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Haematopoietic stem cells and endothelial progenitor cells in healthy men: effect of aging and training

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Summary

The number of hematopoietic stem cells (HSC) and endothelial progenitor cells (EPC) is thought to be a marker for neovascularization and vascular repair. Because physical inactivity and aging are risk factors for cardiovascular diseases, these factors may influence the numbers of HSCs and EPCs. Therefore, we examined baseline and exercise-induced levels of HSCs and EPCs in sedentary and trained young and older men. To study the role of aging in eight sedentary young (19–28 years) and eight sedentary older men (67–76 years), baseline and acute exercise-induced numbers of HSCs (CD34⁺-cells) and EPCs (CD34⁺/VEGFR-2⁺-cells) were quantified by fluorescence-activated cell sorter (FACS) analysis. To examine the effect of chronic training, eight age-matched trained young men (18–28 years) were compared with sedentary young men, whereas older men performed an 8-week endurance training. Older men showed significantly lower baseline and exercise-induced levels of HSCs/EPCs than the young men ($P < 0.05$). In young and older men, acute exercise significantly increased HSCs ($P < 0.01$), but not EPCs. The absolute increase in numbers of HSCs was attenuated in older men ($P = 0.03$). Apart from the lower baseline numbers of EPCs after chronic training in older men, training status did not alter baseline or exercise-induced levels of HSCs/EPCs in young and older men. We concluded that advancing age results in lower circulating numbers of HSCs and EPCs and attenuates the acute exercise-induced

increase in HSCs. Interestingly, in young as well as in older men chronic endurance training does not affect baseline and exercise-induced numbers of HSCs and EPCs.

Key words: aging; endothelial progenitor cell; endurance training; haematopoietic stem cell; single exercise bout.

Introduction

Recent evidence suggests that vascular morphology and function can be modulated by bone-marrow derived haematopoietic stem cells (HSC, CD34⁺) and endothelial progenitor cells (EPC, CD34⁺/VEGFR-2⁺) (Asahara *et al.*, 1997). In preclinical studies, it was shown that HSCs and EPCs can enhance angiogenesis, promote vascular repair, improve endothelial function, and induce neovascularization (Orlic *et al.*, 2001; Hill *et al.*, 2003; Rauscher *et al.*, 2003; Urbich & Dimmeler, 2004). HSCs and EPCs may play a role in the vascular responses and adaptations to exercise. A single exercise bout acutely mobilises circulating HSCs in healthy men (Laufs *et al.*, 2005; Morici *et al.*, 2005) and in subjects with cardiovascular disease (Adams *et al.*, 2004; Laufs *et al.*, 2004; Rehman *et al.*, 2004; Steiner *et al.*, 2005). Based on the fact that advancing age is associated with an impaired vascular function (Celermajer *et al.*, 1994; DeSouza *et al.*, 2000), the acute exercise-induced mobilization of HSCs may be influenced by age. Heiss *et al.* (2005) recently reported that advancing age is associated with a *functional* deficit of stem cells (such as proliferation, survival, and migration). The primary aim of the present study is to examine the effect of advanced age on the *numbers* of HSCs and EPCs in young and older men before ('baseline') and after ('acute exercise-induced') a single exercise bout (Fig. 1). We hypothesize that advancing age reduces baseline and acute exercise-induced levels of HSCs and EPCs.

Increased levels of HSCs and EPCs are associated with an enhanced endothelial function (Hill *et al.*, 2003) and a decrease in cardiovascular risk (Hill *et al.*, 2003; Werner *et al.*, 2005). Interestingly, endurance training is associated with an improved endothelial function (Green *et al.*, 2004) and a decrease in cardiovascular risk (Paffenbarger *et al.*, 1986) as well. As such, one may speculate about a possible influence of the training status (i.e. trained vs. untrained) on the baseline or exercise-induced numbers of HSCs and EPCs. Therefore, the second aim of the study is to examine baseline and exercise-induced numbers of HSCs and EPCs in an age-matched population of sedentary and endurance trained young men. We extended our observations on the possible link between training status and numbers of HSCs and EPCs by training the older men for 8 weeks (Fig. 1). We hypothesize that a higher-training status leads to an elevation in baseline and exercise-induced numbers of HSCs and EPCs in young as well as in older men.

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Table 1 Group characteristics of the sedentary ($n = 8$) and trained ($n = 8$) young men and the older men ($n = 8$) before (sedentary) and after (trained) the 8-week exercise training (mean \pm SE). (HDL, high-density lipoprotein; LDL, low-density lipoprotein; VO_{2max} , maximal oxygen consumption; bpm, beats per minute; RER, respiratory exchange ratio; HSC, haematopoietic stem cell; EPC, endothelial progenitor cell)

	Young men		Older men ($n = 8$)	
	Sedentary ($n = 8$)	Trained ($n = 8$)	Sedentary	Trained
Activity, h/week	0.8 \pm 0.9	10.4 \pm 2.6*	0.5 \pm 0.8	2.5 \pm 0.8*
Body mass index	22.4 \pm 1.7	22.8 \pm 1.2	25.4 \pm 2.6†	25.2 \pm 2.6
Cholesterol, mmol L ⁻¹	4.4 \pm 1.4	4.2 \pm 0.8	4.8 \pm 0.4	4.6 \pm 0.1
Triglycerides, mmol L ⁻¹	1.5 \pm 0.8	0.9 \pm 0.4	1.0 \pm 0.3	1.0 \pm 0.2
HDL, mmol L ⁻¹	1.2 \pm 0.3	1.6 \pm 0.2*	1.2 \pm 0.2	1.2 \pm 0.1
LDL, mmol L ⁻¹	2.5 \pm 1.1	2.2 \pm 0.7	3.2 \pm 0.3	3.0 \pm 0.1
Incremental exercise test				
HR rest, bpm	62 \pm 6	54 \pm 7*	60 \pm 4	52 \pm 3*
HR max, bpm	194 \pm 11	195 \pm 8	165 \pm 7	166 \pm 8
VO_{2max} , mL/kg min ⁻¹	49.0 \pm 4.0	58.5 \pm 7.5*	30.8 \pm 4.8†	33.3 \pm 5.5
Workload max, W	341 \pm 41	393 \pm 53*	171 \pm 32†	201 \pm 39*
Lactate max, mmol mL ⁻¹	9.1 \pm 1.6	12.0 \pm 1.9*	7.7 \pm 2.1	7.6 \pm 1.2
RER	1.24 \pm 0.04	1.19 \pm 0.04*	1.20 \pm 0.13	1.17 \pm 0.06
Resting values stem cells				
HSC, cells mL ⁻¹	412 \pm 70	355 \pm 95	119 \pm 21†	95 \pm 17
EPC, cells mL ⁻¹	154 \pm 43	185 \pm 96	35 \pm 12†	19 \pm 8*

* $P < 0.05$ (sedentary vs. trained).

† $P < 0.05$ (sedentary young vs. sedentary older men).

Results

Effect of aging on HSC and EPC in sedentary healthy men

Maximal oxygen consumption in sedentary young men was significantly higher than in older men, while body-mass index was significantly lower in young than in older men (Table 1). Lipid profile was not different between both groups.

Baseline

Numbers of HSCs and EPCs in young men were significantly higher than in older men (t -test, $P < 0.05$, Table 1). Plasma vascular endothelial growth factor (VEGF) concentration in young men was significantly lower than in older men (Table 2).

Single exercise bout

The single exercise bout in sedentary young and older men significantly increased the numbers of HSCs (Fig. 2A). Numbers of EPCs in young and older men did not change following an acute exercise bout (Fig. 2B). Plasma levels of VEGF in young men were significantly increased by cycling, while older men showed no change (Table 2).

Effect of training status on HSC and EPC in young men

Maximal oxygen consumption, maximal workload, blood lactate production, and HDL in the trained young men were significantly

Table 2 Plasma concentrations VEGF (in pg mL⁻¹) in sedentary ($n = 8$) and trained ($n = 8$) young men and the older men ($n = 8$) before (sedentary) and after training (trained). P -value represents a Student's t -test (in young men) or a Mann-Whitney U -test (in older men) to assess differences between baseline and exercise-induced (post-EX). Values are means (lowest value–highest value) for the young men and median (lowest value–highest value) for the older men

	Baseline	Post-EX
Young men ($n = 8$)		
Sedentary	255 (209–359)	395 (275–754)†
Trained	369 (169–723)	394 (218–551)
Older men ($n = 7$)		
Sedentary	1239 (488–20 316)*	1429 (591–20 872)
Trained	569 (172–18 205)*	590 (244–12 561)

* $P < 0.05$ vs. young men.

† $P < 0.05$ baseline vs. postexercise.

higher than in the sedentary young men. Resting heart rate and the respiratory exchange ratio (RER) were significantly lower in trained than in sedentary young men. Other characteristics did not differ between both groups (Table 1).

Baseline

Circulating numbers of HSCs and EPCs (Table 1), and VEGF plasma levels (Table 2) at baseline were similar between sedentary and trained young men.

Single exercise bout

The acute exercise-induced increase in numbers of HSCs in sedentary and trained young men was not influenced by chronic training (Fig. 3A). Numbers of EPCs in sedentary and trained young men did not change following the single exercise bout (Fig. 3C). Plasma levels of VEGF in trained young men did not change by the acute exercise bout (Table 2).

Effect of training on HSC and EPC in older men

The 8-week endurance training significantly decreased resting heart rate and increased maximal workload (Table 1) in the older men. Maximal oxygen consumption was increased, although not significantly (t -test, $P = 0.09$).

Baseline

Baseline numbers of HSCs were not altered after endurance training (Table 1), while EPCs (Table 1) and plasma levels of VEGF (Table 2) were significantly lower after training.

Single exercise bout

The acute exercise-induced increase in numbers of HSCs in older men is independent of physical fitness (Fig. 3B). Before as well as after the endurance training, the single exercise bout test did not change numbers of EPCs (Fig. 3D) and VEGF concentrations (Table 2).

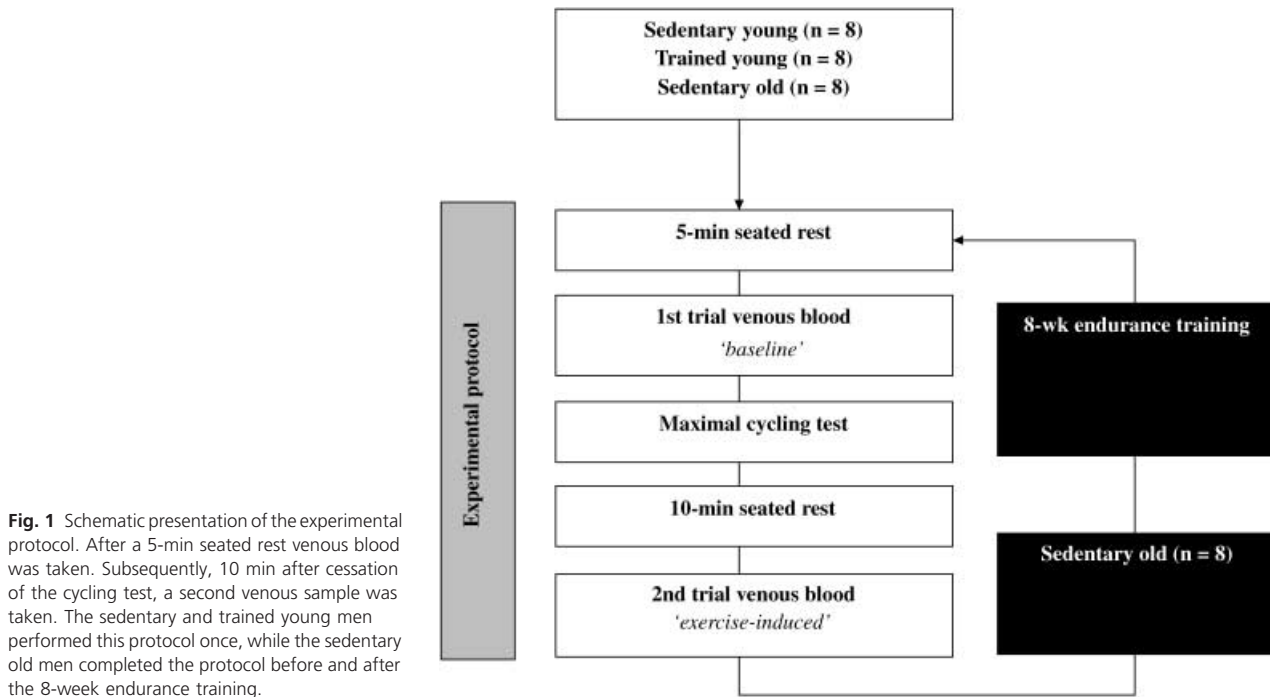


Fig. 1 Schematic presentation of the experimental protocol. After a 5-min seated rest venous blood was taken. Subsequently, 10 min after cessation of the cycling test, a second venous sample was taken. The sedentary and trained young men performed this protocol once, while the sedentary old men completed the protocol before and after the 8-week endurance training.

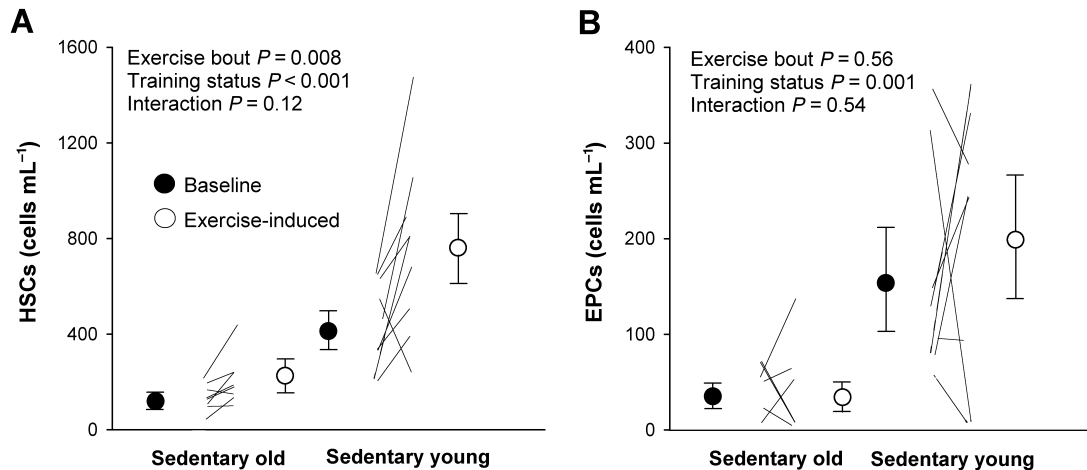


Fig. 2 Individual and average baseline (solid circles) and exercise-induced levels (open circles) of circulating numbers of haematopoietic stem cells (HSCs, A) and endothelial progenitor cells (EPCs, B) in sedentary young and older men (both $n = 8$). Average values represent means \pm SE. The two-way analysis of variance (ANOVA) indicates higher baseline and exercise-induced numbers of HSCs in young men compared with older men, while the exercise-induced increase in HSCs showed an age-dependent tendency.

Linear regression analysis

The linear regression analysis indicated a significant relationship between baseline and exercise-induced numbers of HSCs (Fig. 4A, $P = 0.001$) and EPCs (Fig. 4B, $P < 0.001$) in both sedentary and trained healthy men.

The absolute acute exercise-induced increase in numbers of HSCs in young men (due to the higher baseline numbers of HSCs) was larger than in older men (Fig. 5A). However, the relative acute exercise-induced change in HSCs was similar between young and older men (Fig. 5B). As such, the relative exercise-induced increase in HSCs is independent of age.

The exercise-induced relative increase in numbers of HSCs and EPCs in young ($r^2 = 0.07$ and 0.001 , $P = 0.31$ and 0.93 , respectively) as well as in older men ($r^2 = 0.12$ and 0.04 , $P = 0.20$ and 0.63 , respectively) did not correlate with the relative change in VEGF plasma levels. Maximal oxygen consumption did not correlate with the baseline or exercise-induced levels of HSCs ($r^2 = 0.17$ and 0.07 , $P = 0.12$ and 0.33 , respectively) nor EPCs ($r^2 = 0.10$ and 0.20 , $P = 0.23$ and 0.08 , respectively). Also older men show no correlation between maximal oxygen consumption and baseline or exercise-induced levels of HSCs ($r^2 = 0.001$ and 0.03 , $P = 0.90$ and 0.56 , respectively) nor EPCs ($r^2 = 0.05$ and 0.07 ; $P = 0.42$ and 0.51 , respectively).

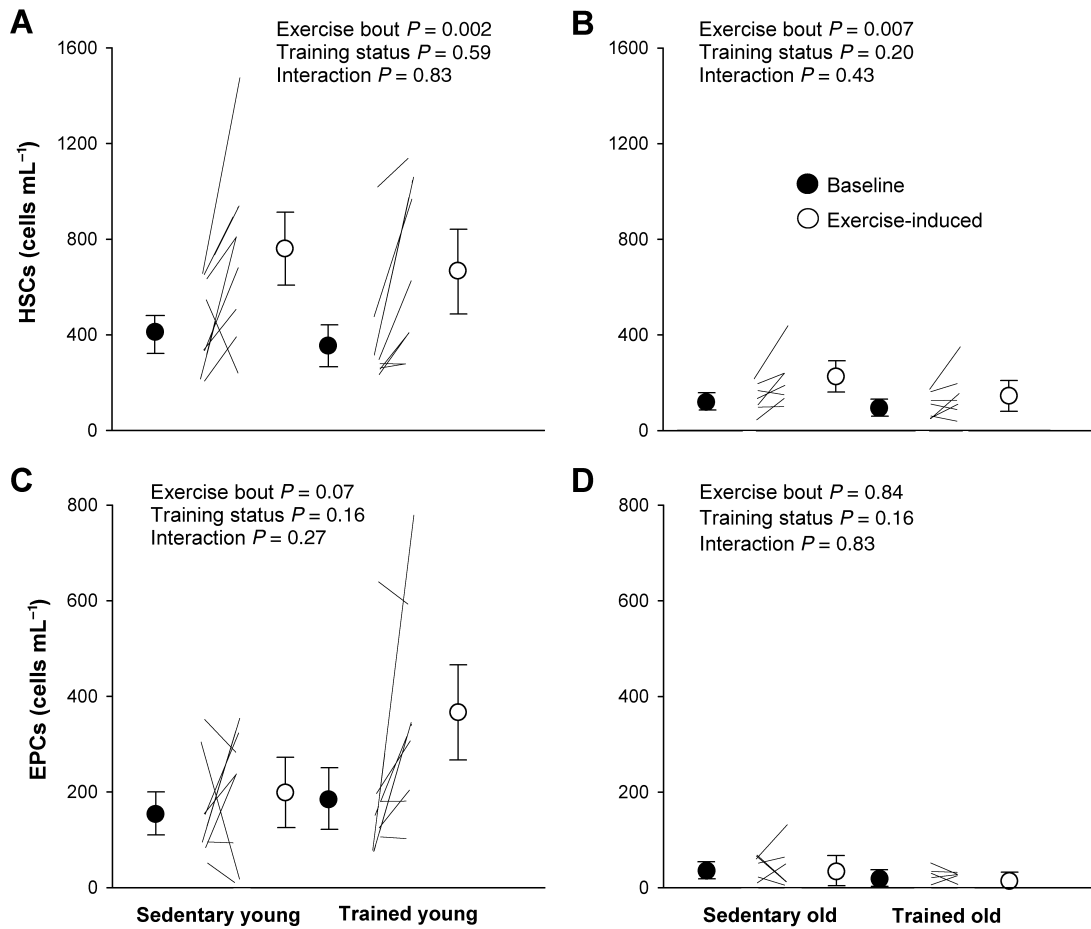


Fig. 3 Baseline (filled circles) and exercise-induced levels (open circles) of circulating numbers of haematopoietic stem cells (HSC, A–B) and endothelial progenitor cells (EPC, C–D). In young men, numbers of HSCs (A) and EPCs (C) were presented in the sedentary ($n = 8$) and trained ($n = 8$) subpopulation. In older men ($n = 8$), numbers of HSCs (B) and EPCs (D) were represented before and after training. Values represent mean \pm SE. Baseline and exercise-induced numbers of HSCs were independent of physical fitness. Also the baseline and exercise-induced numbers of EPCs were independent of physical fitness.

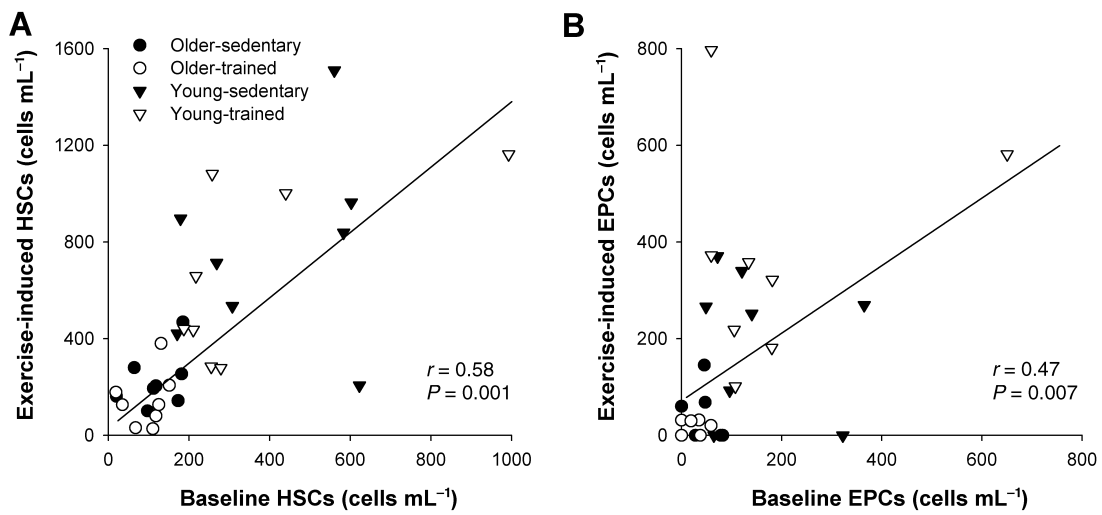


Fig. 4 Linear regression between baseline and exercise-induced numbers of haematopoietic stem cells (HSC, A) and endothelial progenitor cells (EPC, B) in sedentary ($n = 8$, open triangles) and trained young men ($n = 8$, open circles) and older men ($n = 8$) before (solid triangles) and after training (solid circles). This analysis assessed the correlation between baseline and exercise-induced numbers of HSCs and EPCs.

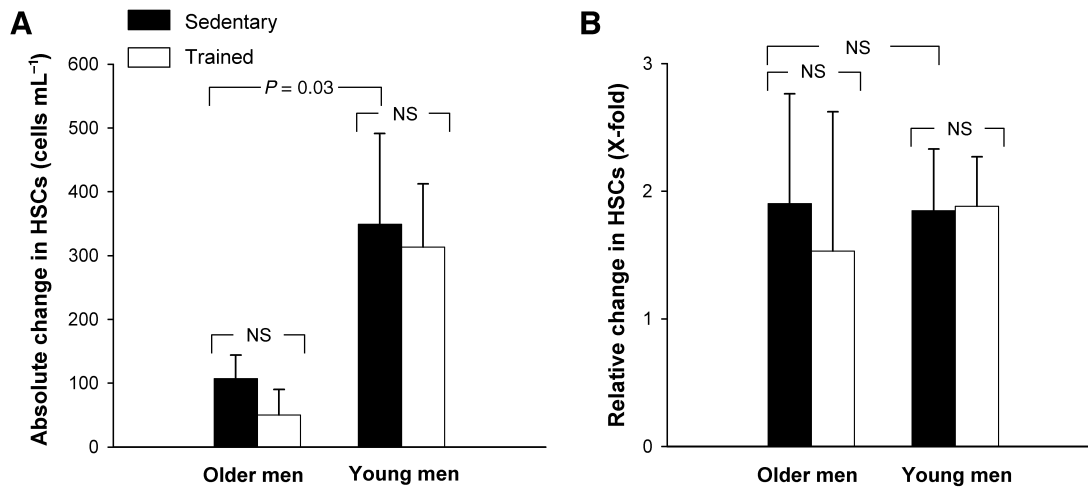


Fig. 5 Exercise-induced change in haematopoietic stem cells (HSC) in sedentary (black bars) and trained (white bars) young and older men, expressed as the absolute (A, in cells mL⁻¹) and relative (B, in X-fold change) from baseline numbers. Because data were not normally distributed, a Mann-Whitney *U*-test (between groups) or Wilcoxon signed rank test (within groups) was used to assess differences between groups. Values are means \pm SE.

Discussion

This study provides several interesting findings. First, the number of HSCs and EPCs in young men is higher than in older men. Second, this inverse correlation in age and HSC numbers was not altered after endurance training in young men, whereas EPC numbers decreased further after 8 weeks of endurance training of older men. Third, a single exercise bout in young as well as in older men acutely mobilizes HSCs, but not EPCs, to the peripheral blood. Fourth, the single exercise bout-induced change in numbers of HSCs is not influenced by training status, while advanced age is associated with an attenuated mobilization in numbers of HSCs. Fifth, numbers of HSCs and EPCs were not correlated to VEGF levels. Thus, our results suggest an age-related decrease in baseline and exercise-induced levels of HSCs and EPCs, which is not influenced by a change in training status.

We compared three well-defined populations, based on their age and training status. The maximal oxygen consumption of the sedentary (Miyachi *et al.*, 2004) and active (Franzoni *et al.*, 2004; Kraus *et al.*, 2004; Laufs *et al.*, 2005) young men and the older men (Tanaka *et al.*, 2000; Monahan *et al.*, 2001; Tanaka *et al.*, 2002; Eskurza *et al.*, 2004) is comparable with previous studies. Although the cycling training in older men did not significantly improve maximal oxygen consumption, seven out of eight subjects increased their maximal oxygen consumption and all men completed a higher maximal workload during the post-training cycling test. Moreover, the ~8% increase in oxygen consumption is larger than reported in previous studies in which older men were trained for 12 weeks (DeSouza *et al.*, 2000; Tanaka *et al.*, 2000; Tanaka *et al.*, 2002).

Effect of aging on numbers of HSCs and EPCs

Sedentary older men demonstrate markedly lower baseline levels of HSCs and EPCs than their younger peers. Because

EPCs correlate inversely with the incidence of cardiovascular events (Werner *et al.*, 2005), the age-related down-regulation in numbers of HSCs and EPCs may contribute to the increased cardiovascular risk in the older population. Although lower baseline numbers of HSCs and EPCs with advancing age is also reported in previous studies (Vasa *et al.*, 2001; Schmidt-Lucke *et al.*, 2005), others (Heiss *et al.*, 2005; Chen *et al.*, 2006; Shaffer *et al.*, 2006) reported no difference between young and older men. This variation among studies likely results from differences in the method used. We analyzed HSCs and EPCs in whole blood, while for studies in which no difference was found (Heiss *et al.*, 2005; Chen *et al.*, 2006; Shaffer *et al.*, 2006), numbers of stem cells was measured in the mononuclear cell fraction, isolated by Ficoll density gradient centrifugation. This method may cause loss of cells and underscores the importance of the choice of the method used.

Effect of a single exercise bout on HSCs and EPCs

The finding that acute exercise increases the level of HSCs is in accordance with a previous study of healthy young subjects (Morici *et al.*, 2005). Given the ability of HSCs to promote angiogenesis and promote vascular repair (Orlic *et al.*, 2001; Hill *et al.*, 2003; Rauscher *et al.*, 2003; Urbich & Dimmeler, 2004), the acute exercise-induced mobilization may serve as a physiological repair or adaptation mechanism. To examine whether the aging process influences the exercise-induced number of HSCs, we assessed this response in young and older men. Young men report a significantly larger increase in exercise-induced absolute numbers of HSCs than older men. However, expressing the increase as a relative change, our data demonstrate no differences between young and older men in the exercise-induced increase in HSCs. This indicates that the larger exercise-induced number of HSCs in young men is primarily caused by the higher baseline numbers of HSCs in young compared with older men.

Acute exercise did not alter the numbers of EPCs in young and older sedentary men, which is consistent with a previous study using a similar exercise protocol for middle-aged healthy subjects and middle-aged patients suffering from nonischemic coronary artery disease (Adams *et al.*, 2004). Interestingly, in ischemic coronary artery disease patients, numbers of EPCs were significantly increased, which coincided with higher levels of VEGF, implying that the ischemic stimulus is the initiating factor for EPC increase. During an ischemic episode, VEGF levels seem to correlate with EPC levels in patients with cardiovascular disease (Shintani *et al.*, 2001; Adams *et al.*, 2004) as well as in animal models (Asahara *et al.*, 1997, 1999), while this was not the case for basic fibroblast growth factor (b-FGF), granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNF- α). Interestingly, we and others (Rehman *et al.*, 2004; Laufs *et al.*, 2005) demonstrated that the exercise-induced increase in stem cells is not accompanied by a change in VEGF plasma levels in healthy men. In the absence of an ischemic stimulus VEGF levels may therefore not predict an exercise-induced change in HSCs and EPCs in healthy men. Although we can only speculate, the well-established exercise-induced increase in nitric oxide bioavailability may be a potential mechanism by which mobilization of EPCs is regulated. This is supported by the finding that in mice the exercise-mediated up-regulation of EPCs was absent in nitric oxide synthase 'knock-out' mice or in the presence of a nitric oxide synthase inhibitor in healthy mice (Laufs *et al.*, 2004). In addition, the hepatocyte growth factor, a potential stimulator of bone marrow derived cells (Ishizawa *et al.*, 2004), may also be involved in the up-regulation of HSCs. Recently, the hepatocyte growth factor was demonstrated as a good marker for progenitor proliferation at rest and after an acute exercise bout in healthy controls and in subjects with chronic pulmonary obstructive disease (Palange *et al.*, 2006).

Effect of training on numbers of HSCs and EPCs

Interestingly, neither baseline numbers nor the exercise-induced numbers of HSCs and EPCs are different between sedentary and age-matched trained young men. This suggests that chronic endurance training in young men does not influence baseline or exercise-induced numbers of HSCs and EPCs. This is supported by the lack of correlation between oxygen consumption and levels of HSCs and EPCs in young men. To further extend this knowledge, we examined the older men after 8 weeks of training. Apart from a small but significant decrease in baseline EPCs levels, there are no differences in baseline and exercise-induced levels of HSCs and EPC in older men after training. This supports our hypothesis that training status does not influence baseline or exercise-induced HSCs and EPCs.

To date, endurance training as an intervention to alter numbers of HSCs and EPCs is only examined in patients with peripheral arterial occlusive disease, coronary artery disease, or with cardiovascular risk factors (Laufs *et al.*, 2004; Sandri *et al.*, 2005; Steiner *et al.*, 2005). Increased numbers of EPCs have

been reported only when patients have symptomatic ischemia during exercise training (Sandri *et al.*, 2005). Our finding of a decrease in numbers of EPCs in healthy older men after endurance training prompts us to question the physiological mechanism, although we can only speculate. It is noteworthy that our participants were free of cardiovascular risk factors, which is in marked contrast with the populations in previous studies (Laufs *et al.*, 2004; Sandri *et al.*, 2005; Steiner *et al.*, 2005). In healthy older men, the EPCs may be involved in exercise-induced angiogenesis and/or repair processes, which consequently could result in lower baseline numbers of EPCs. On the other hand, bioavailability of nitric oxide is decreased during aging (Taddei *et al.*, 2000), which may also affect the number of circulating EPCs. Interestingly, nitric oxide has been shown to differentially support mobilization of endothelial committed progenitors, but did not affect HSCs. Under nitric oxide-deficient conditions, granulocyte-colony stimulating factor (G-CSF) failed to increase EPC numbers, while the HSC population was unaffected (Ozuyaman *et al.*, 2005). This may, at least in part, explain the decrease in EPCs upon chronic training in older subjects. Nevertheless, the responses of HSCs and EPCs to endurance training may differ between health and disease.

Limitations

In addition to the quantity (numbers), the quality of stem cells can also be examined (survival, migration, and proliferation). Advancing age, for example, has recently been associated with functional deficits of the stem cells (Heiss *et al.*, 2005). As such, physical exercise may lead to changes in functional characteristics, rather than in the numbers of HSCs or EPCs. Future studies should examine changes in stem cell function after exercise training.

Comparing the results from the trained young men with the trained older men, one should take the amount of weekly exercise into account. The duration of training in older men is relatively short (8 weeks, three sessions per week), while young men trained chronically (> 8 h a week). Despite these differences, the young as well as older trained men demonstrated baseline and acute exercise-induced values of HSCs and EPCs that are comparable to their sedentary counterparts.

In conclusion, this study demonstrates that advancing age is associated with lower numbers of circulating HSCs and EPCs, while the exercise-induced mobilization of HSCs is attenuated in older compared with young healthy men. Interestingly, in healthy young as well as in older men, endurance training does not affect baseline or exercise-induced numbers of HSCs or EPCs.

Experimental procedures

Subjects

Twenty-four participants, free of any cardiovascular disease and not on any medication known to interfere with the cardiovascular system, were recruited. Subjects had never smoked

or stopped smoking at least 15 years ago. To examine the effect of aging, these healthy participants were parsed into two groups on the basis of age: eight older sedentary men (67–76 years) and eight young sedentary men (19–28 years; Table 1). Sedentary was defined as less than 1 h of exercise per week for the past year or longer. To address the influence of the training status, the group of young sedentary men was compared with an age-matched population of endurance-trained young men ($n = 8$, 18–28 years, ≥ 8 h aerobic exercise per week). In addition, the older men performed an 8-week cycling training. Subjects underwent a physical examination, whereby subjects with abnormalities in a 12-lead electrocardiogram (ECG) at rest and/or an ankle-brachial pressure index of < 0.90 were excluded. The study was approved by the hospital ethics committee and conformed with the principles outlined in the Declaration of Helsinki. All subjects gave their written informed consent before participation.

Experimental design

All participants performed a single maximal exercise bout on a cycling ergometer. Before and directly after the single exercise bout, blood was collected to analyze baseline and acute exercise-induced numbers of circulating HSCs and EPCs. The older men performed an 8-week endurance training. Subsequently, the same experimental protocol was repeated after the final training session (Fig. 1).

Single exercise bout

Experiments started between 08 : 30 and 10 : 00 hours. Subjects refrained from caffeine for at least 18 h and did not perform any strenuous activities at least 24 h before testing. Subjects were advised to have a light breakfast. All tests were performed in a temperature-controlled room (20 ± 1 °C). Before the single exercise bout, subjects were seated in the upright position for at least 5 min before the baseline blood sample was drawn from an antecubital vein. The single exercise bout was performed on an electrically braked leg-cycling ergometer (Lode, Angio300, Groningen, The Netherlands), using a multi-stage protocol. Young men increased their workload by 20 W per minute, starting at 20 W, until exhaustion, whereas older men used steps of 10 W per minute. Oxygen consumption was measured continuously in all groups using a gas-analyzer (Jaeger Benelux BV, Breda, The Netherlands). Maximal oxygen consumption (VO_{2max}) was analyzed as the mean of the last minute of the single exercise bout. In addition, blood lactate level (Roche Diagnostics GmbH, Mannheim, Germany) was measured before and 2 min after cessation of the test using capillary blood. Heart rate (HR) was recorded continuously. Subjects were verbally encouraged to continue for as long as possible. The criterion used to assess VO_{2max} included (1) HR in excess of 90% of age-predicted maximum ($220 - \text{age}$); (2) $RER \geq 1.10$; and (3) identification of a plateau (≤ 150 mL increase) in VO_2 despite a further increase in workload (Gavin *et al.*, 2004). In all tests, at least two of three criteria were met. Finally, 10 min after

cessation of the cycling test, the post-exercise blood sample was taken (Rehman *et al.*, 2004).

Measurements

Enumeration of circulating HSCs and EPCs

For enumeration of circulating HSCs and EPCs, flow cytometric analysis was performed using a multiparametric gating strategy based on the International Society of Hematotherapy and Graft Engineering. This lyse/no wash method uses Trucount tubes (Becton Dickinson, Franklin Lakes, NJ, USA) that contain a defined number of brightly fluorescent microbeads, permitting the acquisition of absolute counts of cells, even at very low numbers. Circulating HSCs are defined as cells with low-expression of CD45, positive for CD34, and located in the lympho-gate on a side- and forward-scatter plot. This gating strategy was extended by calculating the number of CD34⁺ cells that also express vascular endothelial growth factor receptor-2 (VEGFR-2) to define the number of EPCs. This strategy avoids inclusion of mature endothelial cells, which are also positive for CD34 and VEGFR-2, because they are located outside the lympho-gate.

Within 2 h of blood-withdrawal, 50 μ L of EDTA-anticoagulated whole blood was added per Trucount tube (two per subject) by reverse pipetting and directly labeled antibodies were added: CD45-PerCP, CD34-FITC (BD Biosciences, Erembodegem, Belgium) and VEGFR-2-PE (R & D Systems, Minneapolis, MN, USA). After 30 min incubation on ice and in the dark, cells were fixed using FACS-lysing solution (BD Biosciences) and the samples were measured within 24 h using a fluorescence-activated cell sorter (FACS)-Calibur (BD Biosciences). A total of 500 000 CD45⁺ cells were measured (excluding the beads) and the number of HSCs and EPCs per milliliter blood was calculated.

VEGF assay

Plasma VEGF was measured, based on its suggested ability to mobilise EPCs into the blood (Asahara *et al.*, 1999). Plasma VEGF levels were determined with a customized enzyme-linked immunosorbent assay (ELISA; Department of Chemical Endocrinology, Radboud University Nijmegen, The Netherlands). The assay measures VEGF₁₆₅ and VEGF₁₂₁; the main isoforms of VEGF in blood. Details of the assay, including specificity and performance, have been described previously (Span *et al.*, 2000).

Endurance training

The older men performed endurance training for 8 weeks (three sessions per week including cycling exercise) with at least 1 day between subsequent training sessions, under the supervision of a researcher. A cycling ergometer (Lode, Angio300) was used for endurance training. Each session started with a 10-min warming-up at 65% of the individual heart rate reserve (HRR). After muscle stretching, the exercise protocol was followed with

20-min cycling exercise at 65% of the individual HRR. As their exercise tolerance improved throughout the training period, the intensity of the exercise bouts was increased by 5% HRR to a maximum of 85% of the HRR. In the first training session, the average workload was 157 ± 48 kJ and in the twenty-fourth (i.e. last) training session, 234 ± 50 kJ. Adherence to the exercise prescription was documented through the use of HR monitors (s610, Polar, Brooklyn, NY, USA).

Statistics

The main goal of this study is to compare the exercise-induced change in levels of HSCs and EPCs. Based on the average exercise-induced changes from previous studies (Laufs *et al.*, 2004; Morici *et al.*, 2005; Steiner *et al.*, 2005) and an alpha of 0.05, we calculated that eight subjects per group would be necessary to achieve a power of 80%. For the circulating numbers of HSCs and EPCs, a two-way mixed-plot factorial analysis of variance (*single exercise bout* \times *age* or *single exercise bout* \times *training status*) was used [Statistical Package for the Social Science (SPSS), version 12.0] (Ryan *et al.*, 2006). Unpaired Student's *t*-tests were used to compare differences in all other variables between groups (young vs. old and trained vs. sedentary young men), while a paired Student's *t*-test was used to assess differences before and after endurance training in older men. Multiple linear regression was performed to identify relationships between variables. Results are expressed as mean \pm SE. When data are not distributed normally, data are presented as median [lowest value – highest value], and a nonparametric test was used to compare differences between (Mann–Whitney *U*-test) or within groups (Wilcoxon's signed rank test). A two-sided *P*-value of ≤ 0.05 was considered to be statistically significant.

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