2. We found a lower resting heart rate and mean anterial pressure in the athletes compared to the controls, whereas no difference in skin temperature was seen. The lactate concentration was higher in the controls than in the athletes after exercise.

Basal perfusion in the unstimulated state at rest and after exercise

Before exercise perfusion of the forearm skin was significantly higher in the athletes than in the controls [median 5.3 (range 3.6–6.9) vs median 3.1 (range 2.3–4.5), $P < 0.005$]. Both groups had a higher skin perfusion after exercise, compared to the values before exercise ($P < 0.05$). After exercise there were no significant differences between the groups either before the iontophoresis with ACh [median 14.5 (range 6.6–44.0) vs median 10.0 (range 4.3–15.4) $P > 0.3$] or before the iontophoresis with SNP [median 6.9 (range 4.0–15.5) vs median 5.2 (range 4.8–7.0), $P > 0.3$].

Effects of ACh

Iontophoresis with ACh before standardized exercise induced a significant dose-dependent increase in skin perfusion in both the athletes and the controls ($P < 0.05$ for both groups; Fig. 3a). This ACh-induced increase in skin perfusion was higher in the athletes than in the controls (overall $P < 0.05$), giving a significant difference for a dose of 0.75 mC ($P < 0.05$). At a dose of 1.5 mC P equalled 0.08. Iontophoresis with ACh after standardized exercise produced a significant dose-dependent increase in skin perfusion in both athletes and controls ($P < 0.03$ for both groups), but there was no significant difference between the two groups (overall $P > 0.6$) (Fig. 3b).

Effects of SNP

A significant dose-dependent increase in skin perfusion during iontophoresis with SNP was demonstrated in both the athletes and the controls ($P < 0.03$ for both groups), but there was no significant difference between the two groups (overall $P > 0.9$; Fig. 4a). Also after exercise, a dose-dependent increase in skin perfusion was demonstrated in both the athletes and the controls ($P < 0.03$ for both groups). There was no significant difference between the two groups (overall $P > 0.9$; Fig. 4b).

Table 2 Anthropometric and performance data of athletes and controls. $\dot{V}O_{2\,max}$ Maximal oxygen uptake, $\dot{V}O_{2,80\%}$, 80% of $\dot{V}O_{2\,max}$, MAP mean arterial blood pressure, ΔPV plasma volume changes in response to running

	Athletes ($n = 9$)		Controls ($n = 9$)	
	Median	Range	Median	Range
Age (years)	26	18–32	20	19–21[a]
Body mass (kg)	76	70–79	75	70–90
Height (cm)	187	171–192	180	176–197
Heart rate (beats · min^{-1})				
Pre-exercise	51	44–60	57	51–72[a]
15-min post-exercise	63	52–78	95	78–105[b]
At $\dot{V}O_{2\,max}$	189	181–194	199	193–212[b]
MAP (mmHg)				
Pre-exercise	106	87–113	91	79–100[a]
15-min post-exercise	92	82–109	90	80–95
Skin temperature (°C)				
Pre-exercise	33.1	32.3–34.9	33.4	32.1–34.4
Post-exercise	33.9	32.3–35.3	33.7	33.2–34.1
$\dot{V}O_{2\,max}$ (ml · kg^{-1} · min^{1})	68.9	62.0–73.0	51.5	44.4–61.4[c]
Running velocity at $\dot{V}O_{2,80\%}$ (m · min^{-1})	227	217–243	177	143–198[c]
ΔPV (%)	−14.3	−14.6 to −14.3	−13.4	−15.7 to −1.9
Lactate (mmol · l^{-1})				
Pre-exercise	0.7	0.3–1.0	0.5	0.3–0.7
Post-exercise	1.9	0.7–5.6	3.2	1.0–6.2

[a] $P < 0.05$, [b] $P < 0.005$, [c] $P < 0.0001$

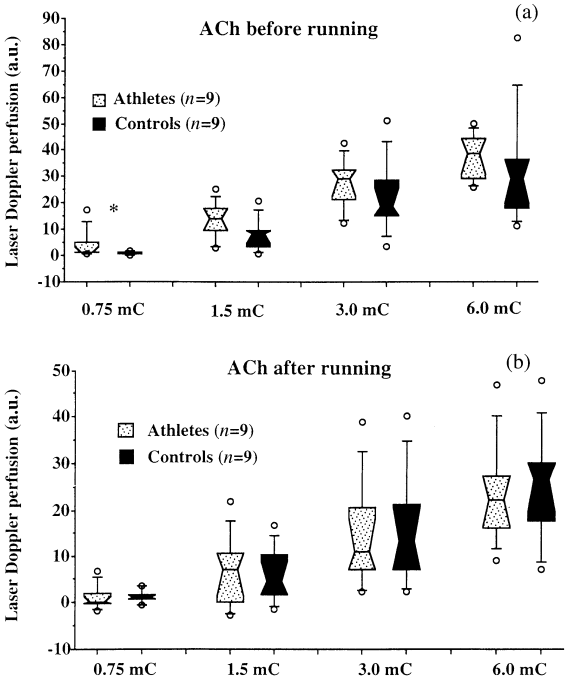

Fig. 3 Laser Doppler perfusion in response to iontophoretic applications of acetylcholine (*ACh*) before (**a**) and after (**b**) running in athletes and controls. The *five horizontal lines on the box* show the 10, 25, 50, 75, and the 90th percentiles. The values above or below the 10th and 90th percentile are represented as data points. * $P < 0.05$ (ANOVA, repeated measure design)

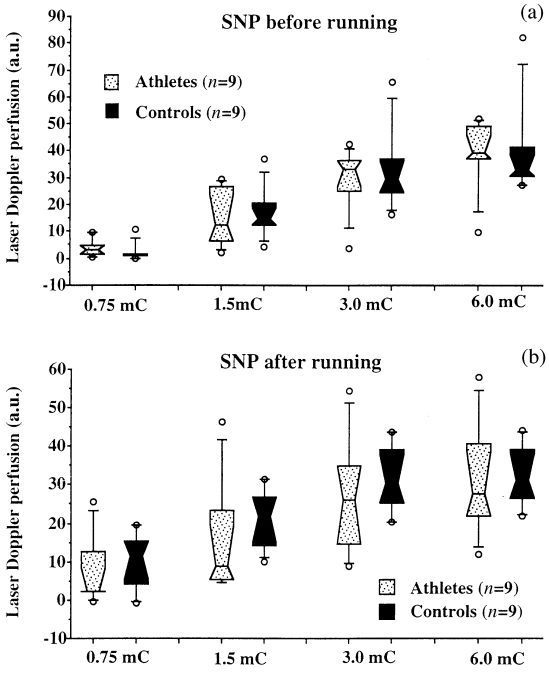

Fig. 4 Laser Doppler perfusion in response to iontophoretic applications of sodium nitroprussid (*SNP*) before (**a**) and after (**b**) running in athletes and controls. The *five horizontal lines on the box* show the 10, 25, 50, 75, and the 90th percentiles. The values above or below the 10th and 90th percentile are represented as data points

Discussion

Assessing endothelium-dependent vasodilatation in forearm skin following iontophoresis with ACh, the present data indicated increased responsiveness of the vascular endothelium in the athletes at rest, compared to the less well-trained controls. Endothelium-independent responses following iontophoresis with SNP, however, were similar to those of the control subjects.

From comparable levels of perfusion in unstimulated skin graded iontophoretic administration of ACh and SNP resulted in successive increases in perfusion in both groups. The observation that ACh-induced skin perfusion responses at rest were significantly higher in the athletes than in the controls was consistent with our hypothesis that the endothelium of the microvascular system becomes more susceptible to stimulation as aerobic capacity increases. These results are in agreement with a recent study using venous occlusion pletysmography in humans (Kingwell et al. 1996), in which intra-arterial infusion of ACh produced higher perfusion responses in athletes than in controls. Enhanced ACh-induced vasodilatation after endurance training has also been demonstrated in the thoracic aorta and pulmonary artery of the rabbit (Chen and Li 1993), as well as in the abdominal aorta of the rat (Delp et al. 1993). Recently ACh-induced vasodilatation in human skin has been shown to correlate closely to peak $\dot{V}O_2$ in heart transplant recipients (Andreassen et al. 1998).

The exact mechanisms underlying the ACh-induced vasodilatation in human cutaneous vasculature may differ from that observed in other vessels. Whereas ACh facilitates vasodilatation indirectly via the conversion of L-arginine to NO in the vascular endothelium of the aorta and arterioles (Moncada et al. 1991), ACh in the cutaneous circulation may in addition induce endothelium-dependent vasodilatation via other pathways. An in vitro study of human subcutaneous resistance vessels has demonstrated that both NO and prostaglandins may be involved in ACh-induced relaxation (Richards et al. 1990). In addition, Morris and Shore (1996) have concluded in their study of the mechanisms underlying ACh-induced responses of cutaneous blood flow that mediators other than prostaglandins and sensory nerve activation may be involved in skin perfusion following iontophoresis with ACh. Kreidstein et al. (1992) in their study of skin flaps have demonstrated the presence of endothelium-dependent and endothelium-independent vasodilatation. They have convincingly shown that the vascular relaxation effect of ACh was significantly reduced by inhibitors of NO synthesis; however, the ACh-induced vasodilatation was not completely blocked. To what extent adenosine, prostaglandins, endothelium-dependent hyperpolarization factor and other substances contribute to the ACh-induced vasodilatation in the cutaneous vasculature remains to be investigated.

To evaluate whether physical conditioning makes smooth muscle cells of the vascular system more sensi-

tive to vasodilators, we have compared vasodilatation induced by SNP in athletes and controls. The SNP has been shown to evoke vascular relaxation by directly increasing guanosine 3′,5′-cyclic monophosphate in vascular smooth muscle cells (Rapoport et al. 1983). Our data showed almost identical SNP-induced increases in skin perfusion in the two groups both at rest and after exercise. This would indicate that the enhanced ACh-induced skin perfusion before exercise was not due to enhanced sensitivity of vascular smooth muscle cells to vasodilators, but rather to increased levels of endothelial factors (most probably NO) reaching vascular smooth muscle cells. This is in agreement with previous studies on animals (Delp et al. 1993) and humans (Kingwell et al. 1996), in which a similar sensitivity to SNP was found at rest for both trained and untrained subjects.

Our results demonstrated enhanced ACh-induced perfusion in athletes compared to controls at rest. No such differences were, however, observed for the responses to SNP. Increased flow and correspondingly increased shear stress to the vessel wall have been shown to be stimuli which elicit vasodilatation in the resistance vessels of skeletal muscles (Miller and Vanhoutte 1988; Koller and Kaley 1991). We hypothesized that the increased muscle blood flow during exercise could be detected indirectly in the perfusion of forearm skin, since increased core temperature provides an increased thermoregulatory contribution to the perfusion. The skin temperature did not differ between the two groups. The observed difference in ACh-induced perfusion at rest therefore would indicate a true difference in the sensitivity of the endothelial cells to inducing vasodilatation, probably as a result of repetitive increases in blood flow during training sessions.

We could find no data after exercise which were comparable to the ACh-responses obtained before exercise. However, a previous study has demonstrated a minor contribution of NO to exercise-induced vasodilatation (Gilligan et al. 1994). In muscles, an increased concentration of metabolic products, altered mechanical forces and changes in the neurohumural milieu, have all been postulated to be important in the development of adaptations in the endothelium which occur with exercise training (Delp 1995). In the present study we demonstrated higher plasma lactate concentrations in the controls than in the athletes after exercise. We therefore speculate that the smaller cutaneous responses to the endothelium-dependent vasodilator ACh in the controls may have indicated an inadequate capacity of the endothelium to induce relaxation of vascular smooth muscle in the small arterioles of exercising muscle, which led to hypoperfusion and increased lactate concentrations.

To what extent lactate or other substances in the plasma contribute to the exercise-induced vasodilatation in the cutaneous vasculature remains unknown. However, there may be factors which make the vessels more sensitive to vasodilator stimuli after repetitive periods of physical exercise. The finding that ACh-induced skin perfusion at rest is significantly higher in athletes than in controls may therefore have relevance not only to the understanding of exercise physiology, but it may also have therapeutic implications for diseases with impaired endothelium-dependent vasodilatation, such as diabetes mellitus, hypertension, hypercholesterolaemia, atherosclerosis and heart failure. Regular exercise may be recommended for sufferers of these diseases as a non-pharmacological approach to restore endothelium-dependent dilatation.

Study limitations

It has been suggested that vasodilatation obtained by iontophoresis with ACh vehicle and SNP vehicle may also stimulate local sensory nerves (Morris et al. 1996; Andreassen et al. in press). However, a study using the same dose-response curve as in the present study has demonstrated that the drug vehicle had significant influence on increases in skin perfusion from the third iontophoresis of ACh and from the second iontophoresis of SNP (Andreassen et al. 1998). Thus, the observed difference between the athletes and the controls in the present study represented a true difference since the difference was observed at the lowest dose of ACh.

We chose to test the subjects at 80% of their individual $\dot{V}O_{2\,max}$, assuming that the exercise intensity would then be equal in the two groups. This standardization resulted in a lower running speed in the controls compared to the athletes. Even though we made this standardization, we found a higher plasma lactate concentration after exercise in the controls than in the athletes. However, the differences demonstrated between the two groups after exercise would probably have been even more pronounced if they had been tested at the same speed. In cross-sectional study designs one also has to bear in mind potential differences in genetic, dietary and other life-style factors between athletes and controls, which may alter endothelial function.

In conclusion, physical conditioning resulted in enhanced endothelium-dependent vasodilatation in the cutaneous vasculature, as demonstrated by the higher ACh-induced perfusion among the athletes compared to the controls. The unaltered response to SNP showed that differences in vascular smooth muscle responsiveness for vasodilatation did not account for this difference. The difference in ACh-induced perfusion and in forearm perfusion in the unstimulated state between the two groups was present only at rest. This finding would indicate that mechanisms are introduced during exercise, which compensate for the lower endothelial sensitivity to stimulation as seen in the controls at rest. The observed difference between the groups illustrates the applicability of cutaneous LDF measurements to investigations such as these.

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