6-1-2010

# Thrombin and Exercise Similarly Influence Expression of Cell Cycle Genes in Cultured Putative Endothelial Progenitor Cells

Michael M. Lockard
*University of Maryland School of Public Health*

Sarah Witkowski
*University of Maryland School of Public Health*, switkowski@smith.edu

Nathan T. Jenkins
*University of Maryland School of Public Health*

Espen E. Spangenburg
*University of Maryland School of Public Health*

Thomas O. Obisesan
*University of Maryland School of Public Health*

*See next page for additional authors*

*J Appl Physiol* 108: 1682–1690, 2010.
First published April 8, 2010; doi:10.1152/japplphysiol.00884.2009.

# Thrombin and exercise similarly influence expression of cell cycle genes in cultured putative endothelial progenitor cells

**Michael M. Lockard,[1,2]* Sarah Witkowski,[1]* Nathan T. Jenkins,[1]* Espen E. Spangenburg,[1] Thomas O. Obisesan,[1] and James M. Hagberg[1]**

[1]*Department of Kinesiology, University of Maryland College Park School of Public Health, College Park, Maryland; and* [2]*Department of Exercise Science, Willamette University, Salem, Oregon*

Submitted 10 August 2009; accepted in final form 5 April 2010

**Lockard MM, Witkowski S, Jenkins NT, Spangenburg EE, Obisesan TO, Hagberg JM.** Thrombin and exercise similarly influence expression of cell cycle genes in cultured putative endothelial progenitor cells. *J Appl Physiol* 108: 1682–1690, 2010. First published April 8, 2010; doi:10.1152/japplphysiol.00884.2009.—Acute exercise and exercise training may influence putative endothelial progenitor cell (EPC) number and colony forming units (CFU-ECs), although the mechanisms remain unclear. This study examined the effects of in vitro thrombin supplementation and acute exercise on CFU-EC gene expression, associated with cellular proliferation and differentiation. The effect of habitual physical activity was evaluated through analysis of EPCs from chronically high- and low-active men. Participants were healthy high- and low-active men ($n = 23$), aged 55–80 yr. Circulating $CD34^+/VEGFR2^+$ number, CFU-ECs, plasma prothrombin fragment (F1+2), and thrombin-antithrombin III were measured at rest and after 30 min of exercise. Gene expression of cyclin A2, cyclin D1, p27, VE-cadherin, and VEGFR2 was assessed in postexercise CFU-ECs and resting CFU-ECs treated with 0, 1, 5, or 10 U/ml of thrombin. Outcomes were compared between high- and low-active participants. F1+2 and thrombin-antithrombin III, but not $CD34^+/VEGFR2^+$ number and CFU-ECs, increased with exercise. Exercise-induced changes in F1+2 correlated with changes in $CD34^+/VEGFR2^+$ number in both groups. Thrombin treatments and acute exercise increased cyclin A2 and cyclin D1 expression and decreased p27 expression. One unit per milliliter thrombin increased VEGFR2 and VE-cadherin expression, whereas 5 U/ml, 10 U/ml, and acute exercise did not elicit any changes. An exercise training effect was observed with greater decreases in p27 expression with 5 and 10 U/ml thrombin and greater increases in VEGFR2 and VE-cadherin expression with 1 U/ml thrombin in high-active men. Exercise-induced changes in putative EPC gene expression are associated with thrombin production and may be modulated by long-term exercise training.

hemostasis; coagulation; endothelial progenitor cell; reverse transcriptase-polymerase chain reaction

REGULAR PHYSICAL ACTIVITY and improved physical fitness are associated with enhanced cardiovascular health and decreased risk for the development of cardiovascular disease (CVD) (30). While exercise training has been repeatedly associated with improvements in risk factors associated with CVD, it appears that training results in an additional reduction in risk, independent of the more conventional risk factors (4). It has been suggested that a causal link between regular physical activity and reduced CVD risk is mediated through the improvement of endothelial health and associated vascular function (7, 18), although a specific mechanism for this relationship has not been established.

Bone-marrow-derived endothelial progenitor cells (EPCs) represent a potential link between exercise training, improved vascular function, and decreased CVD risk. Under conditions of normal cellular turnover and recovery from vascular injury, circulating EPCs actively incorporate into the endothelial layer, where they participate in endothelial maintenance (2) and are a component of blood clot resolution through the process of recanalization (16, 17). Enhancement of these EPC-mediated processes would improve endothelial health and reduce cardiovascular risk. Recent evidence has shown that both an acute bout of exercise (21) and exercise training (14) are associated with an increase in the number of circulating EPCs in certain populations. Changes in circulating EPCs have been associated with reduced neointimal formation, increased lumen circumference, and increased area of neoangiogenesis (15) in mice, as well as increased nitric oxide activity and endothelial function (24) in humans. The specific mechanisms whereby exercise and exercise training influence changes in EPCs are unknown.

During acute exercise, there is a marked increase in vascular shear stress (20) and thrombin production (32). Furthermore, thrombin and its receptor, protease-activated receptor-1 (PAR-1), are required participants in endothelial regulation in adults, as well as angiogenesis during fetal development (8). EPCs, which are believed to direct both adult and fetal endothelial maintenance and vasculogenesis, also express PAR-1 on their cellular surface (22). There is increasing evidence that thrombin signaling through the PAR-1 receptor is directly involved in the regulation of EPC proliferation and differentiation in mice (25) and EPC proliferation in humans (23); however, the details of this regulatory mechanism, as well as the dose-response relationship with thrombin, have not been investigated. Furthermore, recent evidence has suggested that thrombin plays a role in EPC activity within a fibrin clot via the PAR-1 receptor (23). The role that thrombin may play as a signal by which acute exercise affects EPC proliferation and/or differentiation, specifically as it relates to endothelial maintenance and blood clot resolution, has not been investigated.

The purposes of this study were *1*) to investigate the role of thrombin as a signaling molecule for EPC proliferation and differentiation through real-time PCR analysis of endothelial cell cycle genes and cell surface marker expression in response to in vitro thrombin treatments; and *2*) to evaluate EPC gene expression changes related to cell proliferation and differentiation in vivo with a thrombin-stimulating bout of acute exercise. To evaluate the effect of chronic physical activity status,

* M. M. Lockard, S. Witkowski, and N. T. Jenkins contributed equally to this paper.

Address for reprint requests and other correspondence: J. M. Hagberg, 2134 HHP Bldg., Dept. of Kinesiology, Univ. of Maryland, College Park, MD 20742-2611 (e-mail: hagberg@umd.edu).

  

experiments were performed on EPCs cultured from chronically high- and low-active men.

## MATERIALS AND METHODS

The recruitment, screening, and testing procedures for this study have been described previously (31). Participants who qualified for the study completed an informed consent form. The study protocol and written informed consent forms were approved by the University of Maryland College Park Institutional Review Board; all participants provided their written consent before beginning in the study. A maximal graded exercise test was then performed to screen for signs or symptoms of CVD and arrhythmias, as well as to measure maximal oxygen consumption ($\dot{V}O_{2max}$). During a separate visit, a 30-min vigorous treadmill exercise test was completed, as well as measurement of body composition. Each testing session took place ~24 h after the most recent bout of exercise in high-active men. Low-active men were instructed to maintain their normal routine, not exercising during the 24 h before testing. All testing occurred in the morning after a 12-h fast to control for circadian and dietary effects. Participants refrained from the use of antihistamines and nonsteroidal anti-inflammatory drugs for 48 h, and alcohol consumption for 24 h before testing. Blood samples for the hemostatic and EPC variables were collected before, and 30 min after the completion of, the 30-min vigorous exercise test. Baseline samples were collected after at least 15 min of quiet rest.

### *Participants*

Participants included 12 healthy male endurance athletes (high-active), aged 55–80 yr, and 11 healthy low-active age- and body mass index (BMI)-matched men. Participants were nonsmokers, with no history of CVD, bleeding disorders, diabetes, or cancer. Participants were excluded if they were undergoing anti-coagulant therapy, taking medications known to affect EPCs, had an orthopedic condition prohibiting treadmill exercise, had a history of uncontrolled hypertension (systolic > 160 mmHg or diastolic > 99 mmHg), or had elevated cholesterol (>240 mg/dl). High-active men had maintained an aerobic training history of at least 20 yr, greater than three times per week at a moderate-to-vigorous intensity. Low-active participants exercised aerobically less than two times per week for less than 20 min per session for at least the previous 5 yr.

### *Maximal Graded Exercise Test*

After a general physical examination by a physician, participants were monitored with a 12-lead chest electrocardiogram at rest, during exercise, and during recovery. Expired gases were measured using indirect calorimetry (Viasys Healthcare). After a brief warm-up and treadmill familiarization period, the treadmill test was administered using a modified Bruce protocol. Heart rate and blood pressure were monitored at rest, during each stage of the treadmill test, and during recovery. Termination criteria included abnormal electrocardiogram or physiological responses to exercise as defined by the American College of Sports Medicine (1). All tests were determined to be valid maximal exercise tests using standard criteria (1).

### *30-min Vigorous Exercise Test*

Participants exercised on a treadmill for 30 min at 75 ± 5% $\dot{V}O_{2max}$, consisting of a 5-min ramp-up period and subsequent 25 min at the prescribed intensity. At the conclusion of the 30-min exercise period, participants entered a low-intensity active cool-down period for 3 min. Blood samples for $CD34^+$/vascular endothelial growth factor receptor-2 ($VEGFR2^+$) cells and colony-forming unit-endothelial cell (CFU-EC) were obtained immediately before and 30 min after this test. This time point was chosen, based on previously published results, which indicated that a significant peak in circulating EPCs occurs ~30 min after exercise (14). We determined empirically that changes in expression of our target genes were detectable at ~30–60 min following exercise, and chose to use the 30-min time point to minimize subject burden. Furthermore, our laboratory has recently demonstrated substantial changes in the expression of a number of genes in CFU-EC (12).

### *Body Composition*

Height, weight, and BMI ($kg/m^2$) were measured, and body composition was determined via dual-energy X-ray absorptiometry (Hologic, Discovery A, software version 12.7.1).

### *Blood Coagulation*

Plasma levels of thrombin-antithrombin III (TAT) and prothrombin fragment F1+2 (F1+2) were assessed as markers indicative of thrombin production. Plasma TAT level was measured using the Enzygnost TAT micro-enzyme-linked immunoassay (ELISA) (Dade Behring, Deerfield, IL), and F1+2 was measured using the Enzygnost $F_{1+2}$ micro-ELISA (Dade Behring, Deerfield, IL), according to the manufacturer's instructions. Samples were run in duplicate, and values were adjusted for changes in hematocrit with exercise. The intra-assay coefficients of variation were 12.0 and 11.1% for TAT and F1+2, respectively.

### *Putative EPCs*

*Circulating putative EPCs ($CD34^+$/$VEGFR2^+$).* The level of putative EPCs in the circulating blood was assessed by flow cytometry, as described previously (31). Briefly, peripheral blood mononuclear cells were isolated by density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare). Mononuclear cells ($1 \times 10^6$) were immunostained with FITC-conjugated CD34 monoclonal anti-human antibody (BD Biosciences) and biotinylated VEGFR2 anti-human antibody (R&D Systems). Isotype-identical antibodies (mouse IgG2a-FITC, BD Biosciences, and mouse IgG1-PE, R&D Systems) served as controls. All cells were FcR blocked (Miltenyi Biotech), washed with PBS, and fixed in 4% paraformaldehyde before analysis by flow cytometry. Flow cytometry was performed in the Flow Cytometry/Cell Sorting CORE Laboratory at the University of Maryland, Baltimore School of Medicine, with a Beckman Coulter Epics Elite ESP flow cytometer and cell sorter. The forward-side-scatter plot was used to identify the lymphocyte gate. At least 100,000 events per sample were acquired. To assess reproducibility, duplicates from a subset of samples were analyzed ($r = 0.90$, $P < 0.001$).

*Colony-forming unit assay.* Putative EPCs were studied in culture using the colony-forming unit-endothelial cell (CFU-EC) assay (9), as our laboratory has previously described (12, 31). Briefly, mononuclear cells were isolated from peripheral blood by density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare), washed twice, and Resuspended in EndoCult Medium (Stem Cell Technologies, Vancouver, Canada). mononuclear cells ($5 \times 10^6$) were plated in duplicate wells on fibronectin-coated six-well plates (BD Pharmingen, CA) and incubated at 37°C and 5% $CO_2$. After 48 h, nonadherent cells were collected and counted. cells ($1 \times 10^6$) were replated on fibronectin-coated 24-well plates (BD Pharmingen, CA) and incubated at 37°C and 5% $CO_2$. After 3 days, the number of CFU-ECs per well was counted by a trained, independent observer using inverted light microscopy. CFU-ECs were defined as a central cluster of rounded cells with multiple emanating thin, flat cells (9). To assess interobserver variability, CFU-EC counts were performed by two independent observers in a subsample of wells ($r = 0.98$, $P < 0.001$, $n = 36$ wells).

*CFU-EC gene expression with acute exercise and thrombin treatment.* Mononuclear cells were isolated, plated, and incubated for 48 h, and $1 \times 10^6$ nonadherent cells were replated as described above. For thrombin treatments, the incubation medium of the resting samples was supplemented with 0, 1, 5, or 10 U/ml of thrombin (human

α-thrombin, Enzyme Research Laboratory; prepared in PBS + 0.5% bovine serum albumin). The maximum treatment used (10 U/ml) is consistent with the standard levels of thrombin used in vitro and observed in vivo in the related EPC and endothelial cell literature (5, 13, 25, 27). We also examined lower thrombin concentrations of 5 U/ml and 1 U/ml to evaluate responses to a broader range of thrombin and approximate physiological concentrations that may occur with a vigorous exercise stimulus. For cells isolated after acute exercise, standard culture conditions were maintained as described above. Cells were incubated at 37°C and 5% $CO_2$ for 24 h. All culture conditions were measured in duplicate from each subject.

After 24 h of incubation, cells were washed, and total RNA was extracted using TRIzol (Invitrogen), according to previously described procedures (12). RNA concentrations were determined by optical density at 260 nm. RNA quality was confirmed by ethidium bromide staining of 18S and 28S, visualized by gel electrophoresis under ultraviolet light.

*Real-time PCR.* Total RNA was reverse transcribed using a cDNA reverse transcription kit (Applied Biosystems). cDNA was amplified and gene expression assessed via predesigned TaqMan RT-PCR gene expression kits using an Applied Biosystems 7500 RT-PCR System (Applied Biosystems). All samples were amplified in triplicate using RT-PCR. Gene expression was analyzed using Applied Biosystems Sequence Detection Software (Applied Biosystems). To ensure genomic DNA was not amplified, all primer sequences spanned exons. The thermal cycling conditions for each assay included an initial denaturation cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 65°C for 1 min, according to the manufacturer's instructions (Applied Biosystems).

To assess the role of thrombin as a signaling molecule in cellular proliferation, the following cell cycle genes were assessed for changes in expression: cyclin A2, cyclin D1, and p27 (cyclin-dependent kinase inhibitor-1) (Hs00153138_m1, Hs00277039_m1, and Hs00271467_m1, respectively, Applied Biosystems). To assess the role of thrombin in signaling CFU-EC differentiation, the following genes were assessed for changes in expression: VE-cadherin and VEGFR2 (Hs00901463_m1 and Hs00176676_m1, respectively, Applied Biosystems). These membrane-bound cell surface markers are frequently used to identify cells of endothelial lineage (3, 11, 34).

Changes in mRNA levels were assessed through relative quantification (RQ) using the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1, Applied Biosystems, CA). No significant difference was found in gene expression of GAPDH between treatment conditions ($P = 0.494$). Quantification of gene expression in each treatment group was analyzed according to the manufacturer's specifications and is expressed as $\log_{10}$ RQ, indicating the fold difference in gene expression compared with the control sample. Briefly, the threshold cycle of amplification (Ct) for each sample was compared with that of the endogenous control GAPDH. The difference in Ct between the sample and GADPH was expressed as ΔCt. Within each gene assay, the difference in ΔCt between each treatment level (1, 5, 10 U/ml thrombin, and 30 min postexercise) and the control sample (0 U/ml thrombin) was expressed as ΔΔCt. RQ of each sample was calculated as $2^{-\Delta\Delta Ct}$. The fold difference of each sample compared with the control was then expressed as $\log_{10}$ RQ. All gene expression analyses of a given sample were performed on a single plate to eliminate interassay variation. The average intra-assay coefficient of variation for gene expression was 0.65%.

### *Statistical Analysis*

All statistical analyses were performed using SPSS statistical software (SPSS version 16.0 for Windows). Data are presented as means ± SE. Statistical significance was set at $P \leq 0.05$. Variables were tested for assumptions of normality and homogeneity of variance. Acute exercise-induced changes in putative EPC number, CFU-EC, TAT, and F1+2 had nonnormal, positively skewed, single modal distributions. Log-transformed data were analyzed to satisfy the normality assumption, but statistical results were identical to parametric analyses of untransformed data. Therefore, raw data were examined by ANOVA in the final analysis, as recommended by Hinkle et al. (10). For all correlations, Spearman's rank correlation coefficient (*r*) was used.

Comparisons were made between values before and after exercise using repeated-measures ANOVA. The differences in mRNA expression among the five different treatments (0, 1, 5, and 10 U/ml, and 30 min postexercise) were analyzed by ANOVA using simple pairwise comparisons against the 0 U/ml control condition, with follow-up tests for simple effects to assess differences between groups under each treatment condition.

## RESULTS

High-active and low-active participants were successfully matched for age and BMI, with nonsignificant mean differences of 3 yr and 1.5 kg/m$^2$, respectively, between groups (Table 1). Additionally, CVD risk factors, including total cholesterol, LDL-cholesterol, and systolic blood pressure, were similar between the two groups. As expected, aerobic fitness, as measured by $\dot{V}O_{2max}$, was significantly and substantially greater in high-active than in the low-active participants ($P < 0.001$). Likewise, HDL-cholesterol was significantly higher in high-active participants ($P = 0.002$), whereas body fat percentage ($P = 0.019$) and diastolic blood pressure ($P = 0.038$) were significantly lower in high-active participants.

### *Thrombin Production*

To determine the presence of in vivo thrombin production with exercise, blood was collected at rest and 5 min after a 30-min bout of treadmill exercise. In both high-active and low-active participants, there were significant increases in F1+2 and TAT following exercise (Fig. 1). On average, low-active men had ~3.6 times greater exercise-induced increase in plasma F1+2 concentrations than high-active men (37.6 and 10.5 pmol/l, respectively) (Fig. 1); however, this difference was not statistically significant between groups.

### *Gene Expression*

*Cell cycle genes.* To assess the effect of thrombin treatment on CFU-EC gene expression, CFU-ECs were cultured from samples obtained at rest and after exercise. When analyzing

Table 1. *Descriptive data*

| Variables | High Active | Low Active |
|---|---|---|
| *n* | 12 | 11 |
| Age, yr | 62 ± 1.6 | 65 ± 1.5 |
| Height, m | 1.79 ± 0.04 | 1.76 ± 0.02 |
| Weight, kg | 70.1 ± 2.9 | 72.6 ± 1.9 |
| BMI, kg/m$^2$ | 22.0 ± 0.9 | 23.5 ± 0.6 |
| Body composition, %fat | 18.0 ± 1.3 | 23.6 ± 1.8* |
| Total cholesterol, mg/dl | 199 ± 8.9 | 194 ± 10.6 |
| HDL-C, mg/dl | 71 ± 3.3 | 51 ± 4.6* |
| LDL-C, mg/dl | 115 ± 8.4 | 123 ± 11.4 |
| Systolic BP, mmHg | 122 ± 3.0 | 129 ± 3.0 |
| Diastolic BP, mmHg | 79 ± 2.0 | 85 ± 2.0* |
| $\dot{V}O_{2max}$, ml·kg$^{-1}$·min$^{-1}$ | 50.0 ± 1.3 | 28.2 ± 1.8* |

Values are means ± SE; *n*, no. of subjects. BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; BP, blood pressure; $\dot{V}O_{2max}$, maximal oxygen consumption. *Difference between groups was significant ($P \leq 0.05$).

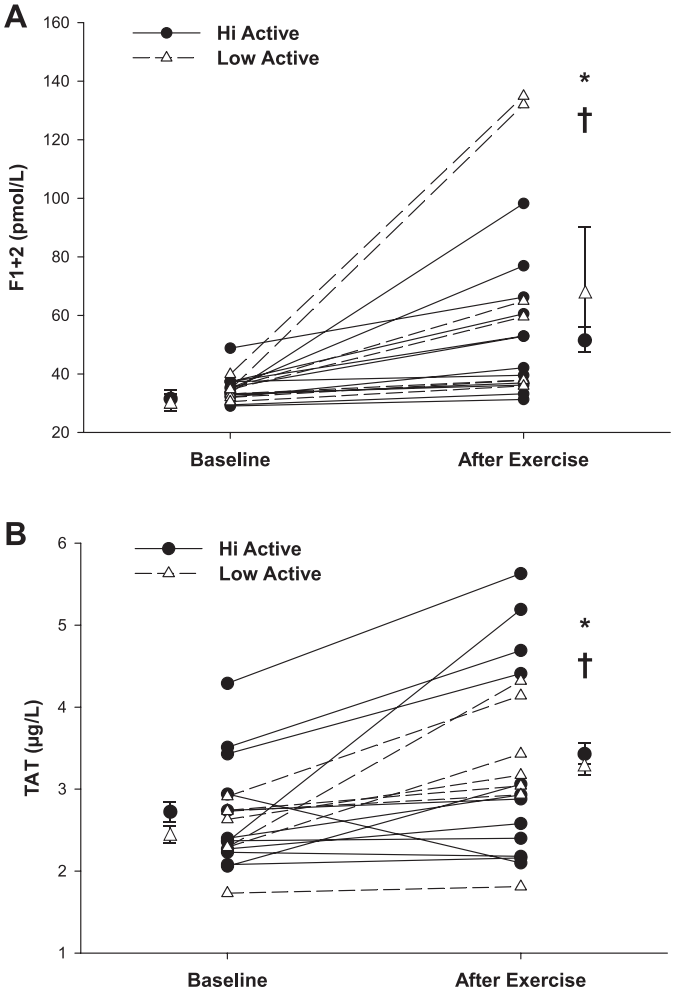


Fig. 1. Prothrombin fragment (F1+2; *A*) and thrombin-antithrombin III (TAT) production (*B*) in response to 30 min of vigorous exercise. Values are means ± SE. *Significant change from baseline in low-active group ($P \leq 0.05$). †Significant change from baseline in high-active group ($P \leq 0.05$).

high-active and low-active groups separately, no significant differences were found between the two groups in cyclin A2 or cyclin D1 gene expression in response to any level of thrombin supplementation or acute exercise (Fig. 2*A*). There was an approximately threefold greater decrease in p27 expression ($P > 0.019$) in high-active participants with 5 U/ml thrombin compared with low-active participants. With groups combined, there were increases in CFU-EC expression of proliferation genes cyclin A2 and cyclin D1 with thrombin treatment, peaking at 5 U/ml thrombin (50%, $P = 0.001$; and 78%, $P = 0.001$, respectively), compared with control cells receiving no thrombin (Fig. 2*B*). This change was mirrored by a decrease in the gene expression of the cell proliferation inhibitor p27 at 5 U/ml compared with control cells (90% decrease, $P < 0.001$). Following 30 min of vigorous exercise, a similar gene expression profile was seen compared with the thrombin treatment (Fig. 2*B*). The exercise stimulus elicited a 58% increase in cyclin D1 ($P = 0.009$) and a 58% decrease in p27 expression ($P = 0.006$) compared with control cells.

*Endothelial differentiation genes.* Gene expression of VE-cadherin and VEGFR2, indicators of endothelial lineage differentiation, increased at 1 U/ml in the high-active group (183%, $P = 0.026$; and 120%, $P = 0.031$, respectively), with no significant change in expression in the low-active group (Fig. 3*A*). The changes in gene expression in response to 30 min of vigorous exercise were not significantly different between groups. With groups combined, VE-cadherin and VEGFR2 mRNA levels increased by 87% ($P = 0.015$) and 66% ($P = 0.004$), respectively, with 1 U/ml thrombin compared with control cells, but did not change significantly from control under all other conditions (Fig. 3*B*). The 30-min exercise stimulus did not significantly change gene expression of VE-cadherin or VEGFR2 compared with control cells.

*Putative EPCs*

No change was observed in either circulating CFU-ECs (Fig. 4*A*) or $CD34^+/VEGFR2^+$ (Fig. 4*B*) in response to vigorous exercise in either the high-active or low-active group. The individual response to exercise varied considerably among participants. The change in circulating $CD34^+/VEGFR2^+$ number with exercise ranged from −98 to +385 cells/100,000 events, while the change in CFU-ECs with exercise ranged from −27 to +142 colonies. When comparing circulating progenitor cells and CFU-ECs between high-active and low-active participants, there was no significant difference between groups either at rest or in response to vigorous exercise.

In high-active men, the change in circulating $CD34^+/VEGFR2^+$ number ($r = 0.711$, $P < 0.005$), but not CFU-ECs ($r = 0.343$, $P = 0.138$), was positively correlated with the change in plasma F1+2 with vigorous exercise. In low-active men, a similar correlation was seen with the change in circulating $CD34^+/VEGFR2^+$ number ($r = 0.771$, $P = 0.036$), but not the change in CFU-ECs ($r = -0.036$, $P = 0.47$) with vigorous exercise. When analyzed across all participants, the change in circulating $CD34^+/VEGFR2^+$ number ($r = 0.698$, $P = 0.001$), but not CFU-ECs ($r = 0.307$, $P = 0.10$), was significantly correlated with the change in plasma F1+2 concentration with vigorous exercise (Fig. 5). No correlation was evident between the change in plasma TAT concentration with exercise and the change in either circulating $CD34^+/VEGFR2^+$ number or CFU-ECs.

## DISCUSSION

We examined the potential link between physical activity, thrombin, and putative EPCs. Our results indicate a correlation between exercise-induced thrombin production and changes in circulating $CD34^+/VEGFR2^+$ number but not CFU-ECs, with vigorous exercise. We also found that putative CFU-ECs treated with thrombin in vitro and CFU-ECs isolated after 30 min of exercise demonstrated similar changes in gene expression patterns of cell cycle genes compared with untreated control cells. Furthermore, we investigated the influence of long-term exercise training on these relationships. In response to acute exercise, we found no difference between high- and low-active men for TAT, F1+2, circulating $CD34^+/VEGFR2^+$ number, or CFU-EC colonies, although there may be an effect of training on CFU-EC gene expression. Together, these results indicate that thrombin may act as a signaling molecule to alter expression of cell cycle genes in putative EPCs. Furthermore, these thrombin-mediated effects may be one mechanism through which acute and chronic endurance exercise affect putative EPC function.

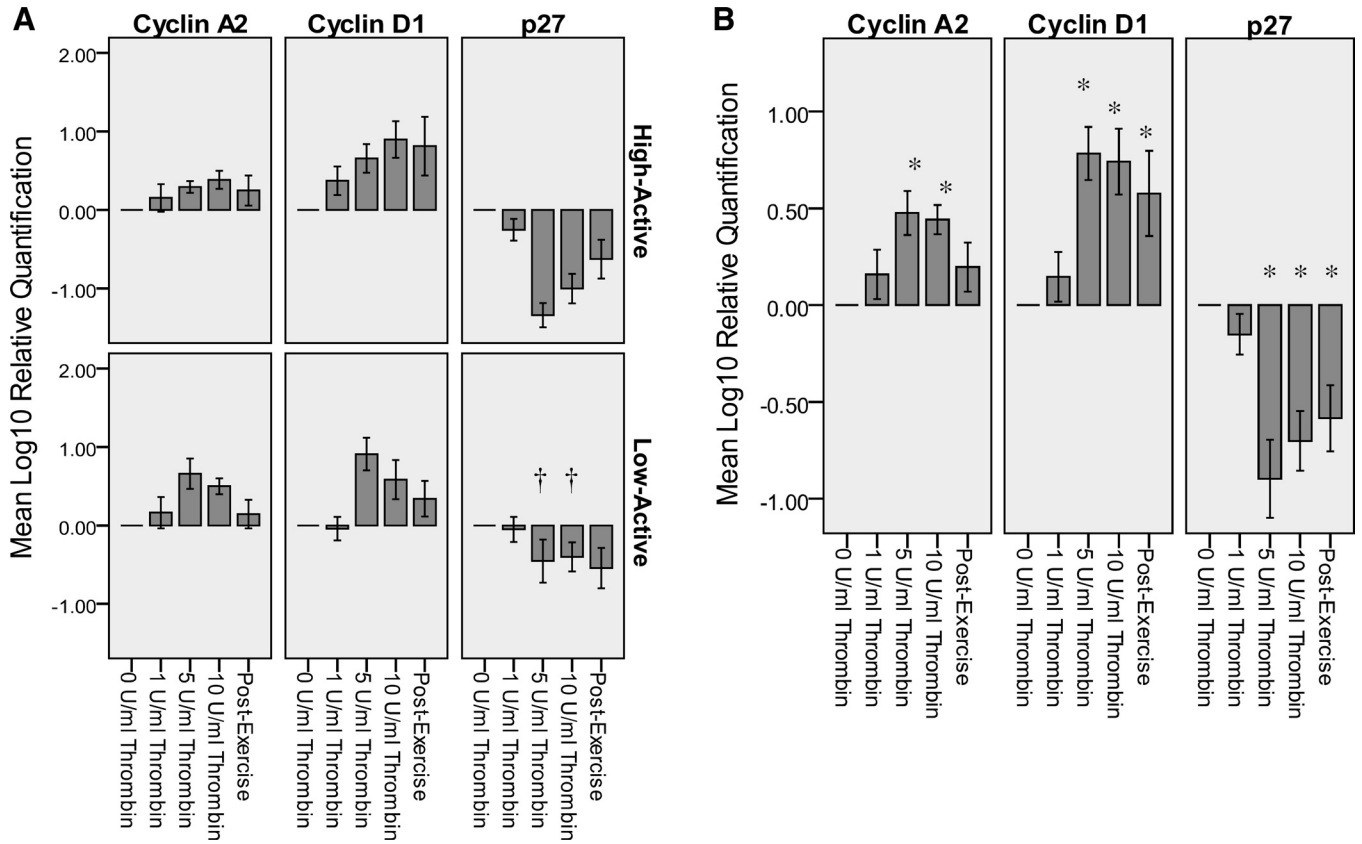


Fig. 2. Cell cycle gene expression in colony-forming unit-endothelial cells (CFU-ECs) supplemented with thrombin and after 30 min of vigorous exercise. Values are means ± SE. *A*: high-active: $n = 6$; low-active: $n = 6$. †Significant difference between groups ($P \leq 0.05$). *B*: $n = 12$: high-active, $n = 6$; low-active, $n = 6$. All comparisons were relative to the control, 0 U/ml thrombin supplementation, set at 0.0. *Significant difference from control ($P \leq 0.05$).

*Exercise-Induced Thrombin Production*

Vascular shear stress, such as that induced by acute exercise (20), gives rise to activation of the thrombotic and fibrinolytic cascades, as well as platelet aggregation associated with endothelial damage and fibrous plaque rupture (19, 32, 33). This investigation confirmed elevated thrombin production in response to the vigorous exercise stimulus within our older male subject population. Our results indicated a significant increase in both markers of thrombin production, F1+2 and TAT. These results have been previously reported by other laboratories (32).

We found no significant differences between high-active and low-active groups in plasma F1+2 or TAT levels at rest or in response to vigorous exercise. Interestingly, the change in plasma F1+2 concentration with exercise in low-active participants was ~3.6 times greater than that of the high-active participants, but this difference was not statistically different. The significant and comparable increase in levels of F1+2 and TAT that we found in both groups, however, does indicate that 30 min of treadmill exercise was a sufficient stimulus to elicit a systemic elevation in thrombin production. This raises the possibility that thrombin may affect cell cycle gene expression in vitro with acute exercise in putative EPCs.

*Gene Expression*

To directly investigate the role of thrombin as a cell signaling molecule in putative EPC proliferation and differentiation, we measured changes in mRNA gene expression in the cell cycle genes cyclin A2, cyclin D1, and p27, as well as endothelial cell surface markers VEGFR2 and VE-cadherin in CFU-ECs supplemented with thrombin in vitro. Previous studies have established the role of PAR-1 in EPC signaling with thrombin (22, 23, 25); we, therefore, focused our study on the dose-response relationship. We further compared these gene expression profiles to those of CFU-ECs isolated after 30 min of vigorous exercise.

CFU-ECs supplemented with thrombin increased gene expression of the cell cycle genes cyclin A2 and cyclin D1 with a plateau in gene expression between 5 and 10 U/ml thrombin. Concurrently, there was a decrease in expression of the cell cycle inhibitor p27, which also showed a plateau in gene expression between 5 and 10 U/ml. This pattern of gene expression supports the role of thrombin as a signal for the proliferation of EPCs, although available literature is somewhat ambiguous. While Tarzami et al. (25) found a thrombin-induced increase in both EPC proliferation and differentiation, Smadja et al. (23) found only a change in proliferation of human EPCs induced by thrombin-PAR-1 signaling. Furthermore, the focus of Tarzami et al. (25) was on EPCs isolated from the bone marrow of mice supplemented with 10 U/ml of thrombin. Aside from the differences in EPC source, definition, species, and conclusions, these studies did not investigate changes in gene expression seen in our present study. Thus our data extend these previous observations by showing a gene expression pattern consistent with thrombin acting as a means

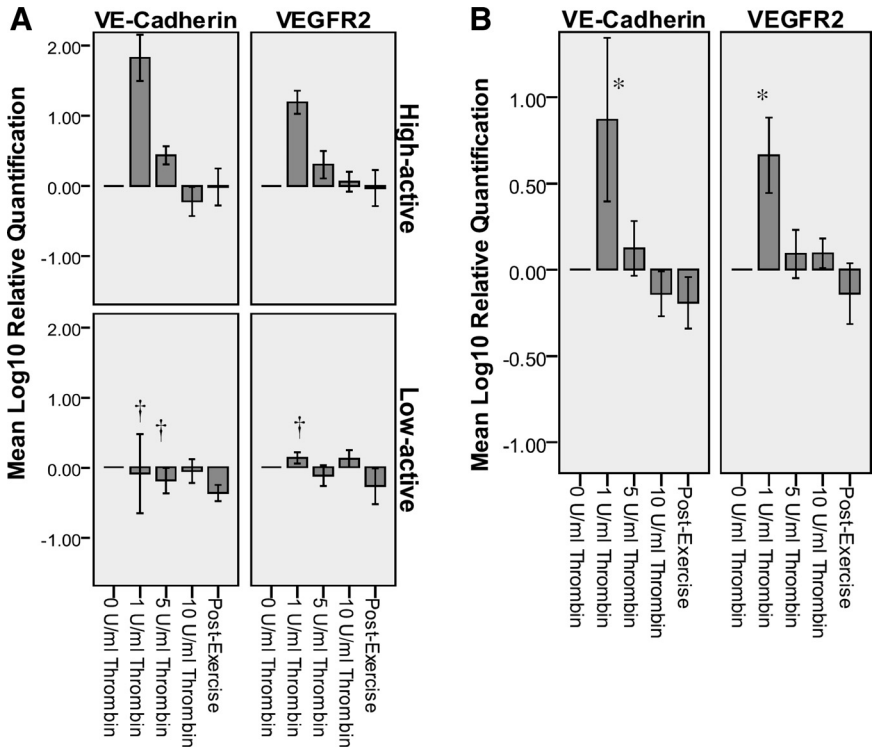


Fig. 3. VE-cadherin and vascular endothelial growth factor receptor-2 (VEGFR2) gene expression in CFU-ECs supplemented with thrombin and after 30 min of vigorous exercise. Values are means ± SE. *A*: high-active: $n = 6$; low-active: $n = 6$. †Significant difference between groups ($P \leq 0.05$). *B*: $n = 12$: high-active, $n = 6$; low-active, $n = 6$. All comparisons were relative to the control, 0 U/ml thrombin supplementation, set at 0.0. *Significant difference from control ($P \leq 0.05$).

to induce proliferation of putative human EPCs at thrombin levels that have been routinely used in the literature (5, 25, 27).

Gene expression of both endothelial cell surface markers, VEGFR2 and VE-cadherin, was significantly elevated in the high-active group with 1 U/ml thrombin supplementation, but returned to baseline at all other thrombin concentrations. Among the low-active participants, there was no significant effect of thrombin on expression of these cell surface markers. The elevated gene expression seen in high-active, but not low-active, participants with 1 U/ml thrombin supplementation may be indicative of a training effect on EPCs with respect to their sensitivity to thrombin-mediated differentiation signals. This effect was seen only at the lowest level of supplementation and was absent at 5 and 10 U/ml supplementation. Interestingly, the cell cycle genes seemed to be more affected by these higher levels of thrombin (discussed above). At present, there are no other data in the literature examining a relationship between either gene expression or EPC differentiation and a low-thrombin supplementation level. These results warrant further investigation into possible mechanistic relationships between thrombin signaling and differentiation of putative EPCs.

Having confirmed earlier data (32) that acute exercise increased circulating markers of thrombin production, we further investigated gene expression changes in putative CFU-ECs isolated from blood drawn 30 min after vigorous exercise. A significant increase in cyclin D1 and decreased p27 expression, without changes in VEGFR2 or VE-cadherin expression, mirrored the gene expression profile of putative EPCs that received thrombin supplementation. These results indicate that, during vigorous exercise, there is apparent signaling to circulating putative EPCs, possibly by thrombin, that results in increased expression of cell cycle genes up to 72 h after exercise and subsequent in vitro incubation. We conclude, therefore, that this is evidence of a possible link between acute exercise and EPC activity.

### *Exercise-Induced Changes in Putative EPCs*

The response of putative EPCs to vigorous treadmill exercise is ambiguous in the current literature, but our data are consistent with emerging evidence that age and training status may modify the acute exercise effect. Van Craenenbroeck et al. (28) found that maximal cycling resulted in a significant increase in EPC number ($CD34^+/VEGFR2^+$), but no change in CFU-ECs in a younger population. Also in a younger population, Laufs et al. (14) found a significant increase in circulating EPC concentration ($CD34^+/VEGFR2^+$) of ~125%, as well as a significant increase in CFU-ECs of ~66% with submaximal running exercise. We recently reported a significant increase in CFU-EC following 30 min of running at ~75% $\dot{V}O_{2max}$ in highly active, but not low-active, young healthy men (12). Finally, Thijssen et al. (26) found that the acute exercise-induced increase in circulating CD34+ progenitor cell number was greater in younger than older men. Interestingly, the magnitude of change for CFU-EC and $CD34^+/VEGFR2^+$ cells with acute exercise in these studies was similar to that in the present study; however, our older population demonstrated a much larger interindividual variability, resulting in a statistically insignificant effect of vigorous exercise on circulating $CD34^+/VEGFR2^+$ number or CFU-ECs. Such a high variability found in our older healthy individuals, compared with the younger healthy individuals in previous studies, suggests that the varying EPC response to acute exercise may be related to age, training status, or some yet unidentified factor.

We observed a correlation between exercise-induced changes in F1+2 and exercise-induced changes in circulating $CD34^+/$

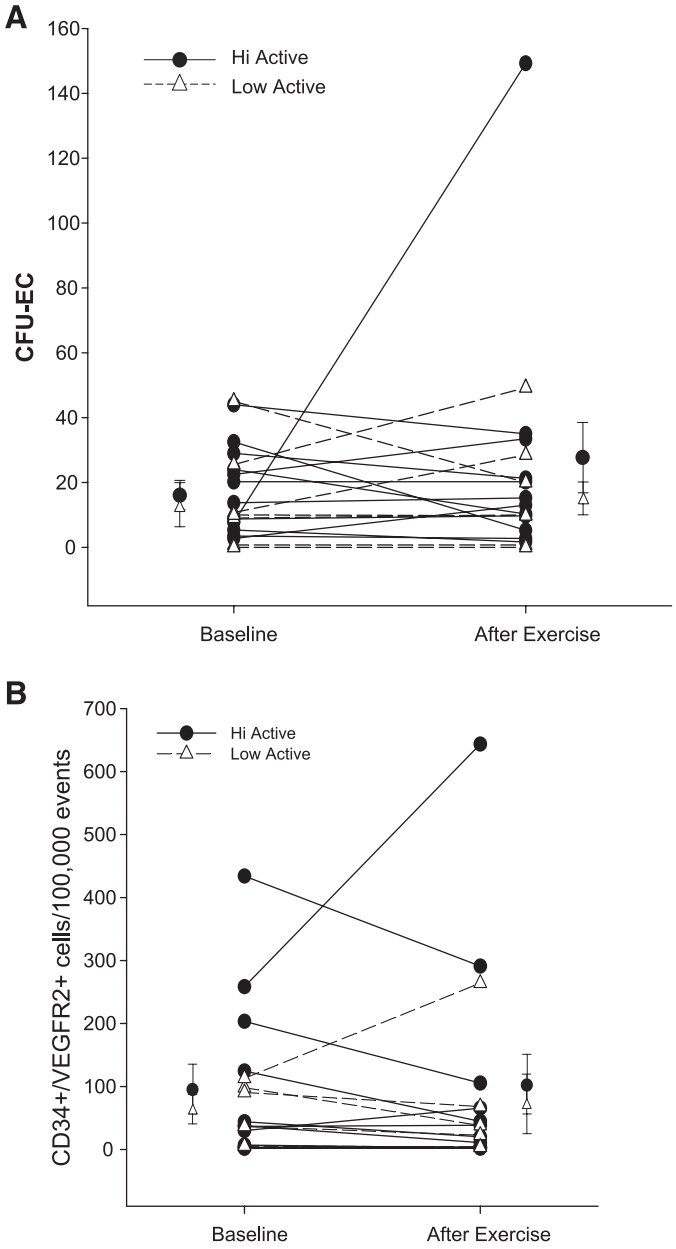


Fig. 4. CFU-EC (*A*) and CD34+/VEGFR2$^+$ (*B*) before and after 30 min of vigorous treadmill exercise. Values are means ± SE. *A*: high-active: $n = 12$; low-active: $n = 7$. High-active before exercise: 17.8 ± 3.9; after exercise: 26.4 ± 9.8 ($P = 0.505$). Low-active before exercise: 14.2 ± 4.1; after exercise: 17.0 ± 12.8 ($P = 0.681$). *B*: high-active: $n = 12$; low-active: $n = 6$. High-active before exercise: 84.0 ± 22.9; after exercise: 132.0 ± 59.7 ($P = 0.326$). Low-active before exercise: 41.3 ± 23.9; after exercise: 80.1 ± 84.4 ($P = 0.471$).

VEGFR2$^+$ number in both high-active and low-active participants. While this correlation does not imply a causal relationship, in this case, there are a number of biologically plausible mechanisms that could result in a cause-and-effect relationship. The coagulation cascade is believed to be activated in response to elevated shear stress (33), which is elevated during exercise (20). Greater shear stress, therefore, results in greater thrombin production. The magnitude of changes seen in circulating EPC concentration within individuals may be the result of the magnitude of shear stress induced by exercise. Alternatively, thrombin may directly act to enhance EPC mobilization. Data from previous studies have indicated a direct signaling mechanism of thrombin in EPCs through the PAR-1 receptor (23, 25). This is further supported by evidence from the present study of thrombin-induced alterations in EPC gene expression, specifically those genes involved in the regulation of cellular proliferation. Alternatively, exercise may induce the appearance of circulating EPCs from previously sequestered cells rather than direct mobilization, as has been observed in hemopoetic stem cells (6).

*Limitations*

The results of our gene expression analysis, in combination with the previous literature, demonstrate a possible role for thrombin in EPC regulation, specifically cellular proliferation. It remains unclear, however, if the changes in gene expression of cell cycle genes with both thrombin supplementation and vigorous exercise will translate directly into increased proliferation of EPCs. Future studies should investigate the detailed pathways by which exercise and/or thrombin may affect cell cycle regulation in putative EPCs. As our ex vivo data, indicating that thrombin and exercise seem to be similarly influencing EPC gene expression, are clearly descriptive, future studies should experimentally investigate, e.g., with in vivo PAR-1 inhibition in an animal model, whether thrombin production is required for exercise-induced promotion of EPC proliferation and/or differentiation. In particular, examination of signaling events downstream of thrombin/PAR-1 will be important. Second, we have interpreted our cell cycle gene expression data as evidence for enhancement of proliferation, but we must be cautious about this conclusion, as we cannot rule out a number of other possibilities. For example, exercise and/or thrombin may promote the differentiation from early-outgrowth EPCs to late-outgrowth EPCs, which could be associated with a similar gene expression pattern that we have

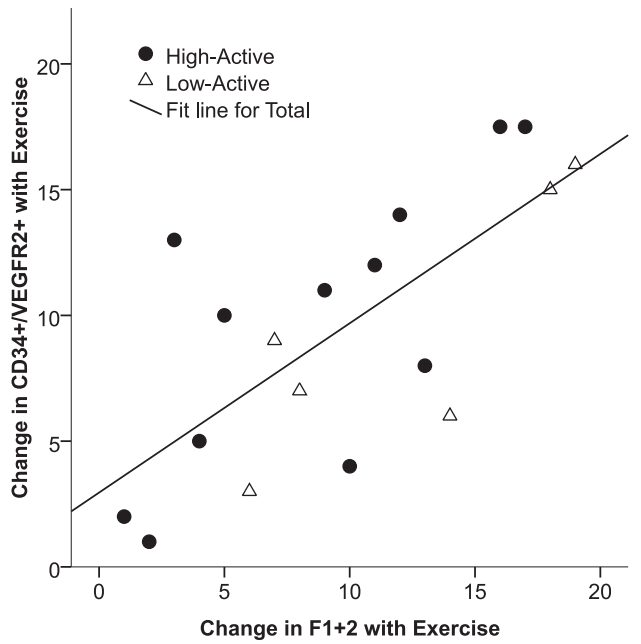


Fig. 5. Relationship between the change in plasma F1+2 and circulating CD34+/VDGFR2$^+$ with 30 min of vigorous exercise. Spearman correlation $r = 0.698$, $P = 0.001$. Variables presented as rank order reflect the nonparametric Spearman correlation test.

observed in the present study. Third, we observed correlations between exercise-induced thrombin production and changes in circulating putative EPC number. While this implies a connection between exercise-induced coagulation activation and the accumulated improvement to endothelial health with exercise training, it does not directly demonstrate the proliferation and incorporation of circulating EPCs at the sites of endothelial cell turnover and repair.

### *Implications and Conclusions*

EPCs have been established as an important regulator of endothelial maintenance and repair (2, 29), as well as fibrin clot resolution through clot recanalization (16, 17, 22, 22). This study is the first to demonstrate a statistical relationship between individual exercise-induced changes in circulating EPCs ($CD34^+/VEGFR2^+$) and thrombin generation. We have further demonstrated similar gene expression profiles among CFU-ECs supplemented with thrombin in vitro and those isolated after 30 min of vigorous treadmill exercise. With an increased expression of cell cycle genes and suppressed expression of a cell cycle inhibitor gene, we speculate that EPC proliferation could be promoted at the site of endothelial repair and clot resolution. Taken together, the results of our study and previously published data suggest a novel mechanism for improved endothelial health and reduced cardiovascular risk with exercise training. While further research is required to elucidate the specific pathways, these thrombin-mediated effects on cell cycle genes in putative EPCs may be a mechanism through which regular exercise leads to more frequent cellular turnover along the endothelium, enhanced fibrin clot resolution through recanalization, and improved endothelial function.

#### ACKNOWLEDGMENTS

The authors acknowledge Keith Tanner, Regina Harley, and Cathy Storrer from the University of Maryland Baltimore Medical School for technical assistance in $CD34^+/VEGFR2^+$ assay optimization and quantification, as well as Caprice Rosato at the University of Oregon for technical assistance with quantitative RT-PCR.

#### GRANTS

This work was supported by National Institutes of Health Pre-Doctoral Training Grant AG00268. T. O. Obisesan was supported by National Institute on Aging K award AG000980.

#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### REFERENCES

1. **American College of Sports Medicine.** *ACSM's Guidelines For Exercise Testing and Prescription*. New York: Lippincott Williams & Wilkins, 2000.
2. **Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM.** Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275: 964–967, 1997.
3. **Bellik L, Ledda F, Parenti A.** Morphological and phenotypical characterization of human endothelial progenitor cells in an early stage of differentiation. *FEBS Lett* 579: 2731–2736, 2005.
4. **Blair SN, Kampert JB, Kohl HW III, Barlow CE, Macera CA, Paffenbarger RS Jr, Gibbons LW.** Influences of cardiorespiratory fitness and other precursors on cardiovascular disease and all-cause mortality in men and women. *JAMA* 276: 205–210, 1996.
5. **Eto M, Rathgeb L, Cosentino F, Kozai T, Luscher TF.** Statins blunt thrombin-induced down-regulation of endothelial nitric oxide synthase expression in human endothelial cells. *J Cardiovasc Pharmacol* 47: 663–667, 2006.
6. **Fleming HE, Janzen V, Lo CC, Guo J, Leahy KM, Kronenberg HM, Scadden DT.** Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell* 2: 274–283, 2008.
7. **Green DJ, Maiorana A, O'Driscoll G, Taylor R.** Effect of exercise training on endothelium-derived nitric oxide function in humans. *J Physiol* 561: 1–25, 2004.
8. **Griffin CT, Srinivasan Y, Zheng YW, Huang W, Coughlin SR.** A role for thrombin receptor signaling in endothelial cells during embryonic development. *Science* 293: 1666–1670, 2001.
9. **Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T.** Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 348: 593–600, 2003.
10. **Hinkle DE, Wiersa W, Jurs SG.** *Applied Statistics for the Behavioral Sciences*. Boston, MA: Houghton Mifflin, 1998.
11. **Imanishi T, Moriwaki C, Hano T, Nishio I.** Endothelial progenitor cell senescence is accelerated in both experimental hypertensive rats and patients with essential hypertension. *J Hypertens* 23: 1831–1837, 2005.
12. **Jenkins NT, Witkowski S, Spangenburg EE, Hagberg JM.** Effects of acute and chronic endurance exercise on intracellular nitric oxide in putative endothelial progenitor cells: role of NAPDH oxidase. *Am J Physiol Heart Circ Physiol* 297: H1798–H1805, 2009.
13. **Kanazawa H, Yoshikawa T.** Up-regulation of thrombin activity induced by vascular endothelial growth factor in asthmatic airways. *Chest* 132: 1169–1174, 2007.
14. **Laufs U, Urhausen A, Werner N, Scharhag J, Heitz A, Kissner G, Bohm M, Kindermann W, Nickenig G.** Running exercise of different duration and intensity: effect on endothelial progenitor cells in healthy subjects. *Eur J Cardiovasc Prev Rehabil* 12: 407–414, 2005.
15. **Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M, Nickenig G.** Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation* 109: 220–226, 2004.
16. **Modarai B, Burnand KG, Sawyer B, Smith A.** Endothelial progenitor cells are recruited into resolving venous thrombi. *Circulation* 111: 2645–2653, 2005.
17. **Moldovan NI, Asahara T.** Role of blood mononuclear cells in recanalization and vascularization of thrombi: past, present, and future. *Trends Cardiovasc Med* 13: 265–269, 2003.
18. **Niebauer J, Cooke JP.** Cardiovascular effects of exercise: role of endothelial shear stress. *J Am Coll Cardiol* 28: 1652–1660, 1996.
19. **O'Brien JR.** Shear-induced platelet aggregation. *Lancet* 335: 711–713, 1990.
20. **Padilla J, Harris RA, Rink LD, Wallace JP.** Characterization of the brachial artery shear stress following walking exercise. *Vasc Med* 13: 105–111, 2008.
21. **Rehman J, Li J, Parvathaneni L, Karlsson G, Panchal VR, Temm CJ, Mahenthiran J, March KL.** Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. *J Am Coll Cardiol* 43: 2314–2318, 2004.
22. **Smadja DM, Basire A, Amelot A, Conte A, Bieche I, Le Bonniec BF, Aiach M, Gaussem P.** Thrombin bound to a fibrin clot confers angiogenic and haemostatic properties on endothelial progenitor cells. *J Cell Mol Med* 12: 975–986, 2008.
23. **Smadja DM, Laurendeau I, Avignon C, Vidaud M, Aiach M, Gaussem P.** The angiopoietin pathway is modulated by PAR-1 activation on human endothelial progenitor cells. *J Thromb Haemost* 4: 2051–2058, 2006.
24. **Steiner S, Niessner A, Ziegler S, Richter B, Seidinger D, Pleiner J, Penka M, Wolzt M, Huber K, Wojta J, Minar E, Kopp CW.** Endurance training increases the number of endothelial progenitor cells in patients with cardiovascular risk and coronary artery disease. *Atherosclerosis* 181: 305–310, 2005.
25. **Tarzami ST, Wang G, Li W, Green L, Singh JP.** Thrombin and PAR-1 stimulate differentiation of bone marrow-derived endothelial progenitor cells. *J Thromb Haemost* 4: 656–663, 2006.
26. **Thijssen DH, Vos JB, Verseyden C, van Zonneveld AJ, Smits P, Sweep FC, Hopman MT, de Boer HC.** Haematopoietic stem cells and endothelial progenitor cells in healthy men: effect of aging and training. *Aging Cell* 5: 495–503, 2006.
27. **Troyanovsky B, Alvarez DF, King JA, Schaphorst KL.** Thrombin enhances the barrier function of rat microvascular endothelium in a PAR-1-dependent manner. *Am J Physiol Lung Cell Mol Physiol* 294: L266–L275, 2008.
28. **Van Craenenbroeck EM, Vrints CJ, Haine SE, Vermeulen K, Goovaerts I, Van Tendeloo VF, Hoymans VY, Conraads VM.** A maximal

exercise bout increases the number of circulating $CD34^+/KDR^+$ endothelial progenitor cells in healthy subjects. Relation with lipid profile. *J Appl Physiol* 104: 1006–1013, 2008.
29. **Walter DH, Dimmeler S.** Endothelial progenitor cells: regulation and contribution to adult neovascularization. *Herz* 27: 579–588, 2002.
30. **Wei M, Kampert JB, Barlow CE, Nichaman MZ, Gibbons LW, Paffenbarger RS Jr, Blair SN.** Relationship between low cardiorespiratory fitness and mortality in normal-weight, overweight, and obese men. *JAMA* 282: 1547–1553, 1999.
31. **Witkowski S, Lockard MM, Jenkins NT, Obisesan TO, Spangenburg EE, Hagberg JM.** Relationship between circulating progenitor cells, vascular function and oxidative stress with long-term training and short-term detraining in older men. *Clin Sci (Lond)* 118: 303–311, 2010.
32. **Womack CJ, Nagelkirk PR, Coughlin AM.** Exercise-induced changes in coagulation and fibrinolysis in healthy populations and patients with cardiovascular disease. *Sports Med* 33: 795–807, 2003.
33. **Wu KK, Thiagarajan P.** Role of endothelium in thrombosis and hemostasis. *Annu Rev Med* 47: 315–331, 1996.
34. **Yamamoto K, Takahashi T, Asahara T, Ohura N, Sokabe T, Kamiya A, Ando J.** Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress. *J Appl Physiol* 95: 2081–2088, 2003.