Endothelial Function Varies According to Insulin Resistance Disease Type

Short Title: Insulin Resistance and Vascular Function

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Joshua A. Beckman, Allison B. Goldfine, Andrea Dunaif, Marie Gerhard-Herman, and Mark A. Creager

Institutions: Cardiovascular Division, Brigham and Women’s Hospital, Boston, MA; Division of Cellular and Molecular Physiology, Joslin Diabetes Center, Boston, MA; and Division of Endocrinology, Northwestern University, Chicago, IL.

Corresponding Author: Joshua A. Beckman, MD; Cardiovascular Division Brigham and Women’s Hospital; 75 Francis Street; Boston, MA 02115 E-mail: jbeckman@partners.org
Abstract

Objective: We examined the relationship between insulin resistance and vascular function in three insulin resistant states (type 2 diabetes mellitus, non-HIV lipodystrophic diabetes, and non-diabetic polycystic ovary syndrome (PCOS)) and in healthy controls.

Research Design and Methods: The population included 12 women with type 2 diabetes, 6 with lipodystrophic diabetes, 10 with polycystic ovary syndrome, and 19 healthy female subjects. Metabolic measures included insulin sensitivity by the homeostasis model assessment, lipids, free fatty acids, and adiponectin. High-resolution B mode ultrasound was used to determine endothelium-dependent and –independent vasodilation.

Results: Type 2 diabetic, lipodystrophic, and polycystic ovary syndrome subjects were insulin resistant compared with control subjects (p = 0.001). Flow mediated vasodilation (FMD) was reduced in diabetic subjects (3.4 ± 1.3%), compared with control (7.3 ± 1.1%) but not in lipodystrophic (7.7 ± 1.2%) or polycystic ovary syndrome subjects (9.9 ± .7%) (p = 0.005). Nitroglycerin-mediated vasodilation (NMD) was attenuated in both diabetic (15.2 ± 2.0%) and lipodystrophic subjects (16.7 ± 3.6%) compared to healthy control subjects (24.6 ± 2.4%) and polycystic ovary syndrome subjects (23.2 ± 1.8%)(p = 0.019). Neither insulin resistance, free fatty acids, adiponectin, nor C-reactive protein associated with vascular dysfunction.

Conclusions: Among these different types of patients with insulin resistance, we found abnormal endothelium-dependent vasodilation only in the patients with type 2 diabetes. We postulate that variations in the mechanism of insulin resistance may affect endothelial function differently than glucose homeostasis.
Insulin resistance, typically defined by vascular dysfunction across insulin resistance impairment of insulin-mediated actions on states, glucose uptake, affects a wide range of tissues including adipose, skeletal muscle, and the vascular endothelium. Insulin, via a sequence of intracellular signals, activates endothelial including 12 with type 2 diabetes, 10 with nitric oxide synthase (eNOS) (1) and polycystic ovary syndrome (PCOS), 6 with increases production of nitric oxide. non-human immunodeficiency virus (HIV)-Reductions in the bioavailability of nitric negative lipodystrophic diabetes, and 19 oxide (NO) are associated with atherogenesis. healthy controls were recruited through Impaired insulin action, when assessed by newspaper advertisement and from the Joslin fasting serum insulin level or the homeostasis Diabetes Center. All subjects underwent model assessment (HOMA-IR) (2, 3), is screening medical history, physical associated with atherosclerosis and an examination, and laboratory analysis increased risk of myocardial infarction. including complete blood count, serum Insulin resistance is associated with electrolytes, glucose, blood urea nitrogen, and endothelial dysfunction (4) and may serve as creatinine, total cholesterol, and low density a link between insulin resistance and lipoprotein (LDL) cholesterol. Among the atherosclerosis.

Accordingly, we sought to investigate the role of insulin resistance on endothelial function in three distinct populations of insulin resistant women (polycystic ovary syndrome (PCOS), type 2 diabetes and lipodystrophic diabetes) compared with healthy subjects. We also measured a marker atherosclerosis or risk factors (except for inflammation (C-reactive protein (CRP)) smoking) for atherosclerosis. The and two adipokines (adiponectin and free lipodystrophic subjects included two with fatty acids) to determine whether these familial partial lipodystrophy (FPLD), two factors, known to be abnormal in insulin with Dunnigan’s FPLD, one with familial resistance, may serve as mechanism of partial lipodystrophy - mandibuloacral

**Research Design and Methods**

**Subjects**

Forty seven non-smoking women, including 12 with type 2 diabetes, 10 with polycystic ovary syndrome (PCOS), 6 with non-human immunodeficiency virus (HIV)-negative lipodystrophic diabetes, and 19 healthy controls were recruited through newspaper advertisement and from the Joslin Diabetes Center. All subjects underwent fasting serum insulin level or the homeostasis Diabetes model assessment (HOMA-IR) (2, 3), is screening medical history, physical associated with atherosclerosis and an examination, and laboratory analysis increased risk of myocardial infarction. including complete blood count, serum Insulin resistance is associated with electrolytes, glucose, blood urea nitrogen, and endothelial dysfunction (4) and may serve as creatinine, total cholesterol, and low density a link between insulin resistance and lipoprotein (LDL) cholesterol. Among the atherosclerosis.

Insulin resistance, however, is not a single entity, those with hypertension, history of single entity and its occurrence as a consequence of a tobacco use, LDL or total cholesterol greater variety of mechanisms and disparate clinic than the 75th percentile for age and gender, presentations, unified phenotypically by cardiovascular disease, use of a impaired insulin-mediated glucose uptake. thiazolidinedione, or other significant disease Because the mechanisms of insulin resistance were excluded. Women with PCOS had 6 or vary in different conditions, its impact on fewer menses per year in addition to other tissues remains unclear. Determining hyperandrogenemia defined by either total the effect of various insulin resistant states on testosterone > 58 ng/dl (2 nmol/L) and/or endothelial function may provide insight in non-sex hormone binding globulin bound NO bioavailability in each disease and testosterone (unbound testosterone or uT) >15 contribute to our understanding of a greater ng/dl (0.5 nmol/L) levels greater than two prevalence of atherosclerosis in insulin standard deviations above the mean value that resistant patients.

Established in reproductively normal women

Accordingly, we sought to investigate aged 18-40 years old in the early follicular the role of insulin resistance on endothelial phase of the menstrual cycle. Other causes of function in three distinct populations of anovulation and hyperandrogenemia were insulin resistant women (polycystic ovary excluded by appropriate laboratory tests. syndrome (PCOS), type 2 diabetes and Patients with lipodystrophic diabetes, because lipodystrophic diabetes) compared with of their rarity, were enrolled despite extant healthy subjects. We also measured a marker atherosclerosis or risk factors (except for of inflammation (C-reactive protein (CRP)) smoking) for atherosclerosis. The and two adipokines (adiponectin and free lipodystrophic subjects included two with fatty acids) to determine whether these familial partial lipodystrophy (FPLD), two factors, known to be abnormal in insulin with Dunnigan’s FPLD, one with familial resistance, may serve as mechanism of partial lipodystrophy - mandibuloacral
dysplasia variety, and one with acquired diastole. After baseline image acquisition, a lipodystrophy after dermatomyositis. One forearm sphygmomanometric cuff was subject was blind, had renal insufficiency, and inflated to suprasystolic pressure (200 mm peripheral arterial disease and died 3 months Hg) for five minutes. Upon cuff release, after the study; one subject had renal reactive hyperemia causes flow to increase insufficiency and died 1 year after the study; through the brachial artery subserving the and one subject had coronary artery disease. forearm. Flow-induced, endothelium-

Two lipodystrophic subjects were taking ACE dependent vasodilation of the brachial artery inhibitors, and they withheld these was determined by acquiring images at one medications for 24 hours prior to vascular minute after cuff deflation. Flow-mediated testing. Diabetes medications were held on vasodilation at this time point is largely the day of study. Other potential subjects endothelium-dependent and nitric oxide taking an angiotensin converting enzyme mediated, and can be inhibited by (ACE) inhibitor, angiotensin receptor blocker, administration of the nitric oxide synthase or statin were excluded. All participants antagonist, N\text{O} \text{-monomethyl-L-arginine} (5). provided written, informed consent. The Ten minutes after cuff release, the brachial protocol was approved by the Human artery was imaged again to re-establish basal Research Committees of the Joslin Diabetes conditions. Then, to determine endothelium-

Center and the Brigham and Women’s independent vasodilation, subjects received Hospital.

Vascular Reactivity Studies

brachial artery was imaged three minutes

All subjects were studied in the later. Brachial artery blood flow velocity was morning in the post-absorptive state, fasting determined via time-velocity integral after the previous midnight. Cyclooxygenase measurement. Nitroglycerin was not inhibitors, alcohol, and caffeine were administered if the systolic blood pressure prohibited for 24 hours prior to the study. was below 110 mm Hg or the subject refused Subjects were studied in a quiet, temperature- nitroglycerin, usually to avoid a severe controlled, dimly-lit room, after resting supine headache during the second and third visits. for a minimum of 5 minutes. High-resolution Laboratory Analyses

B-mode ultrasonography of the brachial artery Total cholesterol, triglycerides, HDL was performed using a Toshiba 270 SSA cholesterol, LDL cholesterol and blood (Toshiba America Medical Systems, Inc., glucose levels were measured by standard Tustin, CA) ultrasound machine and 7.5 MHz laboratory techniques. hs-CRP levels were linear array probe. The brachial artery was imaged using the Beckman LX-20 imaged longitudinally just proximal to the (Beckman Coulter, Brea, CA). This assay has antecubital fossa. Transducer position was been validated against the Dade Behring adjusted to obtain optimal images of the near hsCRP method and has an inter and intra-

and far wall of the intima. Images were assay CV of < 8%. Adiponectin levels were simultaneously recorded on super VHS video measured using a sandwich ELISA (Linco tape. The video output and Research, St. Charles, MO) and had an inter-
electrocardiographic signal of the ultrasound and intra-assay CV of <9%. Insulin levels machine were connected to a computer were measured using a two-site immunoassay equipped with a Data Translation frame- (Linco Research, St. Charles, MO) with an grabber videocard, (Dataviz, Trumbull, CT). inter and intra-assay CV of <11%. Free fatty The ‘R’ wave on the electrocardiogram acids in serum were measured using reagents served as a trigger to acquire frames at end- from Wako diagnostics. Free fatty acids were
measured based the acylation of coenzyme density lipoprotein (LDL) cholesterol levels, A(CoA) by the fatty acids in the presence of blood pressure, and body mass index did not added acyl-CoA synthetase (Wako) differ significantly among the groups. High Chemicals, Richmond, VA). The acyl-CoA density lipoprotein (HDL) cholesterol levels produced is oxidized by added acyl-CoA were lower and triglycerides levels were oxidase (ACOD) generating hydrogen higher in each insulin resistant group peroxide that is measured compared with healthy subjects. Insulin spectrophotometrically. The inter- and intra- resistance, as measured by HOMA-IR, was assay CVs for this assay are between 3-7%. greater in lipodystrophy (27.6 ± 16.4), PCOS The homeostasis model assessment (HOMA- IR) of insulin resistance was calculated as fasting glucose x fasting insulin/22.5 (6).

Statistical Methods

Descriptive measures are reported as means ± standard deviation (SD). Experimental measures are reported as means ± standard error (SE). Demographic data, arterial diameter, reactive hyperemia, and flow-mediated and nitroglycerin-mediated vasodilation were compared using analysis of variance (ANOVA). Post-hoc comparisons were made using Dunnett testing with the healthy control group as the referent. Correlation with Spearman’s rho analysis was performed to assess the effects of baseline characteristics and measured parameters on vascular function and insulin resistance. Statistical significance was accepted at the 95% confidence level (p < 0.05). All statistics were run on SPSS Base 11.0.04 (SPSS, Inc., Chicago, IL).

Results

Baseline characteristics are presented in Table 1. Type 2 diabetic subjects were older than lipodystrophic diabetic, PCOS, and healthy control subjects. Hemoglobin A1c was 7.8±1.9% in the subjects with type 2 diabetes and 8.8±2.3% in the subjects with lipodystrophy. Of the 12 subjects with type 2 diabetes, glucose lowering was achieved in 7 with a sulfonylurea alone, in 2 with insulin alone, and in 3 with metformin and a sulfonylurea. All lipodystrophic subjects were taking high dose insulin and 2 were taking metformin. Total cholesterol and low
not receive nitroglycerin. One PCOS and 3 was preserved in subjects with lipodystrophic healthy control subjects had systolic blood diabetes and with PCOS. Our results do not pressure below our cutoff level of 110 support a direct relationship between insulin mm/Hg, while 2 subjects with type 2 diabetes resistance and endothelial function across a refused.

Free fatty acids (FFA) were surprising, for worsening insulin resistance is significantly higher in each insulin resistant broadly associated with increases in group compared with healthy subjects (p = atherosclerosis across these same disease 0.004, by ANOVA) (Table 1). Adiponectin states, from least in PCOS to most in levels were significantly lower in the type 2 lipodystrophic diabetes (7-9). Phenotypic diabetic and PCOS groups compared with variations in insulin-affected tissues suggest healthy control (p = 0.04, by ANOVA) and that the mechanism of insulin resistance, lipodystrophic subjects. CRP was not instead of the severity of either insulin significantly different among the four groups resistance or glucose disturbance, may be (p > 0.2, by ANOVA).

Insulin resistance, whether measured of these disease states on vascular function. by HOMA-IR or fasting insulin level, In type 2 diabetes mellitus, serine correlated inversely with HDL and phosphorylation of the insulin receptor adiponectin levels and directly with substrate attenuates normal activation of triglycerides levels and BMI (Table 2). Free phosphatidylinositol 3-kinase and AKT fatty acid levels correlated indirectly with attenuating endothelial nitric oxide synthase HDL (r = -0.31, p = 0.04) and directly with activity (10). The molecular basis for defects triglycerides levels (r = 0.39, p =0.008). in insulin signaling differs in lipodystrophy Neither markers of insulin sensitivity, BMI, and PCOS compared with type 2 diabetes. CRP, nor adipokines correlated with For example, lamin or peroxisome endothelium-dependent vasodilation or with proliferator-activated receptor-gamma endothelium-independent vasodilation. mutations in some forms of lipodystrophy but Glucose levels correlated inversely with not common type 2 diabetes or PCOS suggest endothelium-independent vasodilation (r = - a different origin for the resistance to insulin 0.46, p = .009) but not endothelium-action (11). Differing responses to leptin dependent vasodilation.

Conclusions
In this investigation, we evaluated vascular function in subjects with different resistance differently than type 2 diabetes. types of insulin resistance including those Previous investigations by Dunaif and with type 2 diabetes mellitus, lipodystrophic colleagues have demonstrated increased diabetes, and polycystic ovary syndrome, and insulin receptor serine phosphorylation and compared those patients with healthy control decreased IRS-1 tyrosine phosphorylation subjects. Insulin-resistance, as determined by (16, 17) in obese women with PCOS. Several HOMA-IR, was more profound in each studies, including ours, demonstrate preserved insulin resistant group compared with control endothelial function in women with PCOS subjects. Despite the presence of insulin who are either non-obese or without morbid resistance, endothelium-dependent obesity (18, 19), but this remains vasodilation was reduced only in subjects controversial (20-22). The presence of with type 2 diabetes. Endothelial function obesity may contribute importantly to the
attenuation in vascular function in this vasodilation when only diabetic and healthy condition. While overweight, our cohort of controls were examined (data not shown), but PCOS was less obese than those with this only supports the lack of a relationship attenuated vascular function (22), and across insulin resistant states. endothelial function was similarly preserved in lean women with PCOS (18, 19). Adiponectin expression in our cohort Supporting the concept of an effect of obesity was lower in type 2 diabetic and PCOS on vascular function, Baron and colleagues subjects when compared to healthy subjects. found that insulin-mediated increases in leg However, adiponectin levels were not blood flow and skeletal muscle glucose decreased in the lipodystrophic subjects. uptake were reduced in obese subjects and Adiponectin levels in the lipodystrophic those with type 2 diabetes mellitus (4, 23). subjects were similar to the data of Haque and Escobar-Morreale and colleagues found no colleagues (30) in patients with familial difference in plasma inflammatory maker partial lipodystrophy, which were relatively concentrations of C-reactive protein, preserved in comparison to patients with interleukin 6, TNF-a, soluble type 2 TNF congenital generalized lipoatrophy, which receptor, and soluble intercellular cell were not represented in our cohort. adhesion molecule-1 in 35 PCOS and 28 Additionally, while we did find the expected healthy subjects paired for BMI, prevalence strong association between adiponectin and of obesity and smoking (24). Thus, in the HDL, we did not find an association between absence of morbid obesity, vascular function either adiponectin or free fatty acid levels and remains preserved despite evidence of insulin endothelium-dependent vasodilation across resistance in PCOS.

Potential mediators of endothelial dysfunction in insulin resistance

Hyperglycemia

We tested several established mellitus (31). Other investigators have mediators of vascular dysfunction in insulin reported associations between adiponectin resistant states, including glycemia, and vascular function, but a review of the adipokines, and inflammation, to determine if literature reveals an inconsistent link between one consistently modulated vascular function. adipocyte products and vascular function. We have previously demonstrated that Adiponectin has been correlated to reactive hyperglycemia impairs endothelial function in hyperemia in healthy Japanese subjects (32), healthy humans (25, 26). Endothelium- to nitroglycerin-mediated, endothelium-dependent vasodilation correlates with independent vasodilation but not glycemia in healthy subjects, subjects with endothelium-dependent vasodilation in impaired fasting glucose, and subjects with healthy Spanish subjects (33), and weakly type 2 diabetes (27, 28). Improvements in with endothelium-dependent vasodilation (r < glycemia in subjects with type 2 diabetes 0.3) in a large sample of type 2 diabetic and improve endothelial function (29). Despite healthy controls subjects in Hong Kong (34). this evidence, in our cohort, the Likewise, there was no correlation lipodystrophic subjects had the highest fasting between FFA and endothelium-dependent glucose and yet had normal endothelium-vasodilation in our subjects. However, as dependent vasodilation. An expected trend expected free fatty acid levels were increased was noted in the correlation between glucose in each insulin resistance state compared with levels and endothelium-dependent healthy controls; yet, the extent of insulin
resistance did not correlate with FFA levels. adipokines, and inflammation did not. Also, triglycerides were directly associated with vascular smooth muscle and in HDL was inversely associated with function. Glucose levels correlated inversely with insulin resistance. Similarly, across a wide smooth muscle function. The population of healthy control, type 2 diabetic attenuation in endothelium-independent subjects without microalbuminuria, and type 2 vasodilation may relate to chronic subjects with albuminuria, free fatty acid hyperglycemia as reflected in attenuation of levels did not associate with endothelium-nitroglycerin-mediated vasodilation in dependent vasodilation (35). Moreover, diabetes (44, 45). Despite the attenuation in Ballotshafer and colleagues demonstrated no endothelium-independent vasodilation in the association between ambient free fatty acid subjects with lipodystrophic diabetes, flow-levels and flow-mediated vasodilation in first mediated, endothelium-dependent degree relatives of patients with type 2 vasodilation was preserved. These results diabetes (36). Thus, abnormalities in these suggest that endothelial vasodilator adipocyte factors in insulin resistance do not production was sufficient to maintain independently mediate vascular dysfunction endothelium-dependent vasodilation despite across a range of insulin resistance states. attenuations in vascular smooth muscle function.

Inflammation has been demonstrated to associate with endothelial dysfunction in healthy subjects and patients with coronary function in insulin resistance, HOMA-IR was artery disease (37, 38). Moreover, when used to approximate insulin resistance. This inflammation is induced by vaccination, measure, in large part, reflects the relationship endothelial function is depressed and blocking between insulin production and hepatic the increase in inflammatory cytokines glucose output and is reported to correlate prevents endothelial dysfunction (39, 40). well with dynamic measures of insulin. Despite this relationship between resistance, such as a euglycemic clamp or the inflammation and endothelial function in non-minimal model (46). However, HOMA-IR diabetic populations, several studies have may underestimate the severity of insulin demonstrated that CRP does not correlate resistance in subjects with long-standing type with vascular function in diabetic subjects 2 diabetes because of an inability to make (41-43). Similarly, in our cohort, CRP levels insulin. It is unlikely that insulin resistance did not vary significantly across our insulin the type 2 diabetic subjects would approach resistant populations and did not correlate the same severity in subjects with with endothelial function. CRP, as one lipodystrophy. Lipodystrophic subjects were marker of inflammation, however, may not the most insulin resistant and had preserved represent all inflammatory markers. endothelial function unlinking a direct relationship between the two parameters.

Vascular smooth muscle dysfunction The response to nitroglycerin was In studying these populations, the attenuated in subjects with type 2 and number of subjects with lipodystrophy was lipodystrophic diabetes mellitus. We have small compared to the other groups because previously reported an impaired response to of their rarity in the population. Moreover, nitroglycerin in subjects with type 2 diabetes two of the subjects were taking ACE mellitus (44), but this is the first report of a inhibitors which may improve endothelial similar finding in subjects with lipodystrophic function while 3 had microvascular disease or diabetes. Markers of insulin resistance, atherosclerosis which should worsen it.
Although these study conditions were imperfect, the similarity of vascular function atherosclerosis within subjects with lipodystrophy and between them and healthy control subjects suggests our observations are valid and not likely to be altered if a greater number of lipodystrophic patients were included. 

Age varied significantly among the groups. Several investigations have noted Association (1-06-CD-01). Dr Creager is that older subjects have attenuated endothelial function compared with younger controls (47-50). Despite this difference, in this study, Cardiovascular Medicine at Brigham and 50). Despite this difference, in this study, Women’s Hospital. We thank Dr. David endothelial function remained significantly different among the groups after controlling Hospital for his participation in for age.

**Conclusion**

How, then, to explain our findings? Our measure of insulin resistance is strongly correlated with impairment of insulin-mediated skeletal-muscle glucose uptake. Although there is a link between the skeletal muscle defect and vascular defect in obesity/diabetes, different vascular insulin signaling disturbances are likely operational in the other insulin resistance states. Insulin levels in the lipodystrophic diabetic subjects were markedly higher than every other group, possibly stimulating eNOS enough to overcome the disturbances in vascular smooth muscle function demonstrated in our cohort.

Thus, the effects of insulin resistance on vascular function vary according to origin of impaired insulin signaling. Lipodystrophic and PCOS patients have normal endothelial function, indicative of preserved endothelial insulin signaling, despite impaired glucose handling. Abnormal endothelium-dependent vasodilation in subjects with type 2 diabetes mellitus suggests that attenuated endothelial activation of PI-3 kinase and Akt importantly affects endothelial function in this insulin resistance disorder. Mechanisms of insulin resistance that are associated with an increased risk of atherosclerosis require better characterization to explain the variations in

**Acknowledgements:** This work was supported by grants from the National Institutes of Health (HL-48743 and K23- DK02795) and the American Diabetes groups. Several investigations have noted Association (1-06-CD-01). Dr Creager is that older subjects have attenuated endothelial the Simon C. Fireman Scholar in function compared with younger controls (47- Cardiovascular Medicine at Brigham and 50). Despite this difference, in this study, Women’s Hospital. We thank Dr. David endothelial function remained significantly Nathan of the Massachusetts General different among the groups after controlling Hospital for his participation in for age.

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<td>6</td>
<td>10</td>
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<td>Age (years)</td>
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<td>31±6</td>
<td>56±14 *</td>
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<td>Mean Arterial Pressure (mm Hg)</td>
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<td>BMI (kg/m2)</td>
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<td>30±5</td>
<td>31±7</td>
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<td>Total Cholesterol (mg/dL)</td>
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<td>192±31</td>
<td>178±37</td>
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<td>LDL Cholesterol (mg/dL)</td>
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<td>120±26</td>
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<td>HDL Cholesterol (mg/dL)</td>
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<td>49±14</td>
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<td>Triglycerides (mg/dL)</td>
<td>311±165 †</td>
<td>171±72 *</td>
<td>153±95</td>
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<td>Insulin (uU/ml)</td>
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<td>28±22</td>
<td>14±12</td>
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<td>Glucose (mg/dL)</td>
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<td>88±9</td>
<td>156±68 †</td>
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<td>HOMA-IR</td>
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<td>C Reactive Protein (mg/L)</td>
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Abbreviations: BMI: body mass index; LDL: low density lipoprotein; HDL: high density lipoprotein; HOMA-IR: homeostatic model assessment – insulin resistance. † p < 0.01 * p < 0.05 compared to control subjects.
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Abbreviations: BMI: body mass index; LDL: low density lipoprotein; HDL: high density lipoprotein; FMD: flow-mediated vasodilation; NMD: nitroglycerin-mediated vasodilation
Figure Legend
Figure: Vascular Function. (A) The mean percent increase in brachial artery size 1 minute after cuff release compared with baseline is illustrated. (B) The mean percent increase in brachial artery size 3 minutes after sublingual nitroglycerin administration compared with baseline is illustrated.

A: Flow-Mediated Vasodilation

B: Nitroglycerin-Mediated Vasodilation

ANOVA p = 0.005
* p = 0.02 compared to healthy subjects
† p = 0.09 compared to healthy subjects