Overweight and Obese Humans Demonstrate Increased Vascular Endothelial NAD(P)H Oxidase-p47phox Expression and Evidence of Endothelial Oxidative Stress

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Background—Obesity may alter vascular endothelial cell protein expression (VECPE) of molecules that influence susceptibility to atherosclerosis.

Methods and Results—Quantitative immunofluorescence was performed on vascular endothelial cells collected from 108 men and women free of clinical disease who varied widely in adiposity (body mass index 18.4 to 36.7 kg/m²; total body fat 5.8 to 55.0 kg; waist circumference: 63.0 to 122.9 cm). All 3 expressions of adiposity were positively associated with VECPE of the oxidant enzyme subunit NAD(P)H oxidase-p47phox (part correlation coefficient \( r_{\text{part}} \) 0.22 to 0.24, all \( P<0.05 \)) and the antioxidant enzyme catalase (\( r_{\text{part}} = 0.71 \) to 0.75, all \( P<0.001 \)). Total body fat was positively associated with VECPE of nitrotyrosine (\( r_{\text{part}} = 0.36, P = 0.003 \)), a marker of protein oxidation, and, in men, with Ser1177-phosphorylated endothelial nitric oxide synthase (\( r_{\text{part}} = 0.46, P = 0.02 \)), an activated form of endothelial nitric oxide synthase. Overweight/obese subjects (body mass index \( \geq 25 \) kg/m²) had 35% to 130% higher VECPE of NAD(P)H oxidase-p47phox, nitrotyrosine, catalase, and the cytosolic antioxidant CuZn superoxide dismutase (all \( P<0.05 \)), as well as a 56% greater VECPE of the potent local vasoconstrictor endothelin-1 (\( P = 0.05 \)) than normal-weight subjects (body mass index <25 kg/m²). Nuclear factor-κB protein expression was \( \approx 60\% \) to 100% greater in the most obese adults than in the leanest adults (\( P \leq 0.01 \)). These relations were independent of sex but were selectively reduced after accounting for the influence of plasma C-reactive protein, fasting glucose-insulin metabolism, or serum triglycerides.

Conclusions—Compared with their normal-weight peers, overweight and obese adults demonstrate increased vascular endothelial expression of NAD(P)H oxidase-p47phox and evidence of endothelial oxidative stress, with selective compensatory upregulation of antioxidant enzymes and Ser1177-phosphorylated endothelial nitric oxide synthase. Endothelin-1 and nuclear factor-κB protein expression also appear to be elevated in obese compared with lean adults. These findings may provide novel insight into the molecular mechanisms linking obesity to increased risk of clinical atherosclerotic diseases in humans. (Circulation. 2007;115:&NA;-.)

Key Words: endothelium ▪ obesity ▪ endothelin ▪ free radicals ▪ antioxidants ▪ atherosclerosis

The vascular endothelium plays an essential role in the initiation and progression of atherosclerosis. Vascular endothelial cells produce proteins that influence susceptibility to the development of atherosclerosis. In general, an “antiatherogenic” vascular endothelium expresses proteins that act to enhance nitric oxide (NO) bioavailability, inhibit excessive formation of reactive oxygen species and oxidative stress, suppress inflammation, and restrict local production of vasoconstrictor molecules. A “proatherogenic” vascular endothelium typically expresses the reverse phenotype.

Clinical Perspective p ●●●

Experimental animal obesity and human obesity are associated with the development of atherosclerosis and increased prevalence of clinical atherosclerotic diseases. Increasing total body fat and abdominal fat have been linked to impaired NO-dependent endothelial function, oxidative stress, and increased production of vasoconstrictor proteins such as endothelin-1 (ET-1). These changes may in turn be related to chronic low-grade inflammation, insulin resistance, lipid abnormalities, and increases in circulating free fatty acids.
Currently, however, there is no information in humans concerning the effects of adiposity on vascular endothelial expression of proteins involved in atherogenic processes.

Accordingly, the primary purpose of the present study was to determine the relations between adiposity and vascular endothelial cell expression of selective proteins involved in NO production (endothelial NO synthase [eNOS]; phosphorylated eNOS-Ser1177 [P-eNOS]), oxidant enzyme systems [NAD(P)H oxidase-p47phox; xanthine oxidase], oxidative modification of proteins (nitrotyrosine), antioxidant enzymes (catalase; cytosolic CuZn superoxide dismutase [CuZnSOD]; mitochondrial Mn superoxide dismutase), inflammation (nuclear factor-κB [NF-κB]; cyclooxygenase-2), and vasoconstriction (ET-1) in adult humans. To do so, vascular endothelial cells were obtained from adult men and women varying widely in body mass index (BMI), total fat mass, and waist circumference, and protein expression was measured by quantitative immunofluorescence. A total of 108 adults, 73 men and 35 women, were studied. All subjects had resting blood pressure <140/90 mm Hg and were free of clinical cardiovascular diseases, diabetes mellitus, and other major chronic diseases as assessed by medical history, physical examination, resting ECG, and blood chemistries. Men older than 40 years and women older than 50 years of age were further evaluated with ECG and blood pressure responses to incremental treadmill exercise performed to volitional exhaustion. Subjects were nonsmokers, were not regularly exercising (<90 minutes of aerobic activity per week), and were not taking medications or dietary supplements (eg, antioxidants) that could influence the results. All procedures were approved by the Human Research Committee of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation.

Methods

Subjects
A total of 108 adults, 73 men and 35 women, were studied. All subjects had resting blood pressure <140/90 mm Hg and were free of clinical cardiovascular diseases, diabetes mellitus, and other major chronic diseases as assessed by medical history, physical examination, resting ECG, and blood chemistries. Men older than 40 years and women older than 50 years of age were further evaluated with ECG and blood pressure responses to incremental treadmill exercise performed to volitional exhaustion. Subjects were nonsmokers, were not regularly exercising (<90 minutes of aerobic activity per week), and were not taking medications or dietary supplements (eg, antioxidants) that could influence the results. All procedures were approved by the Human Research Committee of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation.

Study Procedures
All measurements were performed at the University of Colorado at Boulder General Clinical Research Center after an overnight fast.

Body Composition
Body weight, BMI, and waist circumference, an index of abdominal fat, were determined as described previously. Total body fat was estimated with the homeostasis model of insulin resistance by the Diagnostic Systems Laboratory, Webster, Tex. Insulin resistance was estimated with the homeostasis model of insulin resistance by the formula [fasting plasma insulin (μU/mL) × fasting plasma glucose (mmol/L)/25]. The homeostasis model of insulin resistance has been validated against other measures of insulin sensitivity (eg, intravenous glucose tolerance test) as a reliable estimate of insulin sensitivity.

Plasma Markers of Oxidative Stress and Antioxidant Status
Serum oxidized low-density lipoprotein was determined by ELISA (ALPCO Diagnostics, Salem, NH), plasma concentrations of thiobarbituric acid reactive substances were determined by a spectrophotometric method (ZeptoMetrix Corp, Buffalo, NY), and 24-hour

Vascular Endothelial Cell Protein Expression

The procedures used for collection of venous vascular endothelial cells and measurement of VECPE have been described in detail. Briefly, endothelial cells were collected from an antecubital vein with 2 sterile J-wires briefly advanced (~4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter. The wires were transferred to a dissociation buffer solution, and cells were recovered by washing and centrifugation. Cells were fixed with 3.7% formaldehyde and plated on poly-l-lysine–coated slides (Sigma Chemical, St Louis, Mo).

For immunofluorescence staining, cells were rehydrated with PBS and rendered permeable with 0.1% Triton X-100 (Alfa Aesar, Ward Hill, Mass). After nonspecific binding sites were blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, Pa), cells were incubated with monoclonal antibodies for one of the following: eNOS (Transduction Laboratories, San Jose, Calif), P-eNOS (Ser1177, Calbiochem, San Diego, Calif), NAD(P)H oxidase-p47phox (Upstate, Billerica, Mass), xanthine oxidase (United States Biological, Swampscott, Mass), catalase (Abcam, Cambridge, Mass), CuZnSOD (Upstate), Mn superoxide dismutase (Research Diagnostics, Concord, Mass), nitrotyrosine (Abcam), ET-1 (Affinity Bioreagents, Golden, Colo); cyclooxygenase-2 (Cayman Chemicals, Ann Arbor, Mich) and NF-κB (Novus, Littleton, Colo). Cells were next incubated with CY3-conjugated secondary antibodies (Jackson ImmunoResearch or Research Diagnostics).

For analysis, slides were viewed with a fluorescence microscope (Eclipse 600, Nikon, Melville, NY) and were digitally captured by a Photometrics CoolSNAPfNx digital camera (Roper Scientific, Inc, Tucson, Ariz). Endothelial cells were identified by the presence of von Willebrand factor staining, and nuclear integrity was confirmed with DAPI (4′,6-diamidino-2-phenylindole hydrochloride) staining. Once endothelial cells with intact nuclei were identified, images were captured and analyzed with Metamorph Software (Universal Imaging Corp, Downingtown, Pa) to quantify the intensity of CY3 staining (ie, average pixel intensity), hence quantifying VECPE. The software program allowed for systematic quantification of staining intensity and eliminated human subjectivity in analysis. Values are reported as ratios of VECPE/human umbilical vein endothelial cell (control cells) VECPE. The reporting of ratios minimizes day-to-day variability resulting from differences in intensity of staining between different staining sessions. Technicians were blinded to subject identity during the staining and analysis procedures. Although slides were not randomly assigned to a staining batch, they were selected so that each batch included subjects with a wide range of adiposity.

The present technique of quantitative immunofluorescence of human endothelial cells was originally validated against immunoblotting by Colombo and colleagues, with an excellent correlation coefficient (r = 0.99), and more recently, by our laboratory, with a similarly impressive correlation coefficient (r = 0.97, P = 0.007; see online Data Supplement).

Plasma Markers of Inflammation, Glucose–Insulin Metabolism, and Lipids
Lipid profiles were determined with the ACE chemistry system (Alfa Wassermann Diagnostic Technologies, West Caldwell, NJ). Serum total cholesterol and triglycerides were analyzed by conventional enzymatic methods. High-density lipoprotein cholesterol was determined with a homogenous 2-reagent method, and low-density lipoprotein cholesterol was calculated with the formula LDL = TC – HDL – (TG/5), in which LDL indicates low-density lipoprotein; TC, total cholesterol; HDL, high-density lipoprotein; and TG, triglycerides. Serum free fatty acids (Waco Chemicals, Neuss, Germany) and high-sensitivity C-reactive protein (Roche Diagnostic Systems, Basel, Switzerland) were measured by enzymatic methods.

Fasting plasma glucose was measured by enzymatic methods (Roche Diagnostic Systems) and plasma insulin by radioimmunoassay (Diagnostic Systems Laboratory, Webster, Tex). Insulin resistance was estimated with the homeostasis model of insulin resistance by the formula [fasting plasma insulin (μU/mL) × fasting plasma glucose (mmol/L)/25]. The homeostasis model of insulin resistance has been validated against other measures of insulin sensitivity (eg, intravenous glucose tolerance test) as a reliable estimate of insulin sensitivity.

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urinary isoprostanes were measured by ELISA (Oxford Biomedical Research, Oxford, Mich). Plasma total antioxidant status and superoxide dismutase-1 were measured with the Cobas Mira Plus Chemistry Analyzer, and glutathione peroxidase was determined with the Mira Method (all Randox Laboratories, Crumlin, Colo).

**Data Analysis**

Statistical analyses were performed with SPSS (version 11.0.3). To determine the relations of total and abdominal adiposity with VECPE independent of sex, multiple linear regression analysis was performed. Separate regression models were used for each measure of VECPE and each measure of adiposity (BMI, total body fat mass, and waist circumference). VECPE was entered as the dependent variable, whereas sex and an expression of adiposity were entered as independent variables. Sex was coded as 0 for men and 1 for women. Part (also known as semipartial) correlation coefficients derived from regression analysis were used to determine the association of adiposity with VECPE, independent of sex. Residual analyses to test the validity of the regression model assumptions were performed for all regression models, and violations were remedied by data transformation. Pearson product-moment correlation coefficients were used to determine bivariate relations in men and women separately. Similar regression analyses were performed to assess the relations between markers of oxidative stress and antioxidant status and the measures of adiposity. Pearson product-moment correlation coefficients were used to determine bivariate relations between markers of oxidative stress and antioxidant status and VECPE.

To further assess associations between adiposity and VECPE, we compared VECPE in a subsample of overweight/obese (BMI >25 kg/m²) and normal-weight (BMI <25 kg/m²) men and women using t tests for independent group comparisons. The groups were composed of subjects from the overall study sample in whom most or all of the proteins were assessed. Further subgroup analyses were performed to compare VECPE between the most obese (highest quartile of BMI) and leanest (lowest quartile of BMI) men and women, as well as between individuals in the highest and lowest quartiles of waist circumference.

**TABLE 1. Subject Characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SE</th>
<th>Minimum–Maximum</th>
</tr>
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<tbody>
<tr>
<td>Sex, m/f</td>
<td>73±35</td>
<td>...</td>
</tr>
<tr>
<td>Age, y</td>
<td>40±2</td>
<td>18–71</td>
</tr>
<tr>
<td>Height, cm</td>
<td>175±1</td>
<td>156–194</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>78.6±1.4</td>
<td>49.7–124.6</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.7±0.4</td>
<td>18.4–36.7</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>28.7±1.0</td>
<td>8.4–52.3</td>
</tr>
<tr>
<td>Body fat, kg</td>
<td>23.1±1.0</td>
<td>5.8–55.0</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>86.2±1.3</td>
<td>63.0–122.9</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.76±0.10</td>
<td>3.32–7.69</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.91±0.08</td>
<td>1.50–5.70</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.25±0.03</td>
<td>0.65–2.56</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.90±0.13</td>
<td>1.22–8.62</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.20±0.23</td>
<td>0.20–8.39</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.04±0.07</td>
<td>3.55–6.72</td>
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<tr>
<td>Insulin, pmol/L</td>
<td>49.13±4.06</td>
<td>6.95–145.85</td>
</tr>
<tr>
<td>Homeostasis model of insulin resistance</td>
<td>3.67±0.34</td>
<td>0.36–10.73</td>
</tr>
<tr>
<td>Free fatty acids, μEq/L</td>
<td>618±38</td>
<td>237–1179</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>115±1</td>
<td>85–139</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>68±1</td>
<td>49–88</td>
</tr>
</tbody>
</table>

Data are mean±SE. m/f indicates male/female. LDL indicates low-density lipoprotein; HDL, high-density lipoprotein; and CRP, C-reactive protein.

To assess inflammation (C-reactive protein), impaired glucose metabolism (fasting glucose, fasting insulin, and the homeostasis model of insulin resistance), resting blood pressure, and blood lipids (total cholesterol, low-density lipoprotein, high-density lipoprotein, triglycerides, and free fatty acids) as potential intermediary mechanisms by which adiposity influences VECPE, multiple linear regression analyses were performed as described above. Each factor was entered as an independent variable in the model, in addition to adiposity and sex. Using this approach, we determined how the relation between adiposity and VECPE was changed by accounting for these factors in addition to sex. Statistical significance for all analyses was set at P<0.05. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Regression Analysis on the Entire Cohort**

Mean values and ranges for sex composition, age, anthropometry, and body composition of subjects in the entire cohort are presented in Table 1. Subjects varied widely in BMI, total body fat, and waist circumference. Subject information, mean values, and ranges for each VECPE are shown in the online Data Supplement, Table I.

All 3 measures of adiposity were positively associated with VECPE of NAD(P)H oxidase-p47phox and catalase (Figures 1, A and B). In addition, a negative correlation was observed between VECPE and CRP, and a positive correlation was noted between VECPE and HDL cholesterol (Figure 1, C). To further assess associations between adiposity and VECPE, we compared VECPE in a subsample of overweight/obese (BMI >25 kg/m²) and normal-weight (BMI <25 kg/m²) men and women using t tests for independent group comparisons. The groups were composed of subjects from the overall study sample in whom most or all of the proteins were assessed. Further subgroup analyses were performed to compare VECPE between the most obese (highest quartile of BMI) and leanest (lowest quartile of BMI) men and women, as well as between individuals in the highest and lowest quartiles of waist circumference. To assess inflammation (C-reactive protein), impaired glucose metabolism (fasting glucose, fasting insulin, and the homeostasis model of insulin resistance), resting blood pressure, and blood lipids (total cholesterol, low-density lipoprotein, high-density lipoprotein, triglycerides, and free fatty acids) as potential intermediary mechanisms by which adiposity influences VECPE, multiple linear regression analyses were performed as described above. Each factor was entered as an independent variable in the model, in addition to adiposity and sex. Using this approach, we determined how the relation between adiposity and VECPE was changed by accounting for these factors in addition to sex. Statistical significance for all analyses was set at P<0.05. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
2A and 2B, and 3; all \( P < 0.05 \). Total body fat was positively associated with nitrotyrosine VECPE (Figure 2C, \( P = 0.003 \)). The relation between total body fat and P-eNOS VECPE differed in men and women (Figure 2D; \( P = 0.04 \) for interaction): There was a positive association in men (\( r = 0.46, P = 0.02 \)) but not in women (\( P = 0.8 \)). There were no other significant relations between BMI, total body fat, or waist circumference and VECPE. Results were similar if total percent body fat was used instead of total body fat mass.

**Group Comparisons of Overweight/Obese and Normal-Weight Adults**

Mean values and ranges for sex composition, age, anthropometry, and body composition of the subjects in the overweight/obese and normal-weight groups are presented in Table 2. All measures of adiposity were greater in the overweight/obese subjects than in the normal-weight subjects (\( P < 0.0001 \)). Subject information, mean values, and ranges for each VECPE in each group are shown in the online Data Supplement, Table II. Although mean age was higher in the overweight/obese group, age was not related to expression of any protein; thus, no correction of the VECPE values for age was necessary.

Consistent with the results of the regression analyses performed on the entire subject cohort, VECPE of NAD(P)H oxidase-p47\textsuperscript{phox}, catalase, nitrotyrosine, and P-eNOS were greater (35% to 130%) in the overweight/obese subjects than in the normal-weight subjects (Figure 4A through 4D; \( P = 0.03 \)). In addition, VECPE of CuZnSOD was \( \approx 40\% \) higher (\( P = 0.04 \); Figure 4E) and VECPE of ET-1 was 56\% higher (\( P = 0.05 \); Figure 4F) in the overweight/obese group compared with normal-weight subjects.

When the most obese subjects were compared to the leanest individuals (i.e., the highest and lowest quartiles of BMI), the group differences in VECPE of catalase, NAD(P)H oxidase-p47\textsuperscript{phox}, nitrotyrosine, CuZnSOD, ET-1, and P-eNOS generally were comparable to the group differences between the overweight/obese and normal-weight individuals indicated above, although they were not always statistically significant because of smaller group numbers (online Data Supplement, Table III). The most notable difference was that...
VECPE of NF-κB was ~60% greater in the most obese group than in the leanest group (0.45 ± 0.06 [n = 10] versus 0.28 ± 0.04 [n = 9] NF-κB/human umbilical vein endothelial cell intensity, P = 0.01).

Similarly, group differences in VECPE of catalase, NAD(P)H oxidase-p47phox, nitrotyrosine, CuZnSOD, ET-1, and P-eNOS between the individuals in the highest and lowest waist circumference quartiles generally were comparable to the group differences between the overweight/obese and normal-weight individuals (online Data Supplement, Table IV). The only notable difference was that VECPE of NF-κB was ~100% greater (0.55 ± 0.09 [n = 9] versus 0.27 ± 0.03 [n = 9] NF-κB/human umbilical vein endothelial cell intensity, P = 0.009) in the group with the highest versus the lowest waist circumference.

### Table 2. Characteristics of a Subsample of Subjects With Lower and Higher Adiposity

<table>
<thead>
<tr>
<th></th>
<th>Normal Weight</th>
<th>Overweight/Obese</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, m/f</td>
<td>23/16</td>
<td>29/13</td>
<td>...</td>
</tr>
<tr>
<td>Age, y</td>
<td>34 ± 3</td>
<td>42 ± 3</td>
<td>0.04</td>
</tr>
<tr>
<td>Height, cm</td>
<td>174 ± 1</td>
<td>174 ± 1</td>
<td>0.8</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>67.0 ± 1.5</td>
<td>88.1 ± 1.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.0 ± 0.3</td>
<td>29.2 ± 0.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>23.4 ± 1.6</td>
<td>33.1 ± 1.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Body fat, kg</td>
<td>15.5 ± 1.1</td>
<td>29.3 ± 1.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>74.6 ± 1.1</td>
<td>94.9 ± 1.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.57 ± 0.19</td>
<td>4.84 ± 0.12</td>
<td>0.2</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.75 ± 0.16</td>
<td>3.02 ± 0.11</td>
<td>0.2</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.33 ± 0.05</td>
<td>1.15 ± 0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.32 ± 0.14</td>
<td>3.35 ± 0.24</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>0.99 ± 0.24</td>
<td>1.41 ± 0.45</td>
<td>0.4</td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>4.97 ± 0.12</td>
<td>5.15 ± 0.12</td>
<td>0.3</td>
</tr>
<tr>
<td>Plasma insulin, mmol/L</td>
<td>37.43 ± 5.67</td>
<td>57.21 ± 7.16</td>
<td>0.04</td>
</tr>
<tr>
<td>Homeostasis model of insulin resistance</td>
<td>2.78 ± 0.49</td>
<td>4.30 ± 0.58</td>
<td>0.05</td>
</tr>
<tr>
<td>Plasma free fatty acids, μEq/L</td>
<td>567 ± 51</td>
<td>661 ± 66</td>
<td>0.3</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>109 ± 2</td>
<td>118 ± 2</td>
<td>0.003</td>
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<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>62 ± 2</td>
<td>70 ± 2</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Data are mean ± SE. m/f indicates male/female. LDL indicates low-density lipoprotein; HDL, high-density lipoprotein; and CRP, C-reactive protein.

### Influence of Inflammation, Glucose-Insulin Metabolism, Lipids, and Blood Pressure

The relations of BMI, total body fat, and waist circumference with VECPE of NAD(P)H oxidase-p47phox were reduced by 46% to 65% after we accounted for the influence of serum triglycerides (Figure 5). There were no other significant influences of plasma lipids, blood pressure, markers of inflammation, or glucose-insulin metabolism on the relations between adiposity and VECPE of NAD(P)H oxidase-p47phox.

The relations of BMI, total body fat, and waist circumference with VECPE of catalase were reduced by 32% to 44% after we accounted for the influence of C-reactive protein, fasting insulin, or the homeostasis model of insulin resistance (Figure 6). Accounting for plasma lipids, resting blood pressure, or fasting glucose alone did not significantly influence the relations between adiposity and VECPE of catalase. The relations between adiposity and VECPE of P-eNOS and nitrotyrosine were not significantly affected after we accounted for each of these factors.

### Relations Between Systemic Markers of Oxidative Stress and Antioxidant Status, Adiposity, and VECPE

Systemic markers of oxidative stress and antioxidant status were not consistently related to the measures of adiposity, nor did they differ between normal-weight and overweight/obese subgroups (online Data Supplement, Table V). Plasma total antioxidant status was positively related to VECPE of CuZnSOD (r = 0.48, P = 0.01), however, and plasma superoxide dismutase-1 was positively related to VECPE of catalase (r = 0.77, P = 0.01).

### Discussion

The primary purpose of the present study was to identify relations between total body and/or abdominal fat and the expression of specific putative atherogenic risk-modulating proteins in the vascular endothelium of adult humans. The key novel findings were that increased adiposity is associated with vascular endothelial oxidative stress, increased expression of NAD(P)H oxidase-p47phox, and upregulation of selective antioxidant enzymes. In addition, endothelial ET-1 and NF-κB protein expression appeared be elevated in obese compared with lean adults. Our findings also suggest that inflammation, insulin resistance, and plasma triglycerides may be among the intermediary mechanisms linking elevated adiposity to altered VECPE. To the best of our knowledge, these are the first observations concerning the effects of...
human obesity on the expression of atherosclerosis-associated proteins in the vascular endothelium.

Adiposity and Vascular Endothelial Expression of Oxidative Stress–Related Proteins

Oxidative stress represents increased production and bioavailability of reactive oxygen species such as superoxide (O$_2^-$) relative to antioxidant defenses and is believed to be a major contributor to the development of atherosclerosis.$^{1,18}$ Obese individuals are at higher risk of clinical atherosclerotic diseases,$^4$ and this is postulated to be mediated in part by oxidative stress.$^{19}$ There also is evidence that oxidative stress impairs vascular endothelial function in obese humans.$^{20}$

NAD(P)H oxidase, the major source of O$_2^-$ in the vasculature, is a multicomponent protein that is strongly implicated in the development of atherosclerosis.$^{18,21}$ NAD(P)H oxidase-p47$^{	ext{phox}}$ is a key cytosolic accessory protein that associates with membrane-bound NAD(P)H oxidase catalytic subunits (eg, p22$^{	ext{phox}}$) to stimulate vascular O$_2^-$ production.$^{21}$ In the present study, BMI, total body fat, and waist circumference all were positively associated with VECPE of NAD(P)H oxidase-p47$^{	ext{phox}}$ in the overall cohort. Moreover, NAD(P)H oxidase-p47$^{	ext{phox}}$ VECPE was ~40% greater in the overweight/obese compared with the normal-weight subjects. In contrast to NAD(P)H oxidase-p47$^{	ext{phox}}$, VECPE of xanthine oxidase, an enzyme that produces equimolar amounts of O$_2^-$ and hydrogen peroxide (H$_2$O$_2$),$^{22}$ was not related to body fatness. Together, these results support the concept that vascular endothelial expression of NAD(P)H oxidase-p47$^{	ext{phox}}$, but not xanthine oxidase, is upregulated in overweight/obese adult humans.

One consequence of oxidative stress is the modification of biomolecules, including proteins, lipids, and DNA.$^{23}$ One such modification of proteins involves the nitration of tyrosine residues.$^{24}$ Thus, elevated nitrotyrosine levels are interpreted as an indicator of vascular oxidative stress.$^{25}$ Nitrotyrosine content is increased in the thoracic aorta of obese rats$^{26}$; however, it is unknown whether this is observed in human obesity. In the present investigation, VECPE of nitrotyrosine was positively associated with total body fat in the entire group. In addition, nitrotyrosine expression was 35% higher in the overweight/obese than in the normal-weight subjects. These observations are consistent with the idea that increased total body fat is associated with oxidative stress in the vascular endothelium in humans.
In the present study, VECPE of catalase, an antioxidant enzyme that catalyzes the conversion of H$_2$O$_2$ to water, was positively associated with all 3 expressions of adiposity. Indeed, the relations between VECPE of catalase and measures of total and abdominal fat ($r_p$ = 0.71 to 0.75) were by far the strongest relations observed. Moreover, VECPE of catalase was ≈130% greater in overweight/obese than in normal-weight subjects. Additionally, VECPE of CuZnSOD, the SOD isoform that converts O$_2^-$ to H$_2$O$_2$ in the cytosol, was ≈40% higher in the overweight/obese group, although no differences were observed in the mitochondrial isoform (Mn superoxide dismutase). Given the increases in NAD(P)H oxidase-p47phox and nitrotyrosine observed with increasing adiposity, one explanation for the present findings is that the greater VECPE of catalase and CuZnSOD in the overweight/obese subjects represents a compensatory adaptation to oxidative stress. Indeed, reactive oxygen species induce expression of antioxidant enzymes in vascular endothelial cells in vitro. Furthermore, endothelial expression of antioxidants is increased in vivo in response to oxidative stress under both normal physiological conditions and until the late stages of atherosclerosis. It is not clear why, in the present study, Mn superoxide dismutase VECPE would not be similarly elevated. One possibility is that elevated body fatness does not cause increased mitochondrial O$_2^-$ production, thus negating the need for augmented expression of this antioxidant enzyme.
It is noteworthy that increased adiposity was associated with increased VECPE of nitrotyrosine, despite selective upregulation of enzymatic antioxidants. It is possible that the increased production of oxidants was greater than the corresponding increased expression of these antioxidants, thus allowing the development of oxidative damage. Alternatively, increased VECPE of catalase and CuZnSOD may not have been associated with increased activity of these antioxidant enzymes.

Finally, we did not observe consistent associations between several conventional systemic markers of oxidative stress/antioxidant status and adiposity in the present study. One possible explanation is that systemic oxidative stress was not present and systemic antioxidants were not upregulated in the otherwise healthy overweight and obese subjects in the present study, despite clear evidence for the presence of endothelial oxidative stress and selective increases in endothelial antioxidant enzymes. An alternative explanation is that the present overweight/obese subjects did have mild to moderate systemic oxidative stress but that the measures used simply were not sensitive enough to depict small differences among healthy adults differing in adiposity.

**Adiposity and Vascular Endothelial Expression of Proteins Involved in NO Production**

Although eNOS protein expression is downregulated in advanced atherosclerosis, eNOS expression is increased in pathophysiological states associated with oxidative stress. The latter is thought to be a compensatory attempt to increase NO production and maintain its bioavailability in the presence of increased vascular \( O_2^- \) levels. Increased eNOS expression has been observed in thoracic aortic homogenates and in small coronary arteries of obese rats. In the present study, however, VECPE of eNOS was not related to adiposity in the entire cohort, and mean values were not different in the overweight/obese compared with normal-weight subjects.

In addition to influencing enzyme concentrations, however, reactive oxygen species can modulate NO production by affecting eNOS activation. For example, \( H_2O_2 \) can activate the enzyme by phosphorylating eNOS at Ser1177. In the present investigation, VECPE of P-eNOS was positively associated with total body fat in men and was 54% greater in the overweight/obese group than in the normal-weight subjects. Taken together, the present results indicate that VECPE of eNOS is not increased in humans with elevated total body fat but that P-eNOS VECPE is greater in overweight and obese men. It is unclear why elevated adiposity was not associated with increased P-eNOS VECPE in women. Differences in sex hormones and/or body fat distribution may contribute to sex-related differences in VECPE. Estrogens exert a strong modulatory influence on the vascular endothelium, and thus, differences in estrogens between the male and female subjects or within the females may have added variability to the VECPE–body fat relations. Females generally demonstrate less abdominal, but greater lower-body adiposity than males. It is possible that this influences circulating factors (eg, other hormones and cytokines) that interact with the endothelium and modulate gene and protein expression. Additional investigations will be required to further elucidate the influence of sex on VECPE and to determine the precise mechanisms by which sex modulates VECPE.

**Adiposity and Vascular Endothelial Expression of ET-1 and Inflammatory Proteins**

ET-1 is a potent vasoconstrictor factor produced by the vascular endothelium and is implicated in processes involved in the development of endothelial dysfunction and atherosclerosis. Plasma concentrations of ET-1 are elevated in obese compared with normal-weight humans and are reduced with weight loss. Furthermore, tonic ET-1 vasoconstriction is increased in obese adults. However, ET-1 protein content is not different in aortas of obese compared with lean mice. In the present investigation, there was no significant relation between adiposity and VECPE of ET-1 in the overall cohort, but ET-1 expression was 54% higher in the present sub-sample of overweight/obese compared with normal-weight subjects. These findings suggest that ET-1 protein expression may be increased in the vascular endothelium of obese humans and that additional work is warranted on this issue.

The important role of inflammation in atherogenesis is widely accepted, and inflammation is considered an important mechanism by which obesity promotes atherosclerosis. Cyclooxygenase-2 is an inducible enzyme that produces eicosanoids believed to promote atherosclerosis, whereas NF-κB is a major transcription factor involved in activation of proinflammatory genes. In the present study, adiposity was not associated with the expression of cyclooxygenase-2. There was, however, a nonsignificant trend for NF-κB VECPE to be greater in the overweight/obese group than in the normal-weight group. Moreover, NF-κB VECPE was significantly (≈60% to 100%) greater in the subjects in the highest versus the lowest quartiles of BMI and waist circumference. As such, the possibility that NF-κB VECPE may be upregulated in human obesity requires further study.

**Associations With Total Body and Abdominal Fat**

Total body fatness and abdominal adiposity are strongly associated with endothelial dysfunction and risk of clinical atherosclerotic diseases. In the present study, dual x-ray absorptiometry–determined total fat mass was more consistently related to VECPE than either BMI or waist circumference. However, more direct measurements of abdominal adiposity (ie, with computed tomography or magnetic resonance imaging) will be necessary to determine the relations between endothelial expression of these proteins and total, subcutaneous, and visceral abdominal fat.

**Role of Inflammation, Glucose-Insulin Metabolism, Lipids, and Blood Pressure in Adiposity-Related Differences in VECPE**

Obesity likely affects the vascular endothelium via multiple mechanisms, including lipid abnormalities, insulin resistance, hypertension, and increases in inflammation. The results of the present study provide initial insight to support the possibility that elevated triglycerides, insulin resistance, and inflammation may be among the mechanisms that contribute...
to associations between adiposity and VECPE of NADPH oxidase-p47<sup>phox</sup> and catalase. Increased plasma triglycerides, insulin resistance, inflammation, and blood pressure are associated with increased atherosclerotic risk. Although insight into the mechanisms by which insulin resistance and particularly inflammation contribute to atherosclerosis is emerging, the mechanisms by which triglycerides influence the atherosclerotic process in humans are not well understood. Recent in vitro data suggest that triglycerides modify endothelial cell expression of atherosclerotic genes; the associations between plasma triglycerides and endothelial cell expression in humans in vivo have not been examined previously, however. The present data provide preliminary insight into a novel mechanism by which triglycerides could affect cardiovascular risk, namely, via altered endothelial expression of a key vascular oxidant enzyme system [ie, NAD(P)H oxidase-p47<sup>phox</sup>]. None of the factors we examined helped explain the relation of obesity with VECPE of nitrotyrosine and P-eNOS. Resting blood pressure, at least within the normal range of the healthy subjects assessed in the present investigation, was not associated with any of the relations between adiposity and VECPE. Future studies will need to determine whether other factors associated with obesity, such as changes in adipokines and renin-angiotensin-aldosterone activity, may be mechanisms linking adiposity with altered VECPE.

**Study Limitations**

We recognize that we studied a limited number of endothelial proteins and that future studies are needed to establish relations between adiposity and expression of proteins involved in other aspects of atherosclerosis, such as cell adhesion, monocyte chemotaxis, thrombosis, and vascular smooth muscle proliferation. Additional studies also will be required to determine the transcriptional, translational, and/or posttranslational mechanisms underlying adiposity-associated differences in endothelial protein expression, as well as their functional consequences.

The measurements of VECPE in the present study were performed on cells obtained from veins as opposed to arteries; the latter would be optimal given that atherosclerosis is an arterial disease. However, the invasiveness of arterial cell collections is limiting in this regard. We believe that our measurements of VECPE in venous cells are valid for several reasons. First, although absolute expression of certain proteins (eg, eNOS) may differ in arteries and veins, the expression of other proteins (eg, nitrotyrosine) appears to be similar. Second, in our laboratory, we find a highly significant positive relation between expression of proteins measured in cells obtained from venous compared with arterial samples collected from subjects on the same day (mean r=0.71, Silver et al, unpublished results). This suggests that although absolute VECPE may differ between arterial and venous cells, group differences or relations between adiposity and VECPE should be observable with venous cells.

In the present study, we measured the expression of NAD(P)H oxidase-p47<sup>phox</sup> but not its phosphorylation status. Although NAD(P)H oxidase-p47<sup>phox</sup> phosphorylation is required for NAD(P)H oxidase activation, the absolute expression of NAD(P)H oxidase-p47<sup>phox</sup> likely influences superoxide production as well. Further studies will be required to identify the influence of adiposity on NAD(P)H oxidase-p47<sup>phox</sup> phosphorylation status.

Finally, we recognize the semiquantitative nature of the immunofluorescence technique; however, quantitative immunofluorescence has been validated against immunoblotting by our laboratory and others (supplemental materials and Colomb et al). Moreover, all techniques for measuring protein expression are semiquantitative. Despite this limitation, we were able to demonstrate significant relations between measures of adiposity and VECPE. Indeed, it is likely that the strength of these associations is underestimated and that some associations may not have been detected owing to the semiquantitative nature of the technique. Intervention studies in which total body and abdominal fat are increased with overfeeding or reduced with caloric restriction may prove more sensitive for establishing the role of adiposity in the regulation of VECPE. Future investigations also are needed to determine the influence of adiposity on gene expression of endothelial-derived factors.

**Conclusions**

The present study provides the first information concerning relations between adiposity and expression of atherosclerosis-linked proteins in the vascular endothelium of humans. Our results indicate that elevated body fatness is associated with increased vascular endothelial NAD(P)H oxidase protein expression and evidence of endothelial oxidative stress. ET-1 and NF-κB protein expression also appear to be elevated in obese compared with lean adults. Antioxidant enzymes, particularly catalase, are selectively upregulated, as is P-eNOS, possibly as compensatory responses to oxidative stress.

The present findings also suggest that elevated triglycerides, insulin resistance, and inflammation may be intermediary mechanisms by which adiposity exerts its influence on VECPE. These observations may provide novel insight into the molecular mechanisms underlying the association between overweight/obesity and increased risk of clinical atherosclerotic diseases in humans.

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**Disclosures**

None.
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CLINICAL PERSPECTIVE

Cardiovascular disease risk is increased in overweight and obese humans compared with their normal-weight peers, but little is known about the molecular mechanisms involved. Vascular endothelial expression of proteins involved in nitric oxide production, the formation of reactive oxygen species and oxidative stress, inflammation, and local vasoconstriction are believed to play an important role in determining the risk of vascular disease. In the present study, we sought to determine whether endothelial expression of such proteins was related to total or abdominal body fatness in adult humans without clinical disease. Endothelial cells were collected from antecubital veins, and protein expression was measured with quantitative immunofluorescence. We found that compared with normal-weight adults, overweight and obese individuals demonstrated increased endothelial expression of (1) NAD(P)H oxidase, an enzyme that produces superoxide anions and is implicated in states associated with vascular oxidative stress; (2) nitrotyrosine, a protein marker of oxidative stress; (3) endothelin-1, an endothelium-derived protein that promotes vasoconstriction; and (4) nuclear factor-κB, a factor that promotes oxidant and inflammatory gene transcription. There also were apparently compensatory increases in expression of an activated form of endothelial nitric oxide synthase and the antioxidant enzymes catalase and CuZn superoxide dismutase. We found that elevated triglycerides, insulin resistance, and inflammation may be among the mechanisms that contribute to associations between adiposity and endothelial cell protein expression. These findings indicate that overweight and obese adult humans demonstrate altered vascular endothelial expression of proteins that may explain, in part, their increased risk of cardiovascular disease.
Overweight and Obese Humans Demonstrate Increased Vascular Endothelial NAD(P)H Oxidase-47phox Expression and Evidence of Endothelial Oxidative Stress
Annemarie E. Silver, Stacy D. Beske, Demetra D. Christou, Anthony J. Donato, Kerrie L. Moreau, Iratxe Eskurza, Phillip E. Gates and Douglas R. Seals

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