
9-1-2014

Circulating Angiogenic Cell Population Responses to 10 Days of Reduced Physical Activity

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Circulating angiogenic cell population responses to 10 days of reduced physical activity

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Submitted 3 February 2014; accepted in final form 4 July 2014

Guhanarayan G, Jablonski J, Witkowski S. Circulating angiogenic cell population responses to 10 days of reduced physical activity. *J Appl Physiol* 117: 500–506, 2014. First published July 10, 2014; doi:10.1152/jappphysiol.00087.2014.—Circulating angiogenic cells (CACs) are a diverse group that have been identified as predictors of cardiovascular health and are inversely proportional to cardiovascular disease (CVD) outcomes. Inactivity is a growing concern in industrialized nations and is an independent risk factor for CVD. There is limited evidence regarding the impact of reduced physical activity (rPA) on different CAC populations. The purpose of this study was to evaluate the effect of objectively monitored rPA with maintained energy balance on two CAC populations (CFU and CD34⁺ cells), intracellular nitric oxide (NOi), and genes related to NO production in active, healthy men. Participants (age 25 ± 2.9 yr) refrained from structured physical activity for 10 days, which was reflected by a significant reduction in time in vigorous + very vigorous intensity activity ($P = 0.03$). Sedentary time tended to increase ($P = 0.06$) with rPA. CFU CACs have been characterized as mainly monocytic and lymphocytic cells. We found significant reductions in both the number of CFU CACs (−35.69%, $P = 0.01$) and CFU CAC NOi (−33.84%, $P = 0.03$). Neither NOi nor the number of CD34⁺ cells, which are hematopoietic and endothelial progenitors, changed with rPA. We found no significant differences in NO-related gene expression or oxidative stress-related gene expression with rPA in either CAC type. Therefore, we conclude that although various CAC populations have been related to vascular health, regular physical activity is necessary to maintain CAC NOi and the vulnerability of CACs to short-term reductions in physical activity is population specific.

endothelium; vascular repair; nitric oxide; cardiovascular regeneration; exercise; sedentary behavior

FEWER THAN 5% OF ADULTS IN the United States meet the current guidelines for physical activity (53). Physical inactivity is a major independent risk factor for cardiovascular disease (CVD) and is associated with diabetes mellitus, high cholesterol, and obesity (1, 5, 7, 8, 52). Endothelial dysfunction underlies the pathophysiology of cardiometabolic disease, and recent studies show that endothelial dysfunction occurs with physical inactivity (9, 10, 15, 24). Reduced nitric oxide (NO) bioavailability is a significant determinant of endothelial dysfunction. A growing body of evidence suggests that circulating cells with angiogenic potential (i.e., circulating angiogenic cells, CACs) contribute to proper endothelial health and function, however, the role of NO within CACs and the influence of lifestyle factors such as physical activity, are largely unknown.

Since the discovery of CD34⁺ endothelial progenitors with vascular regenerative capacity (4), other cell types have been

identified that support blood vessel growth and repair. CACs are a diverse group of cells from both endothelial and hematopoietic lineages (21, 60). Some CACs have the capacity to differentiate into mature endothelial cells, incorporate into preexisting vasculature, and form new blood vessels, whereas other CACs support vascular repair via a paracrine mechanism by which they secrete factors to assist repair of damaged endothelium (21, 41, 59–61). Characterization of individual CAC populations and discovery of the mechanisms by which they specifically contribute to cardiovascular health can improve the efficacy of cell-based treatment.

Evidence suggests that physical activity alters the number and function of some CAC types. For example, fewer colony-forming unit (CFU) CACs are found in sedentary compared with active young men (34), and acute exercise increases the number of CD34⁺/VEGFR2⁺ CACs (38, 54). Only one study has evaluated the influence of reduced physical activity on CACs. Witkowski et al. showed that the number CD34⁺ hematopoietic CACs decreased with 10 days of short-term reduced physical activity (rPA) in older, highly trained men, and that the change in CD34⁺/VEGFR2⁺ cells in peripheral blood significantly predicted the change in the reactive hyperemic forearm blood flow response with reduced activity (57). NO has been shown to be necessary for proper cell migration and neovascularization in some CAC types (22, 27, 55) and is deficient in cardiometabolic disease states (12, 58). CAC NO production can be influenced by activity and expression of endothelial nitric oxide synthase (eNOS), the enzyme that catalyzes the production of NO. Other forms of eNOS regulation include inhibition when it is bound to caveolin 1 (17), and activation via the histone deacetylase sirtuin 1 (40). In addition, superoxide production and the activity of the prooxidant enzyme NADPH oxidase can disrupt the beneficial physiological effects of NO in CACs. (22) Cross-sectional studies have shown that the detrimental effect of a low-active lifestyle on CFU CACs may be related to decreased intracellular NO (NOi), which is at least partially due to higher NADPH oxidase and cellular oxidative stress (34). However, CD34⁺ CAC NOi was higher, and there was greater expression of nitro-oxidative stress-related genes in cells from sedentary young men compared with active young men (33). Therefore, the effect of regular exercise and changes in activity on different CAC populations, NO, and the role of oxidative stress is unclear.

Studies on inactivity and vascular function have used a variety of experimental models, including spinal cord injury, bed rest, and limb immobilization. These studies have revealed drastic changes in cardiorespiratory capacity, vessel structure, and endothelial function with severe inactivity (9, 14, 47, 48). In many of these cases, the results are attributed to long-term inactivity and may be influenced by confounding factors such as inflammation, changes in energy balance, and body mass

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index (28, 50). Longitudinal designs that incorporate short-term transitions from regular, cardioprotective activity patterns to lower activity levels may illuminate early and modifiable factors that are related to deteriorating cardiometabolic health. This information could improve current physical activity prescription and health recommendations. Therefore, the purpose of this study was to use an objectively monitored model of 10 days of rPA in endurance-trained men to determine the effects on two different CAC populations (CFU and CD34⁺), NOi, NO-related CAC gene expression, and oxidative stress-related CAC gene expression. Participants maintained energy balance during rPA to evaluate the independent effect of rPA. We hypothesized that CACs would be fewer in number and demonstrate lower NOi following rPA. We evaluated the expression of genes related to eNOS activity and NADPH-related oxidative stress to identify their potential role in inactivity-induced CAC responses.

METHODS

Subjects. The University of Massachusetts Institutional Review board approved all protocols. Written informed consent was obtained from all participants. Participants were healthy, nonsmoking, physically active men aged 19–47 with a training history of at least 5 yr. Medical and physical activity questionnaires were administered, and resting blood pressure and lipid profile were assessed to verify that participants engaged in moderate to intense endurance exercise at least 3 days a week; were free of diabetes, cancer, and cardiovascular, lung, and liver disease; and were not taking any medications that could influence CAC number or function (46). Prior to testing, participants were required to refrain from caffeine and alcohol for 24 h. Because dietary nitrate may influence blood pressure and endothelial function (30), participants adhered to a low-nitrate diet for 72 h prior to testing. Participants were instructed to engage in a typical exercise session that was to be completed 24 h prior to their baseline visit.

Physical activity and energy expenditure. Physical activity level was assessed via an accelerometer (GT3X; ActiGraph, Pensacola, FL) that was worn on the left hip for 7 days prior to baseline testing. To assess steps/day independent of structured exercise, participants wore an Omron pedometer during the baseline period, which was removed during exercise sessions. Both devices were used to monitor the 10 days of rPA in which participants were instructed to cease structured exercise and maintain their baseline nonexercise step count. Activity logs completed by participants were used to supplement the physical activity data. ActiGraph acceleration signal outputs were evaluated using ActiLife software (version 5.9.1). Freedson cut points (23) were used to evaluate baseline and rPA minutes/day spent in sedentary [<1.5 metabolic equivalent (MET)], light (<2.99 MET), moderate (3.0–5.99 MET), vigorous (6.0–8.99 MET), and very vigorous (>9.0 MET) activity. MET cut point values are widely used in physical activity measurement to align activity monitor counts with absolute activity intensity (3), however, they do not reflect relative intensity or the exact energy cost of activity. To prescribe caloric intake during 10-day rPA, ActiGraph data from the baseline week were analyzed with the nonlinear regression artificial neural network developed by Staudenmayer et al. (49) to calculate the energy expenditure (kcal/day) for all activities >3 MET.

Dietary monitoring and energy intake. Energy intake was assessed via the Automated Self-Administered 24-h dietary recall (ASA24; National Cancer Institute, Bethesda, MD) three times per week (2 weekdays and 1 weekend day) over the course of the baseline week. The data from the dietary recalls were used to calculate each participant's average energy intake (kcal/day). Based on the baseline energy expenditure (described above) and intake data, each

participant was given a goal for daily caloric intake for their 10 days in rPA to maintain energy balance during the reduction in expenditure. Participants logged daily energy intake on myfitnesspal (<http://www.myfitnesspal.com>) and were monitored by study personnel to ensure participants attained their daily goals.

CFU CAC assay. Venous blood (60 ml) was collected into EDTA-coated tubes following 30 min of quiet supine rest during the baseline visit and following 10 days of rPA. CFU cells were cultured after the baseline and final visits. The CFU assay (CFU-Hill; StemCell Technologies, Vancouver, BC, Canada) is a 5-day culture method developed by Hill et al. to assess CACs on the basis of CFU growth (29). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples via density centrifugation with Ficoll-Paque (GE Healthcare, Pittsburgh, PA). PBMCs were placed at a density of 5×10^6 cells per well in fibronectin-coated plates with growth medium. Cells were cultured for 2 days (37°, 5% CO₂, 95% humidity). After 2 days, nonadherent cells were removed and counted. Nonadherent cells (1×10^6) were replated on day 2 into wells of a 24-well fibronectin-coated plate with fresh CFU media. Cells were cultured under the same conditions for 3 more days. On day 5, an investigator blinded to the experimental condition counted the number of CFUs. A CFU was characterized as a cluster of cells that form a round center with outward radiating cells, and quantified as average CFU/well.

CD34⁺ cell isolation. PBMCs were isolated via density centrifugation, then CD34⁺ cells were isolated from the PBMC population with magnetic beads (StemCell Technologies). Briefly, PBMCs were suspended in phosphate-buffered saline (PBS) + 2% fetal bovine serum (FBS) with EDTA at a density of 2×10^8 cells/ml. A CD34⁺ selection antibody cocktail was added at a concentration of 100 μ l/ml and incubated at room temperature for 15 min. CD34⁺ magnetic nanoparticles were added (100 μ l/ml) and incubated for 10 min. PBS + 2% FBS with EDTA was added to a volume of 2.5 ml, then the tube was placed in the separation magnet for 10 min. Following incubation, the supernatant was removed to eliminate non-CD34⁺ cells, and the procedure was repeated to further purify the CD34⁺ population. After the second incubation, the CD34⁺ cells were collected in a hemocytometer and counted. Flow cytometry analysis revealed that this protocol enriched cells to 60% purity, which is similar to the purity reported by Jenkins et al. (33) and greater than CD34⁺ enrichment reported in studies by Asahara et al. (4) and Schattman et al. (26, 43, 44), which ranged from 16% to 50%.

Intracellular NO. NOi was measured in freshly isolated CD34⁺ cells and CFU cultured cells using 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate fluorescent dye (Invitrogen, Carlsbad, CA). DAF-FM diacetate emits fluorescence when reacting with an intermediate of NO as NO is oxidized to NO₂⁻. DAF-FM in PBS (10 μ M) was added to 1.5×10^5 cells and incubated for 60 min at 37°C. NOi was measured with a fluorescence plate reader (PolarStar Optima, BMG Labtech) with excitation and emission wave lengths of 485 and 520 nm, respectively. Each assay included control wells with 1) unloaded cells to control for autofluorescence, 2) DAF-FM dye without cells, and 3) PBS alone. In addition, SIN-1, a nitric oxide donor, was added in known concentrations to each plate as positive controls. Intra-assay and interassay variabilities were 6.5% and 1.1%, respectively.

Extracellular nitric oxide. To evaluate whether rPA influenced NO release by CFU-CAC into the media, nitrate/nitrite content in the media was measured with the Griess Assay (Cayman, Ann Arbor, MI) via the manufacturer's specifications. Briefly, this assay converts nitrate to nitrite with the enzyme nitrate reductase then uses Griess reagents to convert nitrite into the azo chromophore compound, which can be evaluated through reading the absorbance at 540 nm. Media collected from cultured CFU CACs (day 5) at baseline and following rPA was thawed on ice and assayed in triplicate along with standards on a single plate. The intra-assay coefficient of variation was $7.93 \pm 4.45\%$.

Table 1. Subject characteristics

Characteristic	Baseline	Reduced Physical Activity*	
	Means ± SE	Means ± SE	P
Age, yr	25 ± 2.9		
Height, inches	70.8 ± 1.0		
Weight, kg	75.7 ± 2.7	75.7 ± 2.7	0.94
SBP, mmHg	122.4 ± 5.4	121.6 ± 6.3	0.93
DBP, mmHg	72.8 ± 4.4	68.5 ± 2.2	0.39
HR, bpm	78.6 ± 4.0	86.25 ± 4.8	0.24
TC, mg/dl	141.4 ± 12.2	149.3 ± 14.2	0.68
TG, mg/dl	54.7 ± 6.5	54.6 ± 9.5	0.99
HDL, mg/dl	50 ± 4.7	48.4 ± 5.7	0.84
LDL, mg/dl	80.5 ± 9.4	89.9 ± 9.7	0.50

SBP, systolic blood pressure; DBP, diastolic blood pressure, HR, heart rate; bpm, beats per minute; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein. *10 days, $n = 8$.

RNA isolation and gene expression. Gene expression was assessed via real-time PCR (RT-PCR). RNA was isolated from freshly isolated CD34⁺ cells and CFU CACs with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. RNA was quantified on a spectrophotometer (NanoDrop; Thermo Scientific, Agawam, MA) via 260/280 nm, and reverse transcribed with iScript reverse transcriptase and a CFX96 RT-PCR machine (BioRad, Hercules, CA). Primer pairs (Integrated DNA Technologies, Coralville, IA) for each gene were designed and optimized to determine the optimal temperature and concentration for an efficiency of >90% using EvaSsofast PCR mastermix (BioRad). All gene expression analyses were run in triplicate and *GAPDH* was used as a control gene for each sample. We analyzed genes known to contribute to the production of NO (*eNOS*, *iNOS*, *Sirt1*, and *Cav1*), and genes related to NADPH oxidase and the production of oxidative stress (*Nox2*, *Nox4*, *p47phox*, and *SOD1*), which can reduce NO production. Forward and reverse primer sequences are found in Table 3. Messenger RNA expression of the target gene was calculated as $2^{-\Delta\Delta C_T}$, where ΔC_T is the C_T of the target gene minus *GAPDH* control for each condition. Fold change in gene expression is calculated relative to the baseline condition.

Statistical analyses. Paired Student's *t*-tests were used to determine differences from before to after 10 days of rPA in the number of CFU CACs, CFU NOi, CFU extracellular NO (NOe), number of CD34⁺ CACs, CD34⁺ NOi, and gene expression from each CAC type. Statistical significance was accepted at $\alpha \leq 0.05$. Data are presented as means ± SE.

RESULTS

Participant characteristics. Ten participants were recruited and completed this study. One participant was excluded from all analyses because he did not adhere to the caffeine and alcohol restrictions during the 24 h required prior to testing. Adherence to the 10-day rPA protocol was verified with the ActiGraph monitor data. One participant was excluded from all analyses because the ActiGraph data revealed increased moderate to vigorous physical activity (MVPA, 21%) with rPA.

Blood pressure, heart rate, and lipid profile did not change with rPA compared with baseline (Table 1). Subjects had a history of 11.5 ± 1.9 yr of regular physical activity, exercised 5.4 ± 0.3 days/wk, and ran 21.3 ± 3.7 miles/wk. ActiGraph data from their baseline week verified that participants engaged in regular high-intensity physical activity. Neural network analysis revealed that the average energy expenditure for activities >3 MET was 361 ± 77 kcal/day. The average baseline energy intake from dietary recalls was $1,980 \pm 343$

kcal/day. There was no weight change with rPA ($P = 0.98$; Table 1), indicating that the prescribed decrease in energy intake during rPA successfully maintained energy balance in participants.

Physical activity. Table 2 includes data on monitored physical activity separated by category. The data file from one participant was corrupted and could not be evaluated. ActiGraph physical activity data verified that with rPA, participants significantly decreased high-intensity structured exercise (vigorous and very vigorous) by $45 \pm 22\%$ ($P = 0.03$). There were slight but nonsignificant decreases in moderate, lifestyle, and light activities. Sedentary time tended to increase ($P = 0.06$) with rPA. Pedometer data indicated that average steps/day did not significantly decrease between baseline and rPA.

CAC number, NOi, and NOe. Reduced physical activity was associated with a 35.69% reduction in CFU CACs from baseline (16 vs. 10 colonies, $P = 0.01$; Fig. 1). The average number of CD34⁺ CACs did not change with rPA ($P = 0.68$; Fig. 1). CFU-Hill NOi decreased by 33.84% with rPA ($P = 0.03$). CD34⁺ CAC NOi was not significantly decreased with rPA ($P = 0.56$; Fig. 2). NOe was not different between baseline and rPA (18.6 ± 1.44 vs. 17.85 ± 1.68 , $P = 0.33$).

CAC gene expression. In both CAC types, we evaluated genes that contribute to the production of NO (*eNOS*, *iNOS*, *Sirt1*, and *Cav1*) and oxidative stress (*Nox2*, *Nox4*, *p47phox*, and *SOD1*). Reduced physical activity did not significantly alter expression of any gene that contributes to the production of NO or those related to oxidative stress (all $P > 0.05$; Table 3). The largest fold change in gene expression relative to baseline was in CFU CAC *eNOS* and *iNOS* (4.7 ± 2.2 , $P = 0.13$ and 4.8 ± 2.5 , $P = 0.19$, respectively), however, due to individual variability in the response, these changes did not reach significance.

DISCUSSION

The key finding of this study was that the two types of CACs we evaluated—CD34⁺-enriched and CFU CACs—had different responses to 10 days of rPA, with significant decreases in CFU CACs and no change in CD34⁺ CACs. Second, we observed a decrease in intracellular NO only in CFU CACs with rPA. We detected no difference in CAC NO-related gene expression or oxidative stress-related gene expression, and no change in extracellular NO with rPA in CFU CACs. Importantly, in this carefully controlled model of rPA, we objectively measured the physical activity of our participants to quantify

Table 2. Physical activity

Category	Baseline	rPA	
	Means ± SE	Means ± SE	P
Sedentary	654.76 ± 29.18	731.12 ± 35.73	0.06
Light	28.28 ± 3.38	22.03 ± 1.90	0.14
Lifestyle	24.66 ± 2.52	19.50 ± 1.47	0.10
Moderate	64.73 ± 9.84	54.50 ± 7.39	0.16
Vigorous	20.42 ± 4.59	10.50 ± 2.38	0.07
Very vigorous	9.32 ± 3.12	2.10 ± 1.43	0.09
MVPA	94.50 ± 12.44	67.10 ± 8.02	0.11
Vigorous + Very vigorous	29.74 ± 5.02	12.60 ± 3.14	0.03
Steps/day	6640 ± 877.8	6447 ± 1390.4	0.90

Physical activity (min/day) measured via Actigraph; cut points from Freedson et al. (23). $n = 7$. MVPA, moderate to vigorous physical activity.

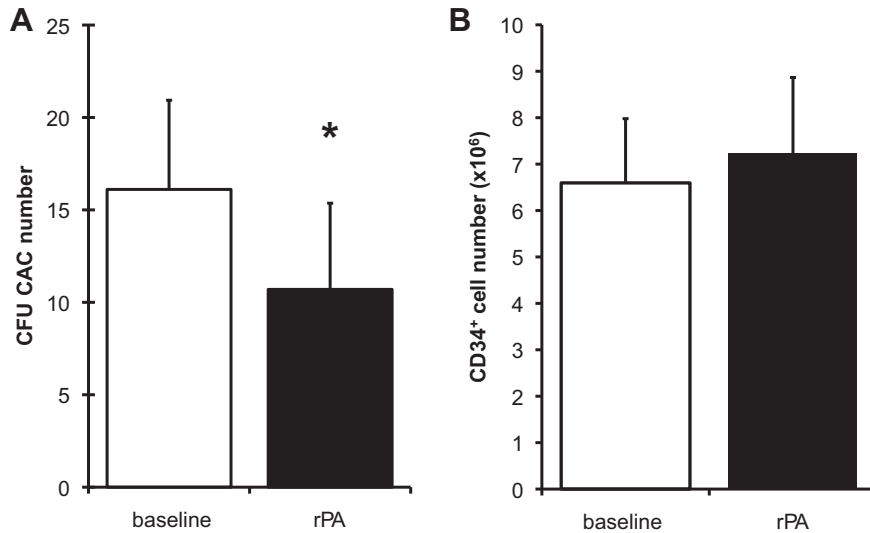


Fig. 1. Number of colony-forming unit (CFU) circulating angiogenic cells (CACs) (A) and CD34⁺ cells (B) at baseline and following reduced physical activity (rPA). *Significantly different from baseline, $P < 0.05$, $n = 8$.

the rPA and we used energy expenditure and caloric intake measures to ensure that participants remained in energy balance during rPA.

Recent evaluations of CACs suggest that the different populations may have varied functions in vascular health and integrity (21). CFU CACs are a mixed population of cells that consist of monocytes and T-lymphocytes, and a few endothelial progenitors (16, 31, 41). Desai et al. characterized these cells via microarray and flow cytometry as >75% CD3⁺/CD45⁺ T-cells (16). These immune cells support endothelial growth and repair via the release of angiogenic cytokines and growth factors because CFU CAC-conditioned media supported the vascular network formation in vitro (31, 41, 59). There is increasing recognition that immune cell populations have a provascular function (21). The inverse relationship between the Framingham risk score for CVD and CFU CACs, and correlations between CFU CACs and vascular function (29) reveal that they have an association with cardiovascular outcomes. Furthermore, Weil et al. recently described that CD3⁺/CD31⁺ angiogenic T-cell migratory capacity was re-

lated to the forearm blood flow response to acetylcholine and Framingham risk score (56). The accumulating evidence suggests that immune cells likely play a role in cardiovascular homeostasis. Therefore, it will be important to continue to evaluate the impact of changes in physical activity behavior on these cells as potential modulators of endothelial function and cardiovascular disease.

CD34⁺ cells are hematopoietic and endothelial progenitors shown to contribute to vessel formation and endothelial repair (6). The number of circulating CD34⁺ cells is an independent predictor of cardiovascular events, and low CD34⁺ number is related to prediabetes and metabolic syndrome (18, 19). CD34⁺ CACs have also been used for their therapeutic potential in a number of CVD clinical trials (20, 45). Therefore, CD34⁺ CACs may be a functional biomarker for cardiometabolic disease. According to our previous work (57) in which we utilized the same 10-day rPA protocol as the current study with highly active older male runners (mean age 62, with a training history of 32 ± 3 yr), we hypothesized that we would observe a significant decline in CD34⁺ in our younger active

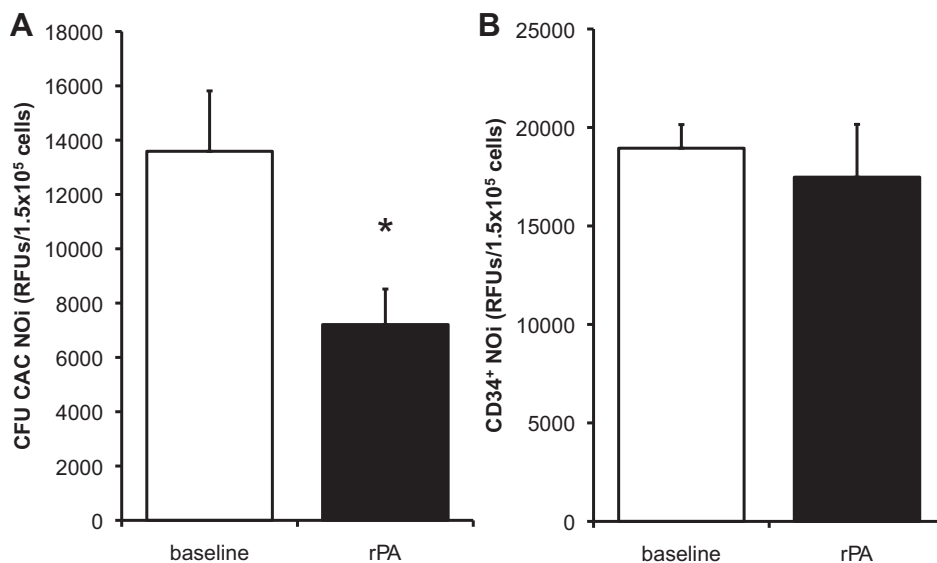


Fig. 2. Intracellular nitric oxide (NOi) measured in relative fluorescence units (RFUs) for CFU CACs (A) and CD34⁺ CACs (B) at baseline and following rPA. *Significantly different from baseline, $P < 0.05$, $n = 8$.

Table 3. RT-PCR genes, primer sequences, and change in gene expression with rPA

Symbol	Gene name	Primer sequence	CFU-CAC		CD34 ⁺ CAC	
			Fold Δ	P	Fold Δ	P
eNOS	Endothelial nitric oxide synthase	F: GAGGGAGCTGTTGTAGGG R: GTGGTAACCAGCACATTTGG	4.7 \pm 2.2	0.13	1.0 \pm 0.2	0.93
Cav1	Caveolin 1	F: AATACTGGTTTACCCTTGCT R: ATGCCGTCAAACTGTGTGTC	2.2 \pm 0.8	0.24	1.4 \pm 0.4	0.47
Sirt1	Sirtuin 1	F: CAGTGGCTGGAACAGTGAGA R: TCTGGCATGTCCCCTATCA	1.5 \pm 0.3	0.43	2.2 \pm 1.1	0.39
SOD1	Superoxide dismutase 1	F: ATGACTTGGGCAAAGGTGGAAATG R: GTTAAGGGGCCTCAGACTACATCC	1.2 \pm 0.2	0.46	1.0 \pm 0.2	0.97
Nox2 (CYBB)	NADPH oxidase 2; Cytochrome b(-245) beta	F: AGGATTGCCTGAAAGGTTCT R: AGGGCTAGCTGGAGAAGACC	1.1 \pm 0.2	0.71	1.2 \pm 0.2	0.56
Nox4	NADPH oxidase 4	F: CAGAAGGTTCCAAGCAGGAG R: GTTGAGGGCATTACCCAGAT	1.7 \pm 0.6	0.28	0.9 \pm 0.2	0.85
P47-phox (NCF1)	Neutrophil cytosolic factor 1	F: CAGGGACAACCAGACAAAAA R: AGAACCACCAACCGCTCTC	1.1 \pm 0.2	0.80	0.9 \pm 0.1	0.84
iNOS	inducible NOS	F: GGCCGCAGAGAAGCTCAGCCTCA R: CTCAAAACAGCCGCTTCCCCAGAA	4.8 \pm 2.5	0.19	0.9 \pm 0.2	0.85

Fold Δ , change in mRNA expression relative to the baseline condition, $n = 8$. F, forward; R, reverse.

men as we observed in the older men. However, 10 days of rPA was not related to lower CD34⁺ number, NOi, or changes in expression of NO stress-related genes or oxidative stress-related genes. Therefore, we interpret these findings that CD34⁺ CACs may be more resistant to short-term rPA compared with CFU CACs in young men, but that the susceptibility to changes in CD34⁺ CACs with rPA may be increased with age; however, further investigations are necessary to confirm this hypothesis.

NO has been shown to be essential for proper function of angiogenic cell populations. NO is important for CAC motility and may be a critical component of paracrine signaling to promote vascular repair and growth (2, 36, 51). In endothelial cells, several factors have been shown to regulate NO, including eNOS expression, phosphorylation, and direct binding of eNOS with cavelolin-1 (17). Also, Sirt1, an NADP⁺-dependent molecule, indirectly increases NO via deacetylation (activation) of eNOS (40). We found that decreases in CFU CAC NOi were independent of changes in *eNOS*, *Cav1*, and *Sirt1* gene expression. However, we assessed gene expression only in CACs, and a complete evaluation of the involvement of these NO regulatory mechanisms necessitates other molecular approaches. Therefore, given the association between CAC function and NO biology, further studies should be conducted to confirm the role of these NO regulatory mechanisms in various CAC populations.

In endothelial cells and CACs, oxidative stress from the enzyme NADPH oxidase has been shown to decrease NO production via eNOS uncoupling (32, 34, 37, 39). Jenkins et al. (34) reported significantly higher CFU CAC NOi in high-active compared with low-active men that was partially recovered in the CFU CACs from low-active men upon NADPH oxidase inhibition. Our data support the importance of regular physical activity to maintain CFU CAC NOi; however, we did not observe any significant changes in gene expression of any measured NADPH subunits in CACs with rPA as observed in other studies. Furthermore, *iNOS*, which is activated with inflammation and oxidative stress (13, 22), has been reported to be greater in CD34⁺ CACs of sedentary (<20 min/day on <2 day/wk) men compared with active men (33). Although we

observed a greater than fourfold increase in CFU CAC *iNOS* expression, the change was variable and not significant. Therefore, overall, the reduction in CFU CAC NOi with rPA observed in our study does not appear to be caused by oxidative stress, which may be due to the relatively short nature of the acute reduced physical activity.

The current study is novel in that it included objective physical activity monitoring, which allowed evaluation of changes in various components of physical activity and confirmation of whether or not all subjects met the inclusion criteria and adhered to the protocol. The greatest changes in physical activity in our participants were reduced time in vigorous and very vigorous activity and increased sedentary time, indicating that as prescribed, they specifically reduced time in structured, intense exercise, and as a result, spent more time being inactive. Recent studies suggest that physical activity and sedentary behavior may have independent and specific contributions to cardio-metabolic disease (1, 5, 11, 35). Therefore, more studies are necessary to explore the independent effects of increased sedentary time and decreased physical activity on cardiovascular outcomes.

Limitations. The study has some limitations including a small sample size, age and sex of participants, and may not be generalizable to other populations. Our study provides a foundation for future studies that may include pharmacologic manipulations of NADPH, NO, and oxidative stress to evaluate the mechanisms related to changes in NOi. CFU CACs in the current study are a mixed population of cells, and their role in vascular function and cardiovascular disease remains unclear. As mentioned previously, these CACs are largely monocytes and T-lymphocytes (16, 41). Monocytes cultured under angiogenic conditions have been shown to mimic the endothelial progenitor phenotype and to express endothelial genes and proteins (42). However, CFU-Hill colonies will not form when monocytes or T-cells are depleted from the culture (41, 42). These data indicate that although monocytes and T-cells in combination possess an in vitro function that appears to be vasculo-protective, the contribution and function of these cells in vivo is largely unknown. We believe that their association with CVD and CVD risk (29) and discovery of more cell types

with cardiovascular potential such as angiogenic T-cells, indicate that further studies on immune cells and vascular function are necessary.

We identified CD34⁺ cells with a single surface marker; this population contains subpopulations (i.e., CD34⁺/VEGFR2⁺), and differences in responses of these subpopulations may have contributed to some variability in our data. CD34⁺ cells are found in small quantities in peripheral blood; therefore, acquisition of enough cells for NOi and gene expression analysis would have required collecting large amounts of blood from participants. CD34⁺ cell marker is ubiquitous to hematopoietic and endothelial progenitor cells and was the original marker that identified endothelial progenitor cells (4). Although it is still currently used as the identifying marker for isolation and use of cells in cardiovascular regenerative therapies (45), isolation of specific CD34⁺ populations may improve the understanding of diversity in circulating angiogenic cells.

Conclusion. The present study used an acute reduction in structured physical activity with maintenance of energy balance to evaluate initial cellular and molecular events related to cardiovascular function. Our results indicate that two types of cells that have both been characterized as circulating angiogenic cells responded differently to our intervention, finding no changes in CD34⁺ CACs but significant reductions in CFU CACs and CFU CAC NOi. These data highlight the diversity of cells that may contribute to cardiovascular homeostasis and that individual populations of CACs may be more vulnerable to the effects of changes in physical activity and sedentary time. Understanding disease-related physiological changes that occur independently with reduced physical activity and increased sedentary behavior have the potential to identify novel targets for the prevention of disease, improve current physical activity and health recommendations, and change leisure time physical activity behavior and workplace physical activity policies (25).

ACKNOWLEDGMENTS

The authors thank the subjects for their participation in this study. We also thank A. Baez and R. Burgess for laboratory assistance, and Dr. L. Troy for careful review of the manuscript.

GRANTS

Support for this study was provided by a University of Massachusetts Amherst Faculty Research grant to S. Witkowski.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: G.G. and S.W. conception and design of research; G.G. and J.J. performed experiments; G.G., J.J., and S.W. analyzed data; G.G., J.J., and S.W. interpreted results of experiments; G.G. and S.W. prepared figures; G.G. and S.W. drafted manuscript; G.G., J.J., and S.W. edited and revised manuscript; G.G., J.J., and S.W. approved final version of manuscript.

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