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

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Running Title: Exercise and paracrine effects of CACs
ABSTRACT

We aimed to determine if chronic endurance exercise habits affected redox status and paracrine function of CD34+ and CD34-/CD31+ circulating angiogenic cells (CACs). Subjects were healthy, nonsmoking, men and women aged 18-35 yrs and categorized by chronic physical activity habits. Blood was drawn from each subject for isolation and culture of CD34+ and 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 HUVEC-based tube assay. CM from CD34+ 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 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 to their endurance-trained counterparts with no significant differences in either protein in the CM of CD34+ 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.
Factors secreted by CD34+ or CD34-/31+ CACs from inactive subjects, but not those with increase chronic exercise behaviors, depress HUVEC tube formation. These findings appear to be at least partially mediated by greater S100A9 present in the CM of inactive compared to endurance-trained individuals’ CD34-/31+ CACs.
INTRODUCTION

Cardiovascular disease (CVD) and the associated dysfunction of the vasculature is the leading cause of death in the 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) (2, 53, 61). CAC is a broad term for subgroups of peripheral blood mononuclear cells 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 hind-limb ischemia, emphasizing the importance of studying non-progenitor cell lines which may also be involved in angiogenesis (31). Indeed, cells characterized by both markers have been previously found to exhibit angiogenic characteristics in vitro (2, 20, 30, 44) and in vivo (32, 37, 41).

Originally believed to exert their reparative functions through direct incorporation into the endothelium (2), recent evidence indicates that CACs accomplish their pro-angiogenic effects through paracrine mechanisms. Specifically, CACs are thought to secrete factors that elicit actions on the pre-existing endothelium and/or signal other circulating cells to home to the endothelium (32, 33, 44, 51, 63). Recently, Hynes et al. (2011) demonstrated that secretome 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 (21). Urbich et al. 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 (51). 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 CV health, including attenuation of many classic CVD risk factors (27, 38) and improvements in endothelial function (9, 48). Our lab and others have previously shown that endurance exercise training increases CAC number and enhances intracellular redox balance in younger and older adults compared to their sedentary counterparts (25, 28, 36, 52-54, 60). Hoetzer et al (2007) found that migratory activity of sedentary individuals’ CACs improved by ~50% after 12 weeks of exercise training (19). 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 to their endurance-trained counterparts (25, 28). It is generally accepted that CACs with less than optimal function cannot contribute properly to vascular repair or neoangiogenesis, 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 anti-angiogenic 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 to 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.
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 yrs 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 VO_{2max}. Specifically, inactive subjects (n=12; 5 women and 7 men) reported performing ≤20 min endurance exercise on ≤2 days/week. The active group (n=15, 5 women and 10 men) reported performing ~4 hours/week of low to moderate intensity activity and the endurance-trained group (n=14, 9 women and 5 men) reported performing >4 hours/week 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 mm Hg, diastolic blood pressure ≥90 mm Hg, serum total cholesterol ≥200 mg/dl; low-density lipoprotein-cholesterol ≥130 mg/dl; high-density lipoprotein-cholesterol ≤ 35 mg/dl; fasting glucose ≥100 mg/dl. Women were all tested during the early follicular phase of their menstrual cycle.

Maximal Graded Exercise Test, Body Composition and Blood Sampling

A screening blood sample was obtained for assessment of fasting serum triglyceride (TG), lipoprotein lipids, and glucose (Quest Diagnostics, Baltimore, MD). Height, weight, seated blood pressure and BMI were measured, and body composition was assessed using the 7-site skinfold procedure (24). Maximal oxygen consumption (VO_{2max}) was assessed using a constant-speed treadmill protocol with 2–3% 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 VO₂max 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 hr) fast. Endurance-trained and active subjects performed their normal exercise routine 16-24 hours prior to the blood sampling. A sample of 50mL of blood was drawn using EDTA-tubes (Becton Dickinson) for isolation of CD34+ and CD34-/CD31+ CACs.

**Immunomagnetic Cell Separation**

Peripheral blood mononuclear cells (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 Ab specific for CD34. CD31+ cells were selected from the CD34- fraction of cells and purified as described above using and Ab specific for CD31 (hereby referred to as CD34-/CD31+). Multiple flow cytometry analyses in our lab have resulted in a CD34+ cell isolation purity of 52 ± 3% in the positively-selected fraction (compared to the 0.1% in total PBMCs prior to selection (Figure 1A and 1B) and virtually no detectable CD34+ cells (<0.1%) in the CD34-depleted fraction (Figure 1C). This isolation approach has been published previously by our lab (25, 26) and the purity of our isolation is equivalent to or greater than other published results also using non-mobilized blood (2, 4, 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 (4, 10, 17, 30, 32, 45).

**CAC Culture, Conditioned Media, and Angiogenesis Assay**

CD34+ and CD34-/CD31+ CACs were re-suspended in un-supplemented endothelial growth medium free of growth factors or serum, (EBM-2, Lonza) with 1% Antibiotic-Antimycotic (Invitrogen) each at a density of 100,000 cells per well. Cultures were maintained for 48 hrs in a humidified incubator at 37°C and 5% CO2. 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 2,500g for 20 min to remove cells and debris from the medium. For the angiogenesis tube formation assay (1, 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. Under these in vitro conditions, HUVECs will form multi-cell cords, which serve as a global indicator of the angiogenic cascade. Each condition was performed in duplicate and each well contained 20,000 human umbilical vein endothelial cells (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 (n.s.). These plates were cultured for 16 hrs at 37°C and 5% CO2. The cultures were then visualized under a light microscope and 5 random images were photographed per well. These images were then coded and blindly assessed for HUVEC tube length and complexity (1, 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 described previously (25, 26), with minor modifications. Briefly, 1.5 × 10^5 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 (H2DCFDA) 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 fluorescence 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 described previously by our lab (25, 26). Briefly, we observe several fold increases in intracellular H2DCF-DA signal in the presence of 3-Morpholinosydnonimine (200 µM, Figure 4A). Pretreatment with PEG-catalase (50U/mL) reduces H2DCF-DA signal by nearly 40% after exposure to hydrogen peroxide (500 µM, Figure 4B). Additionally, we observed several fold increases in DAF-FM signal using NO donor, Diethylenetriamine NONOate (50 µM, Figure 5A) or 3-Morpholinosydnonimine (20 µM, Figure 5B) and substantial reduction in DAF-FM signal with L-NAME treatment (300 µM, Figure 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 nitric oxide 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 S100A8 (Forward primer: 5’-AGCCCTGCATGTCTCTTGTC-3’, Reverse primer: 5’-ACGTCTGCACCCTTTTTCCT-3’) and S100A9 (Forward primer: 5’-TCATCATGCTGATGGCGAGG-3’, Reverse primer: 5’-CCTGGCCTCCTGATTAGTG-3’), which were assessed to confirm that each CAC subtype expressed the mRNA to produce the S100A8 and S100A9 proteins identified in our CM.

**Mass Spectrometry, Western Blot Analysis and S100A8 and S100A9 Expression**

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 of each endurance-trained and inactive groups. Spectral counts for n=1 per group were used as an initial non-labeled method providing semi-quantitative data (39). Subsequently, these findings were quantitatively confirmed using immunoblotting analyses on n=12 per group. Media was concentrated using Amicon Ultra 0.5 3kD centrifugal filter devices (Millipore). 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 with specific antibodies for S100A8 (R&D Systems) and S100A9 (Santa Cruz Biotech). Membranes were washed and the incubated with horseradish peroxidase (HRP)- conjugated anti-mouse IgG (Cell Signaling; 1:1000) or HRP-conjugated anti-goat IgG (Novus; 1:5000) secondary antibodies. Blots were developed using Super Signal ECL reagents (Thermo Scientific) and bands were visualized and quantified using Chemi-Doc Imaging System and software (BioRad). 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 to 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 hrs at 
37°C and 5% CO₂. The cultures were then visualized under a light microscope and 5 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. t-tests were used for 
western blot analyses. Statistical significance was accepted at $P \leq 0.05$. Values are expressed as 
mean ± standard error of the mean.
RESULTS

Subject characteristics (Table 1.)

All subject groups (n= 14 endurance trained, n=15 active and n=12 inactive) were of the same age and BMI. Training status was confirmed through VO_{2max} with the endurance-trained subjects having 15% greater VO_{2max} compared to active and 40% greater VO_{2max} compared to inactive subjects (P<0.05). Active subjects had 30% higher VO_{2max} than inactive (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 cardio-metabolic risk factors including systolic blood pressure, glucose, cholesterol, TG, and lipoprotein lipids.

Angiogenic tube formation with conditioned media

CD34+ cells. Conditioned media (CM) from a purified population of CD34+ CACs (Figure 1) resulted in 29% lower tube length (P<0.05) and 45% lower complexity (P<0.05) in inactive subjects compared to endurance-trained subjects, and 14% lower length (P<0.05) and 20% lower complexity (P=0.05) in active subjects compared to endurance-trained subjects (Figure 2A and 2B). CD34+ CM from inactive subjects produced lower HUVEC length (n.s.) and complexity (P=0.05) than CD34+ CM from active subjects (15% and 26%, respectively; Figure 2A and 2B). Representative images from each condition can be found in Figure 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 to basal conditions whereas CM from inactive individuals CACs resulted in significantly lower length and complexity compared to 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 to 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 (Figure 2C and 2D). 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 (Figure 2C and 2D). 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 Figure 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 to basal
conditions, whereas CD34-/CD31+ CM from inactive individuals’ CACs caused significantly
lower length and complexity compared to 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 (Figures 4A-B and Figure 5A-B) and a purified population of
cells (Figure 1). There were no significant differences in intracellular ROS levels between
endurance trained, active or inactive subjects’ CD34+ CACs (Figure 4C) or CD34-/CD31+ CACs
(Figure 4D). In addition, no significant differences between intracellular NO levels were detected
between CD34+ CACs from endurance trained, active and inactive subjects (Figure 5C) or
CD34-/CD31+ CACs (Figure 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
(Figure 6A) or p47phox or gp91phox mRNA expression (Figures 6B and 6C). 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 to active and endurance-trained subjects (Figure 6D). There were no
significant differences between groups for p47phox or gp91phox gene expression in CD34-/CD31+ CACs (Figure 6E and 6F). There was no significant effect of sex on gene expression for any of our targets for CD34-/CD31+ CACs.

**Detection of Proteins in Conditioned Media**

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

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 conditioned media as a result of chronic exercise training status. Immunoblotting for S100A8 and S100A9 in the conditioned media of CD34+ cells did not show significant differences between endurance-trained and inactive subjects (Figure 9A and 9B). 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; Figure 9C). However, S100A9 content was significantly higher in CD34-/CD31+ conditioned media of inactive subjects compared to endurance-trained subjects (P<0.05; Figure 9D). There was no significant effect of sex on S100A8 or S100A9 content in CM from CD34-/CD31+ CACs.
Using 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 (Figure 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±4.3 μg/mL and S100A9 was estimated to be 16.97±4.6 μg/mL in CD34+ cell CM (Figure 10B). CM for CD34-/CD31+ CACs contained approximately 7.18±0.28 μg/mL of S100A8 and 3.06±0.95 μg/mL of S100A9 (Figure 10C). We next calculated the proportion of S100A8/S100A9 and found that CD34+ CM had an A8/A9 ratio of 0.513, while CD34-/CD31+ CM contained an A8/A9 ratio of 2.343 (Figure 10B and 10C).

**Recombinant human S100A8 and S100A9 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 μg/mL of S100A8 and 3.06 μg/mL of S100A9). Compared to the basal control, addition of S100A8 and S100A9 resulted in an average of 18% lower tube length and 28% lower complexity (P<0.05 for each; Figure 11).
DISCUSSION

Paracrine actions are believed to be the major mechanism through which CACs exert their angiogenic properties on the pre-existing endothelium, but it was previously unknown whether regular endurance exercise affected these actions. In this study we demonstrate that conditioned media generated from inactive subjects’ CD34+ and CD34-/CD31+ 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+ and CD34-/CD31+ 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+ and CD34-/CD31+ CACs on HUVEC-based tube formation as a function of chronic physical activity habits.

Based on previous research from our lab (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 (EPCs), 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 VEGFR2) 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 to our previous work. Recently, Guhanarayan et al. (2014) found that 10 days of
reduced physical activity decreased intracellular NO in CFU-Hill CACs, but they observed no
differences in intracellular NO levels in freshly isolated CD34+ CACs similar to those
investigated in the present study (16). 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 utilized mass spectrometry analyses to assess the secreted protein content of the
conditioned media. 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.
Further, we confirmed that both CAC sub-types 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 our conditioned media. We found that the protein content of S100A9 was twice as
high in the CD34-/CD31+ CM of inactive subjects compared to 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 to 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) demonstrated the enhanced stability of these proteins when functioning as a heterodimer complex (7). 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 less than 20μg of S100A8 and S100A9. Most investigations assessing the *in vitro* effects of S100A8 or S100A9 utilize concentrations between 1-200 μg/mL (3, 35, 55). Thus, our CM content is on the low end of the established *in vitro* range of S100A8 or A9 concentrations. Defining the concentrations of S100A8 or A9 is important since previous publications have shown concentration dependent effects on angiogenic function (56). In additions, both proteins are able to activate signaling in a homodimer, heterodimer or heterotetramer 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 to 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+ CM but not in the CD34+ CM. This also suggests 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 to 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 approximately 18% lower and complexity was 28% lower in this condition compared to 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 percent 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 (43) without being defined as “highly trained” or “elite” as 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 due to 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 pre-menopausal women, possibly due to the difficulty controlling for menstrual cycle status, as 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 to the other groups. However, we do feel that it is an important contribution to the literature to include this population both as a comparison to men and to begin to learn more about the health and function of CACs from pre-menopausal women.

Various sub-populations 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 A9 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 A9 secretion.

Limitations

We were limited in the number of functional assays that we could perform due to the low overall amount of CM that was generated from our isolated cells. Due to 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 approximately 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-/31+ CACs of endurance-trained athletes, 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 pro-inflammatory S100A9 secreted by CD34-/CD31+ CACs is significantly higher in the CM of inactive individuals compared to 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/A9 in inactive individuals are contributing to the reduced HUVEC tube formation associated with CM from CD34-/CD31+ CACs.

**COMPETING INTERESTS**

No competing interests are reported.

**AUTHOR CONTRIBUTIONS**

RQL, NTJ, ERC, EES and JMH conceived and designed the research experiments. RQL, RMS, AEM, and LC performed the experiments and collected the data. Analysis and interpretation of data was performed by RQL, NTJ, ERC, EES, and JMH. RQL drafted the manuscript; RMS, NTJ, AEM, LC, ERC, EES, and JMH edited and revised the paper and provided important intellectual content. All authors approved the final version of the manuscript.
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**FIGURE LEGENDS**

**Figure 1.** Flow activated cell sorting (FACS) plots representing PE-conjugated CD34/V450-conjugated CD45 cells within (A) total PMBCs, (B) CD34+ selected PBMCs using immunomagnetic cell separation procedures and (C) unselected (negative fraction) CD34 cells. Multiple trials have resulted in an isolation purity of 52 ± 3%.

**Figure 2.** Quantification of HUVEC length and complexity after culture with conditioned media from CD34+ (A, B) and CD34-/CD31+ cells (C, D). CD34+ and CD34-/CD31+ CACs were cultured in un-supplemented endothelial growth medium free of growth factors or serum, each at a density of 100,000 cells per well of a fibronectin coated plate. Media was collected after 48hrs of culture from each cell type. 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. Images were taken under 10x magnification. * Indicates statistically significant difference from high endurance trained subjects, # Indicates statistically significant difference from active subjects. Significance accepted at P ≤ 0.05.

**Figure 3.** Representative images of HUVEC cord formation from conditioned media of endurance trained (A, D), active (B, E) and inactive (C, F) circulating angiogenic cells from CD34+ (A, B, C) and CD34-/CD31+ cells (D, E, F).

**Figure 4.** Experiments demonstrating the efficacy of the fluorescent probe H2DCF-DA to detect differences in intracellular ROS in CD34+ PBMCs (A-B, n=3 per experiment). In the presence of peroxynitrile donor SIN-1 (200 μM), H2DCF-DA signal increases by more than 200% (A). The H2DCF-DA signal is increased by over 100% in the presence of hydrogen peroxide (500 μM) but pretreatment with PEG-catalase (50 U/mL) reduces this signal by nearly 40% (B). Effects of exercise training status on intracellular reactive oxygen species content in freshly isolated CD34+ (C) and CD34-/CD31+ (D) circulating angiogenic cells (n=12 endurance-trained, n=15 active, and n=12 inactive).

**Figure 5.** Experiments demonstrating the efficacy of the fluorescent probe DAF-FM diacetate to detect differences in intracellular NO in CD34+ and fractioned PBMCs (n=3 per experiment). Greater than 700% increases in DAF-FM signal in the presence of an NO donor, Deta NONOate (50 μM) (A). Over 3-fold increases in DAF-FM signal in the presence of SIN-1 (20 μM), and 56% reduction in DAF-FM signal with L-NAME treatment (300 μM) compared to control (B). Effects of exercise training status on intracellular nitric oxide content in freshly isolated CD34+ (C) and CD34-/CD31+ (D) circulating angiogenic cells (n=12 endurance-trained, n=15 active, and n=12 inactive).

**Figure 6.** Effects of exercise training status on 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).

**Figure 7.** Total protein gel of CM from cultured CACs (A) showing bands at 10 kDa (S100aA8) and 14 kDa (S100A9). Semi-quantitative analysis of S100A8 and S100A9 in conditioned media of cultured CD34+ cells (B) and CD34-/CD31+ cells (C) using mass spectrometry spectrum counts (n=1) to identify proteins present in conditioned medium.
Figure 8. Effects of exercise training status on S100A8 and S100A9 mRNA expression for freshly isolated CD34+ circulating angiogenic cells (A-B; n= 13 endurance-trained, n=13 active and n=8 inactive) and CD34-/CD31+ circulating angiogenic cells (C-D; n=14 endurance-trained, n=15 active and n=11 inactive).

Figure 9. Secreted S100A8 and S100A9 protein content in cultured CD34+ (A, B) and CD34-/CD31+ (C, D) cell conditioned media (CM) from endurance-trained and inactive subjects (n=12 per group in CD34+ blots and n=11 per group in CD34-/CD31+ blots). Values were normalized to cell number (100,000 cells/well) as equal volumes of CM were loaded for western blot analysis. Representative blots for CD34+ CM (E) and CD34-/CD31+ CM (F). * Indicates statistically significant from endurance-trained subjects. Significance accepted at P ≤ 0.05.

Figure 10. Representative western blots depicting the standard curve (1μg/mL-300μg/mL) of recombinant human S100A8/A9 used to estimate the approximate concentrations of each protein in the CD34+ CM and CD34-/CD31+ CM (A). Approximate concentrations of S100A8 and S100A9 present in n=3 CD34+ CM samples (n=2 inactive subject CM and n=1 endurance-trained subjects’ CM) (B) and n=3 CD34-/CD31+ CM (n=2 inactive subject CM and n=1 endurance-trained subject’s CM) (C) as estimated through western blots using this standard curve.

Figure 11. Effects of recombinant human S100A8 and S100A9 (7.18 μg/mL and 3.06 μg/mL, respectively for a total volume of 150 μL/well) on HUVEC cord length (A) and complexity (B) compared to basal media and vehicle conditions and representative images from each (C). 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). * Indicates statistically significant from endurance-trained subjects. Significance accepted at P ≤ 0.05.
<table>
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<tr>
<th></th>
<th>Endurance Trained (n=14)</th>
<th>Active (n=15)</th>
<th>Inactive (n=12)</th>
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<tr>
<td><strong>Age (yr)</strong></td>
<td>30±1.5</td>
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<td><strong>Triglycerides (mg/dl)</strong></td>
<td>71±8.8</td>
<td>71±7.3</td>
<td>68±8.3</td>
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*Significantly different from Endurance Trained; #Significantly different from Active
Figure 1.

A

B

C

Total PMBCs

CD34+ selected PBMCs

Unselected CD34- PBMCs
Figure 2.

A  CD34+ CM

B  CD34-/CD31+ CM

C  Average Total Length (mm)/Basal

D  Average Number of Nodes/Basal

Endurance Trained  Active  Inactive

* P=0.06

#
Figure 3.

Endurance Trained  Active  Inactive
Figure 4.

A

B

C

D

Endurance Trained | Active | Inactive
---|---|---
Control | | |
SIN-1 (200 uM) | | |
Intracellular ROS (H2-DCF/DAPI)

Endurance Trained | Active | Inactive
---|---|---
Control | | |
H2O2 (500 uM) | 50 U/mL PEG-Catalase | |
Intracellular ROS (H2-DCF/DAPI)

CD34+ Cells

CD34-CD31+ Cells
Figure 5.

A

B

C

D

CD34+ Cells

CD34-/CD31+ Cells

Endurance Trained

Active

Inactive

Intracellular NO (DAF-FM/DAPI)
Figure 6.

A. CD34+ Cells

B. CD34-/CD31+ Cells

C. eNOS mRNA/18s

D. gp91phox mRNA/18s

E. p47phox mRNA/18s

F. eNOS mRNA/18s
Figure 7.

A

B

C

S100A8 10 kDa
S100A9 14 kDa

CD34+ CM Total Spectrum Count

Endurance Trained Inactive

CD34-31+ CM Total Spectrum Count

Endurance Trained Inactive
Figure 8.

A  

CD34+ Cells

Endurance Trained  |
| Active  |
| Inactive  |

S100A8 mRNA/18S

B  

S100A9 mRNA/18S

C  

CD34-/CD31+ Cells

Endurance Trained  |
| Active  |
| Inactive  |

S100A8 mRNA/18S

D  

S100A9 mRNA/18S
Figure 9.

A. CD34+ CM

B. CD34+/CD31+ CM

E. CD34+ CM

F. CD34+/CD31+ CM

G. CD34+ CM

H. CD34+/CD31+ CM

I. CD34+ CM

J. CD34+/CD31+ CM

K. CD34+ CM

L. CD34+/CD31+ CM

M. CD34+ CM

N. CD34+/CD31+ CM

O. CD34+ CM

P. CD34+/CD31+ CM

Q. CD34+ CM

R. CD34+/CD31+ CM

S. CD34+ CM

T. CD34+/CD31+ CM

U. CD34+ CM

V. CD34+/CD31+ CM

W. CD34+ CM

X. CD34+/CD31+ CM

Y. CD34+ CM

Z. CD34+/CD31+ CM

**S100A8 AU**

**S100A9 AU**

**P = 0.08**
Figure 10.

A

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<tr>
<th>1</th>
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<td>S100A9</td>
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B

A8/A9 ratio = 0.513

C

A8/A9 ratio = 2.343
Figure 11.

A

![Graph showing Average Total Length (mm) for different conditions.]

B

![Graph showing Average Number of Nodes for different conditions.]

C

![Images illustrating Basal (EBM-2), EBM-2 + Vehicle, and rhS100A8/S100A9 conditions.]