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ENDOTHELIAL PROGENITOR CELL NUMBER AND COLONY-FORMING CAPACITY IN OVERWEIGHT AND OBESE ADULTS

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Abstract

Objective—To investigate whether adiposity influences endothelial progenitor cell (EPC) number and colony-forming capacity.

Design—Cross-sectional study of normal weight, overweight and obese adult humans.

Subjects—Sixty-seven sedentary adults (age 45–65y): 25 normal weight (BMI ≤ 25 kg/m^2; 12 males/13 females); 18 overweight (BMI = 25–29.9 kg/m^2; 12 males/6 females); and 24 obese (BMI ≥ 30 kg/m^2; 18 males/6 females). All subjects were non-smokers and free of overt cardiometabolic disease.

Measurements—Peripheral blood samples were collected and circulating EPC number was assessed by flow cytometry. Putative EPCs were defined as CD45$^-$/CD34$^+$/VEGFR-2$^+$/CD133$^+$ or CD45$^-$/CD34$^+$ cells. EPC colony-forming capacity was measured *in vitro* using a colony-forming unit assay.

Results—Number of circulating putative EPCs (either CD45$^-$/CD34$^+$/VEGFR-2$^+$/CD133$^+$ or CD45$^-$/CD34$^+$ cells) was lower (P<0.05) in obese (0.0007±0.0001%; 0.050±0.006%) compared with overweight (0.0016±0.0004%; 0.089±0.019%) and normal weight (0.0015±0.0003%; 0.082±0.008%) adults. There were no differences in EPC number between the overweight and normal weight groups. EPC colony-formation was significantly less in the obese (6±1) and overweight (4±1) compared with normal weight (9±2) adults.

Conclusion—These results indicate that: 1) the number of circulating EPCs is lower in obese compared with overweight and normal weight adults; and 2) EPC colony-forming capacity is blunted in overweight and obese adults compared with normal weight adults. Impairments in EPC number and function may contribute to adiposity-related cardiovascular risk.

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Keywords

overweight; obesity; endothelial progenitor cells; colony-forming units

INTRODUCTION

Overweight and obesity are associated with increased rates of cardiovascular morbidity and mortality (1;2)

Endothelial damage and dysfunction is considered to be a major underlying mechanism for the heightened cardiovascular burden with increased adiposity. For example, alterations in endothelial function associated with overweight and obesity that precede and predispose to atherosclerosis and thrombosis include diminished endothelial vasodilator and fibrinolytic function (3;4). Although much attention has focused on factors that contribute to adiposity-related endothelial damage, such as inflammation and oxidative stress, recent studies indicate that endogenous endothelial repair and neovascularization processes also play an important role in vascular health and function.

It is now recognized that endothelial repair/regeneration is not only dependent upon the migration and proliferation of surrounding mature endothelial cells resident in the vascular wall, but also on the availability of circulating endothelial progenitor cells (EPCs) (5). Characterized in 1997 by Asahara and colleagues (6), EPCs possess the ability to proliferate, migrate, differentiate into mature endothelial cells and incorporate into preexisting and newly-forming blood vessels (6–9). Circulating EPCs have also generated interest as a novel biomarker of endothelial function and a prognostic indicator of cardiovascular morbidity and mortality. Two recent clinical studies reported that reduced levels of circulating EPCs independently predict atherosclerotic disease progression and death from cardiovascular causes in patients with established coronary artery disease (CAD) (10;11). Moreover, after adjusting for disease activity and risk factors, low numbers of circulating EPCs were associated with a four-fold increased risk of a future cardiovascular event (10). In addition, Kunz *et al*. (12) reported a strong inverse relation between EPC colony-forming capacity and CAD severity in individuals undergoing diagnostic cardiac catheterization, independent of traditional risk factors. Interestingly, the investigators noted that for every 10 EPC colony-forming unit increase, the likelihood of multivessel CAD declined by 20%. Numerical and functional deficits in circulating EPCs have also been linked to restenosis rates and impaired neovascularization after ischemic events (5;13).

Although reduced number and impaired function of EPCs have been linked to a number of pathologies associated with overweight/obesity, such as hypertension, hypercholesterolemia, diabetes and CAD (14–16), the influence of increased adiposity *per se* on circulating EPCs remains unclear. Accordingly, we tested the hypotheses that: 1) the number of circulating EPCs is lower in otherwise healthy overweight and obese compared with normal weight adults; and 2) EPC colony-forming capacity is also diminished in overweight and obese adults.

METHODS

Subjects

Sixty seven sedentary adults aged 45–65 years participated in the study: 25 normal weight (BMI >18.5 kg/m^2 and <25 kg/m^2; 12 male/13 female); 18 overweight (BMI ≥ 25 kg/m^2 and ≤ 30 kg/m^2; 12 M/6 F); and 24 obese (BMI ≥ 30 kg/m^2; 18 M/6 F). All subjects were normotensive (arterial blood pressure ≤ 140/90 mmHg), non-smokers, non-medicated (including vitamins), and free of overt cardiovascular, metabolic, renal and hematologic disease, as assessed by medical history, resting and exercise electrocardiograms, and fasting blood chemistries. Subjects were excluded from the study if they exhibited plasma glucose >7.0 mmol/L; total cholesterol ≥ 6.0 mmol/L, LDL-cholesterol ≥ 4.5 mmol/L, triglycerides ≥ 2.5 mmol/L. All subjects were sedentary and had not performed regular physical exercise for at least 1 year before the beginning of the study. Female subjects were at least 1 year postmenopausal and had never taken or had discontinued use of hormone replacement therapy at least 1 year before the start of the study. Prior to participation, all of the subjects had the research study and its potential risks and benefits explained fully before providing written informed consent according to the guidelines of the University of Colorado at Boulder. We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research.

Body Composition

Body mass was measured to the nearest 0.1 kg using a medical beam balance. Percent body fat was determined by dual energy X-ray absorptiometry (Lunar Corp., Madison, WI, USA). Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. Minimal waist circumference was measured according to published guidelines (17).

Maximal Oxygen Consumption ($\dot{V}O_2$ max)

To assess aerobic fitness, subjects performed incremental treadmill exercise using a modified Balke protocol. Maximal oxygen consumption ($\dot{V}O_2$ max) was measured using on-line computer-assisted open circuit spirometry, as reported previously (18).

Metabolic Measurements

Fasting plasma lipid, lipoprotein, glucose, and insulin concentrations were determined using standard techniques, as reported previously (3). Insulin resistance (HOMA-IR) was calculated according to the HOMA calculation: fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5 (19).

EPC Isolation and Characterization

Circulating mononuclear cells were isolated from peripheral blood sample by Ficoll density-gradient centrifugation (Histopaque 1077, Sigma Aldrich, St. Louis, MO, USA), washed and resuspended in growth medium (Medium 199, Gibco, Grand Island, NY, USA) supplemented with 20% fetal calf serum (Gibco), penicillin (100 U/mL, Gibco), and streptomycin (100 mg/mL, Gibco). Endothelial phenotype of these cells was confirmed by immunofluorescent staining for the uptake of DiI-ac-LDL (Biomedical Technologies Inc.,

Stoughton, MA, USA) and expression of von Willebrand factor (Dako, Glostrup, Denmark), VE-cadherin, CD31, and VEGFR-2 (Invitrogen, Carlsbad, CA, USA).

EPC Number

Circulating putative EPC number was determined by fluorescence-activated cell sorting (FACS) analysis following guidelines recommended by the International Society for Hematotherapy and Graft Engineering (20). Briefly, 2×10^6 cells were incubated at 4 °C for 30 minutes with monoclonal antibodies for PC7-conjugated CD45 (Beckman Coulter, Fullerton, CA, USA), FITC-conjugated CD34 (Beckman Coulter), PE-conjugated VEGFR-2 (R&D Systems, Minneapolis, MN, USA) and APC-conjugated CD133 (Miltenyi Biotech, Auburn, CA, USA). Non-viable cells were excluded with propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) and appropriate compensation controls were analyzed. Cells were gated for low expression of CD45, then $CD34^+$ cells were analyzed for events double-positive for VEGFR-2 and CD133 and presented as a percent of total viable mononuclear cells. All samples were analyzed using a FC500 flow cytometer (Beckman Coulter) and the data analyzed by CXP software.

EPC Colony-Forming Assay

EPC colony-forming capacity was determined as described previously by our laboratory and others (16;21). Briefly, freshly isolated mononuclear cells were plated on 6-well plates coated with human fibronectin (BD Biosciences, San Jose, CA, USA) for 48 hours at 37 °C. Thereafter, 5×10^5 non-adherent cells from each subject were seeded onto 24-well fibronectin-coated plates (BD Biosciences). Growth medium was changed every 3 days, and the colony-forming units (CFUs) were counted in 4 random wells on day 7 by two independent investigators blinded to sample identification. Only CFUs consisting of multiple thin, flat cells emanating from a central cluster of rounded cells were counted.

Statistical Analysis

Group differences were determined by analysis of variance. Where indicated by a significant F value, Duncan's post hoc test was performed to compare specific group means. Importantly, no main effects of gender, nor interactions of gender with BMI group, were found in any of the key outcome variables. Therefore, the data were combined and presented together. Relations between variables of interest were assessed by Pearson's correlation coefficient and linear regression analysis. All data are expressed as means ± SE. Statistical significance was set *a priori* at $P < 0.05$.

RESULTS

Selected subject characteristics are presented in Table 1. All subjects were normotensive, normolipidemic, and normoglycemic. By design, body mass and body composition values were significantly higher ($P<0.05$) in the overweight and obese groups compared with the normal weight group. Although within clinically normal ranges, obese subjects demonstrated higher ($P<0.05$) resting systolic and diastolic blood pressure, and lower ($P<0.05$) HDL-cholesterol, than the normal weight controls. Obese subjects also had significantly higher plasma insulin concentrations and HOMA insulin resistance values

compared with both the normal weight and overweight subjects. There were no differences amongst the groups in plasma concentrations of total cholesterol, LDL-cholesterol, triglycerides, and glucose.

The number of $CD45^{-}/CD34^{+}/VEGFR\text{-}2^{+}/CD133^{+}$ cells was ~50% lower ($P<0.05$) in the obese (0.0007 ± 0.0001 %) compared with the overweight (0.0016 ± 0.0004 %) and normal weight groups (0.0015 ± 0.0003 %) (Figure 1). Similarly, the number of $CD45^{-}/CD34^{+}$ cells was lower ($P<0.05$) in the obese (0.050±0.006%) compared with overweight (0.089±0.019%) and normal weight subjects (0.082±0.008%). There were no significant differences in either $CD45^{-}/CD34^{+}/VEGFR\text{-}2^{+}/CD133^{+}$ or $CD45^{-}/CD34^{+}$ cells between the overweight and normal-weight subjects. The capacity of EPCs to form colonies was lower (~40%; $P<0.05$) in both the overweight (4 ± 1) and obese (6 ± 1) groups versus the normal weight controls (9 ± 2) (Figure 2). There was no significant difference in EPC CFU number between the overweight and obese subjects.

In the overall study population, there were significant inverse relations between BMI and the number of $CD45^{-}/CD34^{+}/VEGFR\text{-}2^{+}/CD133^{+}$ cells ($r = -0.25$), $CD45^{-}/CD34^{+}$ cells ($r = -0.26$) and EPC colony forming units ($r = -0.24$). Percent body fat was also negatively associated with the number of $CD45^{-}/CD34^{+}$ cells ($r = -0.25$, $P<0.05$). No other significant correlations were observed between either EPC number or colony-forming capacity and any other anthropometric, metabolic or hemodynamic variable.

DISCUSSION

The new findings of the present study are that: 1) the number of circulating EPCs is lower in obese compared with overweight and normal weight adults; and 2) EPC colony-forming capacity is blunted in overweight and obese adults compared with normal weight adults. To our knowledge, this is the first study to demonstrate adiposity-related impairments in EPC number and function in overweight and obese adults free of other cardiometabolic abnormalities.

Many of the cardiovascular complications associated with obesity are due, at least in part, to endothelial damage and/or dysfunction (22;23). Circulating EPCs are considered to be an important hemostatic mechanism for counteracting endothelial injury and the accelerated development of atherosclerosis associated with many cardiovascular risk factors, including obesity (24). For example, circulating EPC number is inversely related to the extent of carotid stenosis and lower extremity atherosclerosis in obese diabetic patients (25). Moreover, in patients with CAD, higher numbers of circulating EPCs have been linked to greater myocardial perfusion and vascularization (26;27) as well as overall better prognosis (10). To date, previous investigations evaluating the influence of adiposity on progenitor cell number have been hampered by design limitations involving the presence of multiple cardiovascular risk factors, overt disease and/or medication use, making it difficult to discern the impact of obesity *per se* on EPC number (28;29). In the present study, we demonstrate that the number of circulating putative EPCs, presented as $CD45^{-}/CD34^{+}/VEGFR\text{-}2^{+}/CD133^{+}$ cells as well as $CD45^{-}/CD34^{+}$ cells, is close to 50% lower in obese compared with overweight and normal weight adults. The absence of other established

cardiometabolic risk factors in our study population suggests a primary negative influence of obesity on EPC number. From a clinical perspective, it is plausible that reduced EPC bioavailability may contribute to the increased incidence of endothelium-related vascular complications and reduced neovascularization with obesity (30–32). Interestingly, EPC number was not lower in the overweight compared with normal weight group. The number of $CD45^{-}/CD34^{+}/VEGFR\text{-}2^{+}/CD133^{+}$ and $CD45^{-}/CD34^{+}$ cells in the overweight adults was almost identical to that of the normal weight controls and, in turn, significantly higher than the obese adults. This finding was unexpected considering we have previously reported no differences in the degree of endothelial vasodilator and fibrinolytic dysfunction between overweight and obese adults similar to those studied herein (3;33). Nevertheless, greater number of EPCs in overweight adults may play a role in their lower risk of recurrent coronary events following acute myocardial infarction compared with obese adults of similar age (34).

The mechanisms responsible for the obesity-related reduction in circulating putative EPC number are not clear. Although we employed strict inclusion criteria in the present study, the obese adults did demonstrate higher, albeit in clinically normal ranges, levels of blood pressure, plasma insulin and insulin resistance. Thus, it is possible that the cumulative effects of these factors, along with other secondary consequences of excess body fatness, such as oxidative stress and inflammation, creates an environment resulting in higher consumption and/or exhaustion of EPC production. For example, the inflammatory cytokine C-reactive protein, although not measured in the present study, is often higher in obese compared with overweight and normal weight adults (35). C-reactive protein has been shown to promote apoptosis in EPCs *in vitro* (36), which could negatively impact circulating EPC number. Differences in body fat distribution between the overweight and obese subjects may also have contributed to our findings. Future studies are needed to determine whether differences in visceral and subcutaneous body fat depots influence EPC number.

There are conflicting reports in the literature regarding the appropriate defining criteria for the quantification of EPCs. Circulating EPCs are thought to be primarily derived from hematopoietic stem cells, however, non-hematopoietic mesenchymal stem cells and other monocytic cells from peripheral blood have also been shown to cross-differentiate into functionally active endothelial progenitors, or assume an endothelial-like phenotype (37;38). It has been suggested that the best strategy for enumeration is to isolate cells positive for both a hematopoietic stem cell surface membrane protein, such as CD34, and a marker of endothelial lineage, such as VEGFR-2, by flow cytometry (39). However, the antigen CD34 can also be weakly expressed in mature endothelial cells (40), that are also present in the circulation (41). As a result, it has been proposed that the immature hematopoietic stem cell marker CD133 also be used for enumeration (42;43). Peichev *et al.* demonstrated that circulating $CD34^{+}/VEGFR\text{-}2^{+}/CD133^{+}$ cells differentiate into endothelial cell clusters *in vitro* and may also contribute to neoangiogenesis (40). In contrast, Case *et al.* (44) recently reported that $CD34^{+}/VEGFR\text{-}2^{+}/CD133^{+}$ cells do not differentiate into endothelial cells, raising some doubt regarding their EPC designation. However, because the cells were cultured in isolation, devoid of interaction with other cell lines (a situation never encountered *in vivo*), it is possible that the culture conditions influenced the results. Of note,

the same study also reported that $CD34^+$ cells with low expression of the common leukocyte antigen CD45 do, give rise to proliferative endothelial cells even under the same restrictive culture conditions, demonstrating the endothelial potential of the $CD45^-/CD34^+$ cell populations. Recently, a statement from the EULAR Scleroderma Trials and Research Group recommended the use of CD34, VEGFR-2 and CD133 antigens to quantify circulating EPCs (45). In the present study, we believe, we employed antigenic profiles to identify putative EPCs (i.e. $CD45^-/CD34^+$ and $CD45^-/CD34^+/VEGFR\text{-}2^+/CD133^+$ cells) that encompass a cellular phenotype with confirmed endothelial differentiation capacity (44;46) and that, importantly, have been linked to cardiovascular disease risk and outcome (47–49). Given the lack of consensus in enumerating EPCs, our results must be viewed within the context of our antigenic profile. Other enumerating criteria, such as quantifying cells double positive for CD34 and VEGFR-2, may yield different results.

In contrast to circulating EPC number, the number of EPC colony-forming units was ~40% lower in both obese and overweight compared with normal weight adults. To our knowledge, this is the first study to demonstrate an adiposity-related reduction in EPC colony-forming capacity. Previous studies that have failed to find a relation between adiposity and EPC colony-forming units were either underpowered to detect a difference (16), or included a study population that was compromised by statin-use and documented CAD (50), factors which have been shown to influence EPC function (12;51). Once thought to be a surrogate marker of circulating EPC number, it is now generally accepted that the number of EPC colony-forming units does not correspond to the number of circulating EPCs measured by flow cytometry (52;53). The discrepancy in our results between EPC number (determined by FACS) and the number of colony-forming units further confirm the lack of association between these two measures. Nevertheless, clinical interest in EPC colony-forming units has increased due to the consistently observed relation between colony number and both endothelial function (16) and cardiovascular risk in healthy and diseased populations (12;21;54). These findings have prompted its use as a novel cardiovascular biomarker (11;55). Whether the number of EPC colony-forming units in overweight and obese adults is associated with endothelial dysfunction and adverse cardiovascular events remains to be determined.

The reasons for the reduced EPC colony-formation in overweight and obese compared with normal weight adults are not clear. Recent data indicate that the central clusters of cells that make up these colonies are comprised mainly of $CD3^+/CD31^+/CXCR4^+$ T cells (56;57). Thus, it is possible that reduction in this subpopulation of T cells with adiposity is the underlying cause. However, in a currently ongoing study focused on these so-called “angiogenic T cells,” we have not observed a decline in their number in either overweight or obese adults compared with normal weight controls (unpublished observations). It is important to emphasize that the defining characteristic of an EPC colony-forming unit is the presence of spindle-shaped cells emanating from a central cluster of cells (16). These spindle-shaped cells, more so than the central colony clusters, have been shown to demonstrate immunologic and morphologic characteristics consistent with the identification of putative EPCs (58). It is possible that the same milieu of factors that negatively affect circulating EPC number *in vivo* may also impair their ability to contribute to cluster

formation *in vitro*. Indeed, inflammatory and oxidative substances, such as CRP and oxidized LDL-cholesterol, have been shown to impair EPC function *in vitro* (59;60).

In conclusion, the results of the present study indicate that increased body fatness, independent of other traditional cardiovascular risk factors, adversely affects EPC biology. Obesity, but not overweight, is associated with lower levels of circulating EPCs compared with normal weight adults; whereas, both overweight and obesity are associated with blunted EPC colony-forming capacity. While the differential effects of overweight and obesity on circulating EPCs require more attention, diminished EPC bioavailability and impaired function may contribute to adiposity-related cardiovascular morbidity and mortality.

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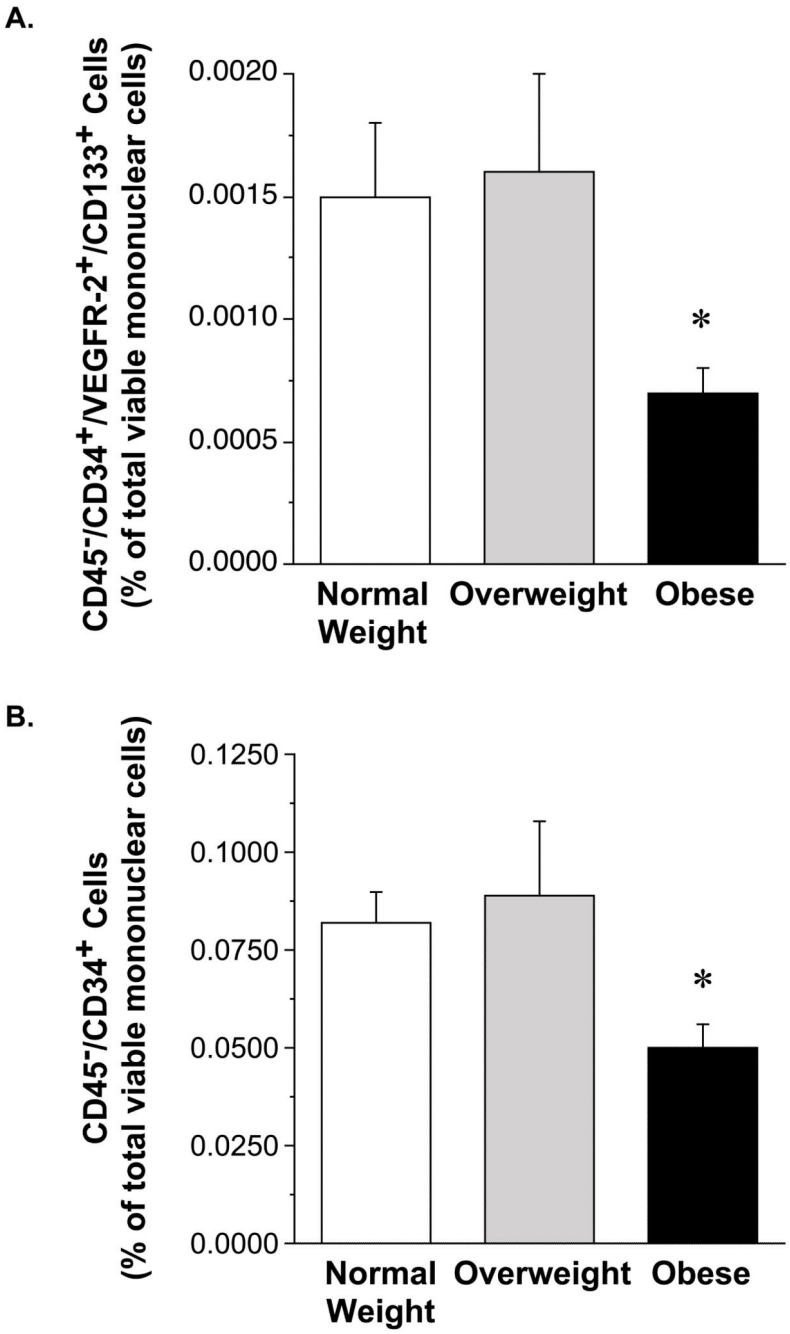

Figure 1.

Circulating levels of $CD45^{-}/CD34^{+}/VEGFR\text{-}2^{+}/CD133^{+}$ and $CD45^{-}/CD34^{+}$ cells in normal weight, overweight, and obese adults.

Values are mean ± SE. *P< 0.05 vs. normal weight and overweight.

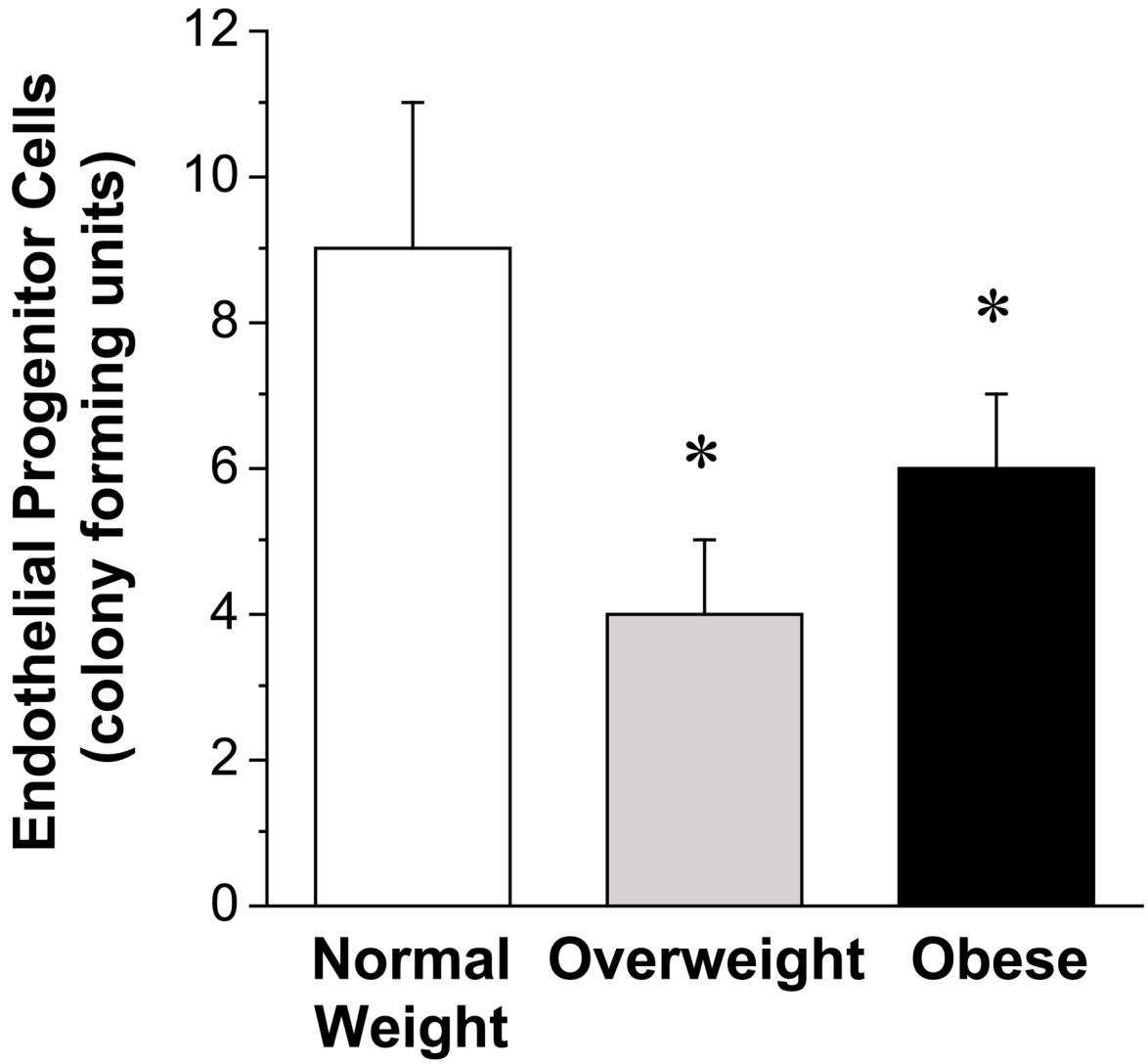

Figure 2.

Endothelial progenitor cell colony forming units for normal weight, overweight, and obese adults.

Values are mean ± SE. *P< 0.05 vs. normal weight

Table 1

Selected subject characteristics

Variable	Normal Weight (N=25)	Overweight (N=18)	Obese (N=24)
Age (years)	56±1	58±1	56±1
Body mass (kg)	70.3±2.1	80.1±2.4*	97.5±2.8*†
BMI (kg/m^2)	23.4±0.4	27.6±0.2*	32.9±0.5*†
Body fat (%)	28.8±1.6	31.2±2.1	37.4±1.4*†
Waist circumference (cm)	81.9±2.0	93.0±1.7*	107.8±1.5*†
Systolic BP (mmHg)	118±2	125±2	126±2*
Diastolic BP (mmHg)	72±1	78±1*	81±1*
$\dot{V}O_2$ max (L/min)	2.2±0.1	2.7±0.2	2.6±0.1
$\dot{V}O_2$ max (mL/kg/min)	31.5±1.3	32.4±1.9	27.9±1.4
Maximum heart rate (bpm)	172±3	173±2	171±2
RER at $\dot{V}O_2$ max	1.19±0.01	1.14±0.01	1.16±0.02
Treadmill Time (min)	10.6±0.2	10.5±0.4	9.6±0.3
Total Cholesterol (mmol/L)	5.1±0.1	5.4±0.1	5.3±0.1
HDL-Cholesterol (mmol/L)	1.5±0.1	1.3±0.1	1.2±0.1*
LDL-Cholesterol (mmol/L)	3.1±0.1	3.5±0.1	3.4±0.1
Triglycerides (mmol/L)	1.1±0.1	1.3±0.1	1.4±0.1
Glucose (mmol/L)	4.6±0.1	5.2±0.1	5.1±0.1
Insulin (pmol/L)	32.0±3.5	43.9±3.9	57.0±5.0*
HOMA IR	1.2±0.1	1.7±0.2	2.2±0.2*

BMI, Body Mass Index; BP, blood pressure; $\dot{V}O_2$ max, maximal oxygen consumption; RER, respiratory exchange ratio; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment-insulin resistance.

Values are mean ± SEM.

*P<0.05 vs. Normal Weight.

†P<0.05 vs. Overweight.