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Chronic endurance exercise affects paracrine action of CD31+/ and CD34+/ cells on endothelial tube formation

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Landers-Ramos RQ, Sapp RM, Jenkins NT, Murphy AE, Cancre L, Chin ER, Spangenburg EE, Hagberg JM. Chronic endurance exercise affects paracrine action of CD31+ and CD34+ cells on endothelial tube formation. Am J Physiol Heart Circ Physiol 309: H407–H420, 2015. First published June 8, 2015; doi:10.1152/ajpheart.00123.2015.—We aimed to determine if chronic endurance-exercise habits affected redox status and paracrine function of CD34+/CD31+ circulating angiogenic cells (CACs). Subjects were healthy, nonsmoking men and women aged 18–35 yr and categorized by chronic physical activity habits. Blood was drawn from each subject for isolation and culture of CD34+/CD31+ CACs. No differences in redox status were found in any group across either cell type. Conditioned media (CM) was generated from the cultured CACs and used in an in vitro human umbilical vein endothelial cell-based tube assay. CM from CD34+/CD31+ cells from inactive individuals resulted in tube structures that were 29% shorter in length (P < 0.05) and 45% less complex (P < 0.05) than the endurance-trained group. CD34+/CD31+ CM from inactive subjects resulted in tube structures that were 26% shorter in length (P < 0.05) and 42% less complex (P < 0.05) than endurance-trained individuals. Proteomics analyses identified S100A8 and S100A9 in the CM. S100A9 levels were 103% higher (P < 0.05) and S100A8 was 97% higher in the CD34+/CD31+ CM of inactive subjects compared with their endurance-trained counterparts with no significant differences in either protein in the CM of CD34+/CD31+ CACs as a function of training status. Recombinant S100A8/A9 treatment at concentrations detected in inactive subjects’ CD34+/CD31+ CAC CM also reduced tube formation (P < 0.05). These findings are the first, to our knowledge, to demonstrate a differential paracrine role in CD34+ and CD34+/CD31+ CACs on tube formation as a function of chronic physical activity habits and identifies a differential secretion of S100A9 by CD34+/CD31+ CACs due to habitual exercise.

NEW & NOTEWORTHY

Factors secreted by CD34+ or CD34+/CD31+ circulating angiogenic cells from inactive subjects, but not those with increased chronic exercise behaviors, depress human umbilical vein endothelial cell tube formation. These findings appear to be at least partially mediated by greater S100A9 present in the conditioned media of inactive compared with endurance-trained individuals’ CD34+/CD31+ circulating angiogenic cells.

CARDIOVASCULAR DISEASE (CVD) with the associated dysfunction of the vasculature is the leading cause of death in developed countries (18). Studies suggest that the repair and maintenance of the vascular endothelium is critically dependent on the number and function of circulating angiogenic cells (CACs) (3, 53, 61). CAC is a broad term for subgroups of peripheral blood mononuclear cells (PBMCs) with angiogenic properties that stimulate vascular repair and contribute to neovascularization. CD34 is the most commonly described cell surface marker on CACs that are known for their progenitor cell properties (41). CD31 is a cell surface marker most commonly found in mature endothelial cells but has also been identified in circulating cells with angiogenic properties (26, 30, 31, 34). Although not as commonly studied as CD34+ CACs for therapeutic neovascularization, CD34+/CD31+ CACs have been found to exhibit similar angiogenic potential as CD34+ CACs in terms of improving mouse hindlimb ischemia, emphasizing the importance of studying non-progenitor cell lines that may also be involved in angiogenesis (31). Indeed, cells characterized by both markers have been previously found to exhibit angiogenic characteristics in vitro (3, 20, 30, 44) and in vivo (32, 37, 41).

Originally believed to exert their reparative functions through direct incorporation into the endothelium (3), recent evidence (32, 33, 44, 51) indicates that CACs accomplish their proangiogenic effects through paracrine mechanisms. Specifically, CACs are thought to secrete factors that elicit actions on the preexisting endothelium and/or signal other circulating cells to home to the endothelium (32, 33, 44, 51, 63). Recently, Hynes et al. (21) demonstrated that secretope from cultured CACs successfully treated a porcine model of myocardial infarction, suggesting that paracrine factors secreted by CACs may be more important for vascular repair than the cells alone. Urbich et al. (51) found that CACs expressed and released a number of different factors that assist with the growth of mature endothelial cells to accelerate revascularization in ischemic tissues. However, CD34+ or CD34+/CD31+ CAC paracrine actions and their susceptibility to modulation by lifestyle factors (e.g., physical activity) have received minimal investigation.

Physical activity is associated with improvements in cardiovascular (CV) health, including attenuation of many classic...
CVD risk factors (27, 38) and improvements in endothelial function (9, 48). Our laboratory and others (25, 28, 36, 52–54, 60) have previously shown that endurance-exercise training increases CAC number and enhances intracellular redox balance in younger and older adults compared with their sedentary counterparts. Hoetzer et al. (19) found that migratory activity of sedentary individuals’ CACs improved by ~50% after 12 wk of exercise training. We previously documented that CACs from young, sedentary but otherwise healthy individuals exhibit an unfavorable balance between reactive oxygen species (ROS) and nitric oxide (NO) compared with their endurance-trained counterparts (25, 28). It is generally accepted that CACs with less than optimal function cannot properly contribute to vascular repair or neangiogenesis, thus hindering their therapeutic use (13).

Previous studies have examined the effects of endurance-exercise habits on CAC function through measures such as migration and adhesion (19, 52), but the paracrine function of CD34+ and CD34+/CD31+ CACs on angiogenesis has not been considered. The purpose of this study was to determine whether there is a differential response in paracrine function of CD34+ and CD34+/CD31+ CACs as a result of chronic endurance-exercise habits in younger men and women and to identify differences in CAC intracellular redox pathways that may be eliciting angiogenic or antiangiogenic actions. We hypothesized that both CD34+ and CD34+/CD31+ CACs from endurance-trained individuals will have enhanced paracrine function that will result in superior angiogenic capacity compared with those from inactive individuals and that this will be linked to a healthier redox balance in the endurance-trained group. A secondary aim of our study was to identify proteins present in the secretome of CD34+ and CD34+/CD31+ CACs and to determine whether these proteins are differentially expressed as a function of chronic exercise training status.

METHODS

Ethical Approval

The University of Maryland College Park Institutional Review Board approved all study procedures and subjects provided written informed consent. The study procedures conformed to the standards set by the Declaration of Helsinki.

Screening

Subjects in this study were healthy, nonsmoking men and women aged 18–35 yr with no history of CV or metabolic disease. Potential subjects were initially screened by telephone or email and reported to the laboratory following an overnight fast for a screening visit to verify eligibility. Subjects were categorized based on their reported physical activity over the last 5 years and confirmed via VO₂ max. Specifically, inactive subjects (n = 12; 5 women and 7 men) reported performing ≤20 min endurance exercise for ≤2 days/wk. The active group (n = 15, 5 women and 10 men) reported performing ~4 h/wk of low- to moderate-intensity activity, and the endurance-trained group (n = 14, 9 women and 5 men) reported performing >4 h/wk of moderate- to high-intensity endurance exercise. Groups were matched for age and body mass index (BMI). Exclusion criteria were as follows: systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 90 mmHg, serum total cholesterol ≥ 200 mg/dl, low-density lipoprotein cholesterol ≥ 130 mg/dl, high-density lipoprotein cholesterol ≤ 35 mg/dl, and fasting glucose ≥ 100 mg/dl. Women were all tested during the early follicular phase of their menstrual cycle.

Maximum-Graded Exercise Test, Body Composition, and Blood Sampling

A screening blood sample was obtained for assessment of fasting serum triglyceride, lipoprotein lipids, and glucose (Quest Diagnostics, Baltimore, MD). Height, weight, seated blood pressure, and BMI were measured, and body composition was assessed using the seven-site skinfold procedure (24). Maximum oxygen consumption (VO₂ max) was assessed using a constant-speed treadmill protocol with 2 to 3% increases in incline every 2 min until exhaustion. The treadmill speed was based on the subject’s experience, typical run speed, and heart rate such that VO₂ max was achieved within 6–12 min. Pulmonary ventilation and expired gas concentrations were analyzed in real time using an automated computerized indirect calorimetry system (Oxycon Pro, Viasys). VO₂ was considered maximum if a plateau was achieved (increase in VO₂ of <250 ml/min with increased work rate). In the absence of a clear plateau, tests had to meet at least two of the following secondary criteria: a respiratory exchange ratio > 1.10, a rating of perceived exertion > 18, and a peak heart rate within 10 beats/min of the age-predicted maximum. On the testing day for blood sampling for CACs, the subjects reported to the laboratory in the morning after an overnight (~12 h) fast. Endurance-trained and active subjects performed their normal exercise routine 16–24 h before the blood sampling. A sample of 50 ml of blood was drawn using EDTA tubes (Becton Dickinson) for isolation of CD34+ and CD34+/CD31+ CACs.

Immunomagnetic Cell Separation

PBMCs were isolated from the venous blood samples using density gradient centrifugation (Ficoll, GE Healthcare). The CD34+ fraction was purified using multiple rounds of immunomagnetic cell separation according to the manufacturer’s instructions (EasySep Immunomagnetic Cell Separation Kits, STEMCELL Technologies), using an antibody specific for CD34. CD34+ cells were selected from the CD34− fraction of cells and purified as described above using and antibody specific for CD31 (hereby referred to as CD34+/CD31+). Multiple flow cytometry analyses in our laboratory have resulted in a CD34+ cell isolation purity of 52 ± 3% in the positively selected fraction compared with the 0.1% in total PBMCs before selection (Fig. 1, A and B) and virtually no detectable CD34− cells (<0.1%) in the CD34− depleted fraction (Fig. 1C). This isolation approach has been previously published by our laboratory (25, 26), and the purity of our isolation is equivalent to or greater than that in other published results also using nonmobilized blood (3, 4a, 17, 46, 57). The different surface antigen combinations were chosen on the basis of previous research indicating the involvement of stem/progenitor (i.e., CD34+) and endothelial antigen-expressing (i.e., CD31+) PBMC subsets originating from bone marrow or the vessel wall in the maintenance and repair of the vascular endothelium (4a, 10, 17, 30, 32, 45).
CAC Culture, Conditioned Media, and Angiogenesis Assay

CD34⁺ and CD34⁻/CD31⁺ CACs were resuspended in unsupplemented endothelial growth medium free of growth factors or serum, [endothelium basal medium-2 (EBM-2), Lonza] with 1% antibiotic-antimycotic (Invitrogen) each at a density of 100,000 cells per well. Cultures were maintained for 48 h in a humidified incubator at 37°C and 5% CO₂-95% room air. After incubation, the conditioned media (CM) from all wells of the same cell type was withdrawn, combined into one tube and clarified by spinning at 2,500 g for 20 min to remove cells and debris from the media. For the angiogenesis tube formation assay (2, 8, 15, 44), culture plates were coated with reduced-growth factor Matrigel (BD Biosciences), and the Matrigel was left to solidify for 30 min at 37°C and 5% CO₂-95% room air. Under these in vitro conditions, human umbilical vein endothelial cells (HUVECs) will form multicell cords, which serve as a global indicator of the angiogenic cascade. Each condition was performed in duplicate, and each well contained 20,000 HUVECs and equal volumes of CM from either CD34⁺ or CD34⁻/CD31⁺ CACs. Control wells were prepared with a similar amount of fresh EBM-2. The average HUVEC passage used in the angiogenesis assay for endurance-trained, active, and inactive subjects was 4.4 ± 0.4, 4.6 ± 0.3, and 4.2 ± 0.3, respectively (not significant). These plates were cultured for 16 h at 37°C and 5% CO₂-95% room air. The cultures were then visualized under a light microscope, and five random images were photographed per well. These images were then coded and blindly assessed for HUVEC tube length and complexity (2, 5, 29) by two individuals. Tube length of each segment was quantified using ImageJ and averaged over the total number of images. Complexity was quantified as the number of nodes present, with a node being a site with ≥3 branching points. Results are presented as each condition normalized to the basal (EBM-2) condition to control for daily variability in HUVEC growth and passage number with each assay. Average readings of the two individuals were used for statistical analyses.

Measurement of Intracellular NO and ROS

These experiments were performed on freshly isolated cells in duplicate as we have previously described (25, 26), with minor modifications. Briefly, 1.5 × 10⁵ cells were stained with 10 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate for determination of intracellular NO levels or 2 μM 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCF-DA) for determination of intracellular ROS levels (Molecular Probes). 4’,6-Diamidino-2-phenylindole (DAPI; 750 ng/ml) was used to identify cell nuclei (Molecular Probes). Cells were incubated with fluorescent dyes in a final volume of 150 μl serum-free PBS for 30 min at 37°C. NO and ROS fluorescences were quantified using a fluorescent microplate reader (Biotek), using excitation and emission filters of 488 and 535 nm, respectively. DAPI fluorescence was measured using excitation and emission filters of 355 and 460 nm, respectively. NO and ROS fluorescence values were divided by DAPI fluorescence values to normalize for cell number. All fluorescent probes were validated using positive and negative controls as previously described by our laboratory (25, 26). Briefly, we observe severalfold increases in intracellular H₂DCF-DA signal in the presence of 3-morpholinosydnonimine (200 μM, Fig. 4A). Pretreatment with polyethylene glycol catalase (50 U/ml) reduces H₂DCF-DA signal by nearly 40% after exposure to hydrogen peroxide (500 μM, Fig. 4B). Additionally, we observed severalfold increases in DAF-FM signal using NO donor diethylenetriamine NONOate (50 μM, Fig. 5A) or 3-morpholinosydnonimine (20 μM, Fig. 5B) and substantial reduction.

Fig. 1. Flow-activated cell sorting plots representing phycoerythrin (PE)-conjugated CD34/V450-conjugated CD45 cells within total peripheral blood mononuclear cells (PBMCs; A), CD34⁺-selected PBMCs using immunomagnetic cell separation procedures (B), and unselected (negative fraction) CD34 cells (C). Multiple trials have resulted in an isolation purity of 52 ± 3%.
in DAF-FM signal with $NG$-nitro-l-arginine methyl ester treatment (300 μM, Fig. 5B). Intra-assay coefficients of variation for ROS and NO were 5.4 and 4.8%, respectively.

**Assessment of Gene Expression by RT-PCR**

RNA was extracted from freshly isolated CACs using the TRIzol reagent DNase treated using TURBO DNA-free kit (Life Technologies) and reverse transcribed to cDNA. Expression of angiogenic [endothelial NO synthase (eNOS)] and pro-oxidant (NADPH oxidase subunits gp91phox and p47phox) genes were assessed as previously described (28) to confirm our hypothesis that ROS and NO were mediating differences in CAC paracrine function. Additional gene targets include S100A8A9 complex (35, 55) and to quantitatively assess levels present in the standard curve based on concentrations reported in the literature (30).

Previously clarified CM from CD34+ and CD34-/CD31+ cells was divided into aliquots and stored at −80°C until further analyses. Proteomics techniques were used to determine proteins present in the CD34+ and CD34-/CD31+ CM from each endurance-trained and inactive groups. Spectral counts for $n = 1$ per group were used as an initial nonlabeled method providing semiquantitative data (39). Subsequently, these findings were quantitatively confirmed using immunoblotting analyses on $n = 12$ per group. Media was concentrated using Amicon Ultra 0.5 3-kDa centrifugal filter devices (Milipore). The soluble proteins in the supernatant were subjected to digestion with trypsin Lys-C. Samples were analyzed using a ThermoFinnigan LTQ Orbitrap XL mass spectrometer and matched to the UniProtKB database. Western blot analyses were used to assess differences in two identified S100 proteins for S100A8 (R&D Systems) and S100A9 (Santa Cruz Biotechnology). Membranes were washed and then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling; 1:5,000) or horse-radish peroxidase-conjugated anti-goat IgG (Novus; 1:5,000) secondary antibodies. Blots were developed using Super Signal ECL reagents (Thermo Scientific), and bands were visualized secondary antibodies. Blots were developed using Super Signal ECL reagents (Thermo Scientific), and bands were visualized using Chemi-Doc Imaging System and software (Bio-Rad). Values were normalized to 100,000 cells/well used to generate the CM. Recombinant human S100A8/A9 complex (R&D Systems) was used in Western blots to establish a standard curve based on concentrations reported in the literature (35, 55) and to quantitatively assess levels present in the CM of each CAC subtype.

**Recombinant S100A8/A9 Treatment**

To confirm the direct effects of S100A8 and S100A9 on HUVEC tube formation, the concentrations and proportions estimated in the CM of the inactive subjects’ CD34+/CD31+ CACs were used in an angiogenesis assay. As estimated, 7.18 μg/ml of recombinant human S100A8 (ProSpec Bio) and 3.06 μg/ml of recombinant human S100A9 (Life Technologies) were added to a HUVEC-based tube formation assay and compared with the positive control prepared with EBM-2 and vehicle control. In these experiments, each condition was assessed in samples collected from six independent cell culture wells from multiple culture plates collected on different days. All experiments were conducted on cells from the same passage number (P4). As described above, each well contained 20,000 HUVECs. The plates were cultured for 16 h at 37°C and 5% CO2-95% room air. The cultures were then visualized under a light microscope, and five random images were photographed per well.

**Statistics**

Statistical analyses were completed using IBM SPSS Statistics 21. Assumptions of homoscedasticity and normality were verified for all outcome measures. Data were analyzed using multivariate ANOVA with pairwise comparisons where appropriate. For Western blot analyses, t-tests were used. Statistical significance was accepted at $P \leq 0.05$. Values are expressed as means ± SE.

**RESULTS**

**Subject Characteristics**

Subject characteristics can be found in Table 1. All subject groups ($n = 14$ endurance trained; and $n = 15$ active and $n = 12$ inactive) were of the same age and BMI. Training status was confirmed through VO$_2$max with the endurance-trained subjects having 15% greater VO$_2$max compared with active and 40% greater VO$_2$max compared with inactive subjects ($P < 0.05$). Active subjects had 30% higher VO$_2$max than inactive subjects ($P < 0.05$). Despite the three groups being matched for BMI, inactive subjects had significantly higher body fat percentage than both the active and endurance-trained subjects ($P < 0.05$). Importantly, subjects were all matched for the majority of cardiometabolic risk factors including systolic blood pressure, glucose, cholesterol, triglyceride, and lipoprotein lipids.

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Values are means ± SE; $n$, number of subjects. VO$_2$max, maximum oxygen consumption; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; C. cholesterol, TC, total cholesterol. *Significantly different from endurance trained; #significantly different from active.
Angiogenic Tube Formation with CM

CD34+ cells. CM from a purified population of CD34+ CACs (Fig. 1) resulted in 29% lower tube length ($P < 0.05$) and 45% lower complexity ($P < 0.05$) in inactive subjects compared with endurance-trained subjects, and 14% lower length ($P < 0.05$) and 20% lower complexity ($P = 0.05$) in active subjects compared with endurance-trained subjects (Fig. 2, A and B). CD34+ CM from inactive subjects produced lower HUVEC length (not significant) and complexity ($P = 0.05$) than CD34+ CM from active subjects (15 and 26%, respectively; Fig. 2, A and B). Representative images from each condition can be found in Fig. 3. CM from both endurance-trained and active subjects’ CD34+ CACs did not result in statistically different tube length or complexity ($P > 0.05$) compared with basal conditions, whereas CM from inactive individuals’ CACs resulted in significantly lower length and complexity compared with basal ($P < 0.05$).

CD34+/CD31+ cells. HUVEC tube length was 26% lower ($P < 0.05$) and complexity was 42% lower ($P < 0.05$) with CM from CD34+/CD31+ CACs of inactive subjects compared with endurance-trained individuals. CD34+/CD31+ CM from endurance-trained subjects was not significantly different in terms of tube length and complexity than CD34+/CD31+ CM from active subjects (Fig. 2, C and D). Although not statistically significant, CM from CD34+/CD31+ CACs of inactive subjects resulted in 16% shorter tube length and 28% less complexity than active subjects (Fig. 2, C and D). There was no significant effect of sex on tube formation for either CD34+ or CD34+/CD31+ CM ($P > 0.05$). Representative images from each condition can be found in Fig. 3. The CD34+/CD31+ CM from endurance-trained and active subjects’ CACs did not result in significantly different tube length or complexity ($P > 0.05$) compared with basal conditions, whereas CD34+/CD31+ CM from inactive individuals’ CACs caused significantly lower length and complexity compared with basal ($P < 0.05$).

Intracellular ROS and NO

To determine if the differences in CM-induced tube formation were associated with differences in intracellular redox pathways, we assessed intracellular ROS and NO levels using previously validated techniques (Figs. 4, A and B, and 5, A and B) and a purified population of cells (Fig. 1). There were no significant differences in intracellular ROS levels between endurance-trained, active, or inactive subjects’ CD34+ CACs (Fig. 4C) or CD34+/CD31+ CACs (Fig. 4D). In addition, no significant differences between intracellular NO levels were detected between CD34+ CACs from endurance-trained, active, and inactive subjects (Fig. 5C) or CD34+/CD31+ CACs (Fig. 5D). There was no significant effect of sex on intracellular ROS or NO levels for either cell type.

Gene Expression

CD34+ cells. There were no significant effects of training status on CD34+ eNOS (Fig. 6A) or p47phox or gp91phox mRNA expression (Fig. 6, B and C). There was no significant effect of sex on expression of any of our genes of interest in CD34+ CACs.

CD34+/CD31+ cells. eNOS gene expression was not statistically different in inactive subjects compared with active and...
endurance-trained subjects (Fig. 6D). There were no significant differences between groups for p47phox or gp91phox gene expression in CD34+/H11002/CD31+/H11001 CACs (Fig. 6, E and F). There was no significant effect of sex on gene expression for any of our targets for CD34+/H11002/CD31+/H11001 CACs.

Detection of Proteins in CM

In an attempt to identify secreted paracrine factors from CACs, CM from CD34+/H11001 and CD34+/H11002/CD31+/H11001 cells was analyzed by mass spectrometry. Two targets from the S100 family
of proteins, S100A8 and S100A9, were identified in the secre-
tome of both CD34− and CD34+/CD31+ cells (Fig. 7). Be-
cause of our detection of S100A8 and S100A9 in the CM of
each CAC subtype, we sought to confirm that CACs express
genes. S100A8 and S100A9 were expressed in both CAC sub-
types (Fig. 8). In CD34+ CACs, there were no significant
differences between groups in S100A8 mRNA or S100A9
mRNA (Fig. 8, A and B). S100A8 and S100A9 mRNA in
CD34−/CD31+ CACs were also not significantly different as a
function of training status (Fig. 8, C and D).

The S100A8 and S100A9 proteins are known mainly for
their role in inflammation but their role in the regulation of
angiogenesis is not well understood, especially as a secreted
factor from our cells of interest. As such, we sought to
determine if differences existed in the levels of these proteins
present in CAC CM as a result of chronic exercise training
status. Immunoblotting for S100A8 and S100A9 in the CM of
CD34+ cells did not show significant differences between
endurance-trained and inactive subjects (Fig. 9, A and B).
There was also no significant sex-related difference in the
content of either protein in CD34+ CM. In CD34−/CD31+
cells, there were no significant differences in S100A8 content
between endurance-trained and inactive subjects ($P = 0.08$;
Fig. 9C). However, S100A9 content was significantly higher
in CD34−/CD31+ CM of inactive subjects compared with endur-
ance-trained subjects ($P < 0.05$; Fig. 9D). There was no
significant effect of sex on S100A8 or S100A9 content in CM
from CD34−/CD31+ CACs.

With the use of recombinant human S100A8/A9, a standard
curve was established using typical concentrations employed in
the literature (35, 55) to allow us to estimate total content of
each protein that the HUVEC cells were exposed to in a subset

of remaining CM samples (Fig. 10A). For this experiment, we
used $n = 3$ samples from the inactive groups’ CD34−/CD31+
CACs to estimate the concentrations of each protein that could
potentially be responsible for depressed tube formation. As no
differences in either S100A8 or S100A9 were noted in the CM
of CD34+ CACs, we used $n = 2$ inactive CM and $n = 1$
endurance-trained subjects’ CM to estimate the concentrations
of each of these proteins. The total amount of S100A8 was
estimated to be $8.69 \pm 4.3 \mu g/ml$ and S100A9 was estimated
to be $16.97 \pm 4.6 \mu g/ml$ in CD34+ cell CM (Fig. 10B). CM for
CD34+/CD31+ CACs contained $\sim 7.18 \pm 0.28 \mu g/ml$ of
S100A8 and $3.06 \pm 0.95 \mu g/ml$ of S100A9 (Fig. 10C).
We next calculated the proportion of S100A8/S100A9 and found
that CD34+ CM had an A8-to-A9 ratio of 0.513, whereas
CD34+/CD31+ CM contained an A8-to-A9 ratio of 2.343 (Fig.
10, B and C).

Recombinant Human S100A8 and S100A8 Treatment

Recombinant human S100A8 and S100A9 were added to a
HUVEC-based tube formation assay in the concentrations and
proportions that were estimated to be present in the CD34−/
CD31+ CM of inactive subjects ($7.18 \mu g/ml$ of S100A8 and
$3.06 \mu g/ml$ of S100A9). When compared with the basal con-
trol, addition of S100A8 and S100A9 resulted in an average of
18% lower tube length and 28% lower complexity ($P < 0.05$
for each; Fig. 11).

DISCUSSION

Paracrine actions are believed to be the major mechanism
through which CACs exert their angiogenic properties on the
preexisting endothelium, but it was previously unknown
whether regular endurance exercise affected these actions. In this study we demonstrate that CM generated from inactive subjects’ CD34+/H11001 and CD34+/H11002/CD31+/H11001 CACs depresses paracrine-mediated tube formation. These data strongly suggest that endurance exercise training-mediated alterations in CAC paracrine activity influence angiogenesis. Other studies have demonstrated the role of paracrine signaling of CACs in diseased states (21, 44, 62). Our data provide further support for a paracrine role for CD34+/H11001 and CD34+/H11002/CD31+/H11001 CACs on angiogenesis in younger, healthy individuals. More importantly, to our knowledge, we are the first to report of a differential paracrine role in CD34+/H11001 and CD34+/H11002/CD31+/H11001 CACs on HUVEC-based tube formation as a function of chronic physical activity habits.

Based on previous research from our laboratory (25), we hypothesized that differences in intracellular ROS and NO concentrations as a result of exercise-training status would at least partially explain our observed differences in paracrine function in both cells types. Surprisingly, the present data suggest that intracellular ROS and NO are not playing a major role in regulating the differential paracrine responses observed.

We found no significant effects of training status on intracellular NO or ROS levels or in mRNA expression for eNOS as well as p47phox and gp91phox for either cell type. Although some studies have found an effect of endurance exercise on improved redox balance in both healthy (25, 59) and diseased populations (12, 47, 49), others have found improvements in endothelial function with endurance exercise independent of changes in oxidative stress (38). It is important to note that these previous studies have focused mainly on endothelial progenitor cells, which share some of the same characteristics as the two CAC subtypes that we studied, but generally they express both the progenitor cell marker (CD34) and the endothelial marker (CD31 or VEGF receptor 2) simultaneously and are negative for CD45. As these cells can have very different properties, caution must be used when comparing studies using different cell types as a result of selection or culture conditions. Importantly, our previous cross-sectional studies included only men (25, 28), whereas in the current study we included both men and women, potentially accounting for the different findings in the current study compared with our previous work. Recently, Guhanarayan et al. (2014) (16) found that 10 days of

**Fig. 6. Effects of exercise-training status on endothelial NO synthase (eNOS), p47phox, and gp91phox mRNA expression for freshly isolated CD34+ circulating angiogenic cells (**A–**C; n = 13 endurance trained, n = 13 active, and n = 8 inactive) and CD34+/CD31+ circulating angiogenic cells (**D–**F; n = 14 endurance trained, n = 15 active, and n = 11 inactive).**
reduced physical activity decreased intracellular NO in CFU-Hill CACs, but they observed no differences in intracellular NO levels in freshly isolated CD34+/H11001 CACs similar to those investigated in the present study. Additionally, they found no significant changes in NO or ROS-related gene expression with reduced physical activity in either CAC type. Together, these data suggest that the effects of intracellular ROS and NO may be specific to certain populations of CACs and emphasize the need for future investigations.

In an attempt to elucidate potential contributors to our observed differences in tube formation, we used mass spectrometry analyses to assess the secreted protein content of the media. Figure 7 shows total protein gel of CM from cultured circulating angiogenic cells showing bands at 10 kDa (S100A8) and 14 kDa (S100A9). Figure 8 shows semiquantitative analysis of S100A8 and S100A9 in CM of cultured CD34+ cells (B) and CD34+/CD31+ cells (C) using mass spectrometry spectrum counts (n = 1) to identify proteins present in CM.
We identified two members of the S100 family of calcium-modulated proteins, S100A8 and S100A9, in the secretome of both CD34⁺ and CD34⁻/CD31⁺ CACs. Furthermore, we confirmed that both CAC subtypes expressed mRNA species specific for both of these proteins, indicating that both cell types possess the necessary molecular machinery to produce the proteins. As our observed differences in tube formation appeared to be a graded effect, we focused on the two extreme groups, endurance trained and inactive, for protein analyses of the proteins. As our observed differences in tube formation appeared to be a graded effect, we focused on the two extreme groups, endurance trained and inactive, for protein analyses of the CM. We found that the protein content of S100A9 was twice as high in the CD34⁻/CD31⁺ CM of inactive subjects compared with their endurance-trained counterparts. These findings are the first, to our knowledge, to demonstrate differential secretion of S100A9 by CD34⁻/CD31⁺ CACs as a function of an individual’s exercise-training status. Although not significant, S100A8 was also roughly twice as high in the CD34⁻/CD31⁺ CM from inactive subjects compared with endurance-trained subjects. S100A8 and S100A9 proteins are abundantly expressed in both myeloid and vascular cells (3) and are most commonly known for their role in regulating inflammatory processes (3, 11, 56). Several studies have linked systemic concentrations of the S100A8/S100A9 heterodimer complex to CVDs (3) with plasma levels predicting future CV events in middle-aged healthy individuals (6, 7). Thus the higher levels of S100A9 in inactive individuals are consistent with these previous findings, indicating these proteins as a risk factor for CVD. Croce et al. (2009) (7) demonstrated the enhanced stability of these proteins when functioning as a heterodimer complex. Given the biology of the S100A8/A9 complex, it is possible that our S100A8 findings in CD34⁻/CD31⁺ CM paired with the significant differences in S100A9 may be physiologically relevant despite the lack of statistical significance.

In our hands, we found that our CM from both cell types contained <20 µg of S100A8 and S100A9. Most investigations assessing the in vitro effects of S100A8 or S100A9 use concentrations between 1 and 200 µg/ml (3, 35, 55). Thus our CM content is on the low end of the established in vitro range of S100A8 or S100A9 concentrations. Defining the concentrations of S100A8 or S100A9 is important since previous publications have shown concentration-dependent effects on angiogenic function (56). In addition, both proteins are able to activate signaling in a homodimer, heterodimer, or heterotramer form (56), thus adding more complexity to their mechanism of action. When the proportion of S100A8/S100A9 was calculated in the CM, there was a greater proportion of S100A8/S100A9 in the CD34⁻/CD31⁺ CM compared with that of the CD34⁺ CM. These results suggest that CM from the different cells types are fundamentally different from each other even though both showed similar effects on the HUVEC cells. The differences in the ratios of these proteins in the CM of the two different cell types may provide one explanation as to why we observed differences in these proteins as a function of training status in the CD34⁻/CD31⁺ CACs. We found that other secreted factors may better explain our observed differences in CD34⁺ CM-mediated HUVEC tube formation as a function of training status.

As we found significantly greater levels of S100A8/A9 in the CD34⁻/CD31⁺ CM of inactive individuals compared with endurance-trained individuals, we sought to empirically test the effects of these proteins on HUVEC tube formation. We added recombinant human S100A8 and S100A9 using the estimated concentrations of each of these proteins that were present in the CM of CD34⁻/CD31⁺ CACs. We found that HUVEC length was ~18% lower and complexity was 28%
lower in this condition compared with the positive control. Importantly, these findings confirm the role of each of S100A8 and S100A9 in the discovered concentrations as a depressor of HUVEC tube formation.

As only a small percentage of the population are high-level endurance athletes, a strength of our study is the inclusion of a regularly active group that met the guidelines for physical activity (1) without being defined as “highly trained” or “elite,” since this group is more applicable to the general population. In the current study, we observed a graded effect of exercise with CM from endurance-trained athletes contributing best to tube formation and CM from inactive subjects having a negative effect on tube formation. We observed no significant differences in tube formation between the active and endurance-trained groups in the CD34+/CD31+ CM conditions, suggesting that the range of physical activity influences the function of the CACs. In this study, we only examined levels of S100A8 and S100A9 in the CM from the two extreme groups, so future studies should also follow up on habitually physically active individuals to determine the concentrations on these proteins being secreted by their CACs.

This study is also unique in that it includes data from both young men and women. We did not detect significant differences between any variable measured because of sex, although we acknowledge that with such a small sample of women, the ability to detect statistically significant differences is low. Regardless, very little research in the CAC field focuses on premenopausal women, possibly because of the difficulty of controlling for menstrual cycle status, since estrogen and other female sex hormones have known effects on CAC function (22, 23, 42). In our study, we attempted to control for the effects of estrogen throughout the menstrual cycle by testing all women during the follicular phase when estrogen is typically at its lowest levels (50). We acknowledge that there are individual fluctuations in estrogen levels throughout the menstrual cycle, and we did not directly measure estrogen levels, which could have contributed to variations in some of our findings. It is also important to mention that our subject groups were heterogeneous with respect to representation of men and women, with the endurance-trained groups being comprised of proportionally more women compared with the other groups. However, we do feel that it is an important contribution to the

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literature to include this population both as a comparison with men and to begin to learn more about the health and function of CACs from premenopausal women.

Various subpopulations of CACs have been used in autologous cell therapies as a means for treating ischemic CVD. It is now well accepted and our results support that CACs work through paracrine functions (14, 31, 44, 58). A sedentary lifestyle is associated with future CVD risk through diseases such as obesity and type 2 diabetes. In the absence of lifestyle changes, it is possible that differences in the secretion of these proteins in sedentary but otherwise young, healthy individuals will become more exacerbated with age and progression of CVD. As such, our findings of greater S100A8 and S100A9 secretion by CD34+/CD31+ CACs of young, inactive individuals provide rationale for future studies to determine whether the dysfunctional properties of CACs from CVD patients are linked to altered S100A8 and S100A9 secretion.

**Limitations**

We were limited in the number of functional assays that we could perform because of the low overall amount of CM that was generated from our isolated cells. Because of this limitation, we performed an in vitro assay that has been widely used as a global readout of the entire angiogenesis cascade. We acknowledge that the use of the HUVEC-based angiogenesis tube formation assay does not individually assess all the critical regulatory steps during angiogenesis. Future studies should include measures such as HUVEC proliferation and migration as a complement to the angiogenesis assay to more comprehensively assess angiogenic activity in endothelial cells. Additionally, further research on the different proportions of S100A8 and S100A9 as a function of training status would provide more understanding into function of these proteins. In the current study we used recombinant S100A8 and S100A9 to reproduce the depressed tube formation observed when using CD34+/CD31+ CM from inactive subject’s CACs. We acknowledge that other secreted factors are potentially contributing to our observations. Future experiments should employ a loss of function approach to confirm that S100A8 and S100A9 are mediating the differences in tube formation or determine whether other secreted proteins present in the CM are affecting HUVEC function as well. Neutrophils and other myeloid cells are major producers of S100A8 and S100A9. Our CAC isolations yield a purity of ~50%, and, therefore, we cannot exclude the possibility of other cells contributing to the levels of S100A8 and S100A9 in our CM. It is unlikely that one individual cell type would make up the majority of the other 50% consistently across training groups. Rather, this is more likely comprised of a heterogeneous mix of cell types. As such, it is probable that the differences observed in our study are predominantly due to each of the purified CAC subtypes, although further characterization of other cell types present after CAC purification are necessary to confirm this. Furthermore, rates of cell proliferation over the culture period among different training groups should be considered in future work to confirm that the observed differences are due to greater secretion of S100A8 and S100A9 and not a greater number of cells.

All measures in this study were taken under basal cell conditions, which is not always representative of the in vivo environment. Under resting conditions, these cells are less active and many studies have found that pharmacological challenges such as LPS or PMA stimulate cytokine/growth factor secretion from various cell types (40). We have previously shown that in vivo consumption of a high-fat meal alters the intracellular ROS levels of CACs (26); thus it is our belief that a similar stimulation may also alter the paracrine profile of CACs. Follow-up experiments investigating how an in vivo or in vitro challenge affect CAC paracrine functions will be necessary to fully elucidate the paracrine and autocrine behaviors of these cells.

**Conclusions**

In summary, we demonstrate, for the first time, a differential effect on tube formation when HUVECs were cultured with CM from either CD34+ or CD34−/CD31+ CACs of endurance-trained athletes and active or inactive individuals. CM from inactive subject’s CACs appears to produce an inflammatory effect that depresses tube formation, and this effect is not evident with increased chronic exercise behaviors. We found that proinflammatory S100A9 secreted by CD34−/CD31+ CACs is significantly higher in the CM of inactive individuals compared with their endurance-trained counterparts, and culture with recombinant S100A8 and S100A9 in concentrations estimated to be present in the CD34+/CD31+ CM significantly reduced HUVEC tube formation. These results support the possibility that increases in secreted S100A8/S100A9 in inactive individuals are contributing to the reduced HUVEC tube formation associated with CM from CD34−/CD31+ CACs.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


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