Endothelial Dysfunction in Men With Small LDL Particles

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Background—It is unknown whether LDL particle size is, independent of other lipids and lipoproteins, associated with endothelial dysfunction in vivo.

Methods and Results—We determined in vivo endothelial function in 34 healthy men by measuring forearm blood flow responses to intrabrachial artery infusions of acetylcholine (ACh, an endothelium-dependent vasodilator) and sodium nitroprusside (an endothelium-independent vasodilator). LDL peak particle size was measured with gradient gel electrophoresis. Men with small LDL particles (LDL diameter ≤25.5 nm, n = 10) had a 39% lower blood flow response to ACh than men with large LDL particles (LDL diameter >25.5 nm, n = 24, blood flow 6.9 ± 3.6 versus 11.4 ± 5.1 mL/dL·min, P = 0.006). The groups had comparable LDL cholesterol concentrations (3.9 ± 0.6 versus 3.7 ± 1.0 mmol/L, men with small versus large LDL particles), blood pressure, glucose concentrations, and body mass indexes. LDL size (r = 0.45, P = 0.01) but not HDL cholesterol (r = 0.31, P = 0.09) or triglycerides (r = −0.19, P = 0.30) was significantly correlated with endothelium-dependent vasodilation. Serum triglyceride concentrations and LDL size were inversely correlated (r = −0.44, P = 0.01). In multivariate regression analysis, LDL size was the only significant determinant of the ACh-induced increase in blood flow. Sodium nitroprusside–stimulated endothelium-independent vasodilation was similar in both groups.

Conclusions—Small LDL particles are associated with impaired in vivo endothelial function independent of HDL and LDL cholesterol and triglyceride concentrations. LDL size may therefore mediate adverse effects of hypertriglyceridemia on vascular function. (Circulation. 2000;102:716-721.)

Key Words: lipoproteins • atherosclerosis • vasodilation • blood flow • nitric oxide

Low-density lipoprotein particles are heterogeneous with respect to size, density, and lipid composition. According to several cross-sectional and prospective epidemiological studies, individuals with small, dense LDL particles have a higher risk for coronary artery disease (CAD) than subjects with large, buoyant LDL particles. Possible mechanisms mediating this increased atherogenicity of small LDL particles include increased oxidation, decreased binding to LDL receptor, and increased binding of small LDL to arterial wall. Small LDL particle size also associates with other atherogenic changes in lipids and lipoproteins, especially elevated serum triglyceride and decreased HDL cholesterol concentrations, and insulin resistance.

Because of its easy accessibility, the forearm vascular bed has been used as a surrogate for studying atherosclerosis. In vivo endothelial function is often assessed by comparing blood flow responses to agents such as acetylcholine (ACh), which stimulates endothelial nitric oxide production from l-arginine, and direct nitric oxide donors such as sodium nitroprusside (SNP), which causes smooth muscle relaxation and vasodilation independent of endothelium. Endothelium-dependent vasodilation is diminished in atherosclerotic coronary arteries and characterizes individuals who are at high risk of developing atherosclerosis, as such as those with hypercholesterolemia. Although increased LDL cholesterol concentrations have consistently been associated with endothelial dysfunction, it is controversial whether individuals with hypertriglyceridemia and normal or low LDL cholesterol concentrations have impaired endothelial function. Two studies found endothelial function to be normal and 1 study abnormal in hypertriglyceridemic subjects. The latter study compared obese hypertriglyceridemic subjects with normal-weight normotriglyceridemic subjects, and it remained unclear whether obesity or hypertriglyceridemia explained endothelial dysfunction. The impact of LDL particle size on endothelial function has not been analyzed in previous studies. Although serum triglycerides are an important determinant of LDL size, it is also regulated by other factors such as the activities of hepatic lipase and cholesteryl-ester transfer protein. Since LDL size may mediate the atherogenicity of triglycerides, it seems possible that small LDL particle size rather than triglycerides is associated with endothelial dys-
function. In the present study, we determined whether small LDL particles are independent of other cardiovascular risk factors associated with endothelial dysfunction in forearm resistance vessels in healthy men without clinical evidence of atherosclerosis.

Methods

Subjects

Thirty-four healthy men were recruited for the study. All subjects were nonsmokers and did not use medications affecting glucose or lipid metabolism or endothelial function. Subjects with diabetes or overt hypertension (blood pressure >160/90 mm Hg) were excluded. The subjects underwent a complete history, physical examination, and laboratory tests including an ECG to exclude diabetes, renal, hepatic, and hematological disease and clinically significant CAD. Subjects were classified into 2 groups on the basis of LDL peak particle size, with 25.5 nm used as the cutoff diameter, as suggested by Austin et al.2,3 The purpose, nature, and potential risks of the studies were explained to the subjects before their informed consent was obtained. The experimental protocol was designed and performed according to the principles of Helsinki Declaration and was approved by the ethical committee of the Helsinki University Central Hospital.

In Vivo Endothelial Function Test

In vivo endothelial function was determined by measurement of forearm blood flow responses to intraarterial infusions of endothelium-dependent and endothelium-independent vasodilators. The study was begun after a 10- to 12-hour fast at 8 AM. Venous blood samples were withdrawn for measurement of plasma glucose and serum insulin concentrations and for the other laboratory analyses. A 27-gauge unmounted steel cannula (Coopers Needle Works), connected to an epidural catheter (Portex, Hythe), was inserted into the left brachial artery. Drugs were infused at a constant rate of 1 mL/min with an infusion pump (Braun AG). Subjects rested supine in a quiet environment for 30 minutes after needle placement before blood flow measurements. Normal saline was first infused for 18 minutes. Drugs were then infused in the following sequence: SNP (Roche), 3 (low dose) and 10 (high dose) µg/min, and ACh (Iolab Corp), 7.5 (low dose) and 15 (high dose) µg/min. Each dose was infused for 6 minutes, and the infusions of SNP and ACh were separated by infusion of saline for 18 minutes, during which time blood flow returned to basal values. Forearm blood flow was recorded for 10 seconds at 15-second intervals during the last 3 minutes of each drug and saline infusion period with mercury-in-rubber strain-gauge venous occlusion plethysmography (EC 4 Strain Gauge Plethysmograph, Hokanson) combined with a rapid cuff inflator (E 20, Hokanson), an analog-to-digital converter (McLab/4e, AD Instruments Pty Ltd), and a personal computer, as previously described.25 The measurement was performed simultaneously in the infused (experimental) and uncanculated control arm. Blood flow in the control arm remained unchanged during the entire study. The mean of the final 5 measurements of each recording period was used for analysis.

Insulin Sensitivity

Whole-body insulin sensitivity of glucose uptake was measured by the euglycemic hyperinsulinemic clamp technique.26 The study began at 8 AM after a 10- to 12-hour fast. Insulin and glucose were infused through an 18-gauge catheter (Venflon, Viggo-Spectramed) inserted into the left antecubital vein. The left hand was kept in a heated chamber (65°C), and arterialized venous blood samples were withdrawn from a catheter inserted retrogradely into a heated dorsal hand vein. Insulin (Actrapid Human, Novo Nordisk) was infused in a primed continuous fashion for 120 minutes.26 The rate of the continuous insulin infusion was 1 mL·kg⁻¹·min⁻¹. This increased serum free insulin concentrations from 50±19 to 438±84 pmol/L in men with small LDL particles and from 47±26 to 438±54 pmol/L in those with large LDL particles. During hyperinsulinemia, normoglycemia (5 mmol/L) was maintained by adjusting the rate of a 20% glucose infusion on the basis of plasma glucose measurements performed from arterialized venous blood at 5-minute intervals. The glucose infusion rate needed to maintain normoglycemia (M value) during the final 30 minutes (90 to 120 minutes) of hyperinsulinemia was used as the measure of whole-body insulin sensitivity.26

Quantification of LDL Size

LDL particle size was measured in whole serum samples stored at −80°C. Nondenaturing polyacrylamide gel electrophoresis was performed on the samples with the use of gels cast in our laboratory, as previously described in detail.27-28 Gels were stained with Sudan black B, lipid stain and scanned with a computer-assisted laser scanning densitometer (Personal Densitometer, Molecular Dynamics) with a 50-nm pixel size and 12-bit signal resolution. The particle diameter of the major LDL peak was determined by comparing the mobility of the sample with the mobility of 2 reference LDL preparations run of each gel. The particle diameters of the reference LDL preparations were determined by electron microscopy.27-28 The coefficients of variation for intergel and intragel precisions of control samples were 2.0% and 1.2%, respectively.

Analytical Methods

Serum lipoprotein subfractions were isolated as previously described29,30 by sequential ultracentrifugation with the use of the following densities (d): VLDL d <1.006 g/mL, LDL d = 1.006 to 1.019 g/mL, HDL d = 1.019 to 1.063 g/mL, IDL d = 1.063 to 1.210 g/mL. Triglyceride (Unimate 7 TRIG, kit No. 0736805, Hoffman-La Roche) and cholesterol (Unimate 7 CHOL, kit No. 0736643) concentrations in serum and lipoprotein subfractions were determined with enzymatic spectrophotometric methods with the use of an autoanalyzer (Cobas Mira, F Hoffman-La Roche). Plasma glucose concentrations were measured in duplicate with the glucose oxidase method with the use of the Beckman Glucose Analyzer II (Beckman Instruments). Serum free insulin concentrations were determined by double-antibody radioimmunoassay (Pharmacia Insulin RIA kit) after precipitation with polyethylene glycol.31 HbA₁c was measured by high-performance liquid chromatography with the use of the fully automated Glycosylated Hemoglobin Analyzer System (BioRad).

Statistical Analyses

Variables with a skewed distribution (total and VLDL triglyceride and VLDL cholesterol concentrations, blood flow values) were log₁₀-transformed before statistical analyses. Data between the individuals with small and large LDL particles were compared by means of the Student’s unpaired t test or repeated-measures ANOVA (blood flow responses to different doses of vasoactive agents). Simple correlations were calculated by means of Pearson’s correlation coefficient. Multiple regression analyses were performed by means of stepwise and enter procedures. Calculations were made with the use of the SPSS 7.5 statistical package. Data are expressed as mean±SD for text and tables and mean±SEM for the figures. A value of P<0.05 (2-tailed) was considered significant.

Results

Subject Characteristics

The small and large LDL particle groups had comparable age, body mass index, blood pressure, serum glucose, and total and LDL cholesterol concentrations (Table 1). Subjects with small LDL particles had impaired insulin sensitivity (4.2±1.3 versus 5.9±2.0 mg · kg body wt⁻¹ · min⁻¹, P=0.018). Their lipoprotein phenotype was characterized by high total and VLDL triglyceride and VLDL cholesterol concentrations, a low HDL/total cholesterol ratio, and a trend toward a lower LDL cholesterol concentration. There were no significant differences between the groups in other lipoprotein variables.
significantly lower in men with small LDL particles (mean increase in flow 266 ± 60% vs men with large LDL; 418 ± 60%, men with small versus large LDL particles, F 3.0, P = 0.004), whereas SNP-induced fold-increases were similar in both groups (389 ± 161 vs 424 ± 166%, respectively, NS) (Figure 1, bottom).

In Pearson’s correlational analysis, the fold ACh-induced increase in blood flow was significantly correlated with LDL particle size but not with LDL cholesterol, HDL cholesterol, HDL/total cholesterol ratio, log10-transformed triglycerides, or insulin sensitivity (Figure 2 and Table 2). To analyze the impact of different atherosclerosis risk factors on endothelial function, stepwise multivariate regression analysis was performed with LDL particle size, LDL and HDL cholesterol, log10-transformed triglycerides, age, systolic blood pressure, and insulin sensitivity index (M value) in the model. Of these variables, only LDL particle size could be fitted in the model (R²=0.204, P=0.010). When all the aforementioned variables were forced in the model, the LDL particle size remained the only significant determinant of ACh-induced increase in forearm blood flow (P=0.031).

**Discussion**

The atherogenic lipoprotein phenotype consisting of small, dense LDL particles, elevated triglycerides, and low HDL cholesterol has been associated with coronary artery disease in cross-sectional and prospective epidemiological studies.3-5 The novel finding of the present study is that small LDL peak particle size was associated with impaired endothelium-dependent vasodilation in apparently healthy men. As expected, subjects with small LDL particles were insulin resistant and had a higher serum triglyceride concentration and a lower HDL/total cholesterol ratio than men with large LDL particles. LDL and total cholesterol, blood pressure, and glucose concentrations were similar in both groups.

LDL peak particle size was the only variable significantly associated with ACh-induced endothelium-dependent increase in forearm blood flow in univariate analysis. Because we wanted to further study whether this association was truly independent of other atherosclerosis risk factors, such as LDL and HDL cholesterol, triglycerides, and insulin resistance, multivariate regression analysis was performed. None of these additional variables significantly contributed to varia-

**TABLE 1. Subject Characteristics**

<table>
<thead>
<tr>
<th></th>
<th>LDL Size ≤25.5 nm (n=10)</th>
<th>LDL Size &gt;25.5 nm (n=24)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>49.6±11.9</td>
<td>54.8±7.6</td>
<td>0.23</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.0±2.2</td>
<td>26.7±3.2</td>
<td>0.80</td>
</tr>
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<td>Systolic blood pressure, mm Hg</td>
<td>129±17</td>
<td>131±23</td>
<td>0.79</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>80±8</td>
<td>80±9</td>
<td>0.99</td>
</tr>
<tr>
<td>Plasma glucose, mg/dL</td>
<td>103±6</td>
<td>104±12</td>
<td>0.81</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.5±0.3</td>
<td>5.4±0.3</td>
<td>0.43</td>
</tr>
<tr>
<td>M value†, mg/kg body wt⁻¹ · min⁻¹</td>
<td>4.2±1.3</td>
<td>5.9±2.0</td>
<td>0.018</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.80±0.73</td>
<td>5.51±1.11</td>
<td>0.45</td>
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<tr>
<td>VLDL cholesterol, mmol/L</td>
<td>0.64±0.22</td>
<td>0.34±0.23</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.87±0.58</td>
<td>3.68±0.96</td>
<td>0.56</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.07±0.35</td>
<td>1.30±0.32</td>
<td>0.081</td>
</tr>
<tr>
<td>Total triglycerides, mmol/L</td>
<td>1.92±0.55</td>
<td>1.24±0.56</td>
<td>0.013</td>
</tr>
<tr>
<td>VLDL triglycerides, mmol/L</td>
<td>1.40±0.51</td>
<td>0.77±0.51</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Values are mean±SD. *Unpaired t test between subjects with small LDL vs large LDL. †Insulin-stimulated whole-body glucose infusion rate.
tion in endothelium-dependent blood flow in the study subjects, whereas LDL size remained a statistically significant variable. Insulin resistance and serum triglyceride concentrations were strongly correlated, which could reflect resistance of anti-lipolysis or hepatic VLDL production to insulin.32–34 Although the inverse correlation between serum triglycerides and LDL particle size was statistically significant, it was weakly consistent with the ability of other factors, such as the activities of lipolytic and lipoprotein-modifying enzymes (eg, hepatic lipase and cholesteryl-ester transfer protein),23 to regulate LDL size. Assuming that LDL size is a more important determinant of endothelial function than serum triglycerides and confirming that insulin resistance is more strongly correlated with triglycerides than LDL size,35 it is not surprising that insulin resistance was not significantly correlated with endothelial function (Figure 2).

The mechanisms linking small LDL particles to endothelial dysfunction are not precisely understood. It is possible that small LDL size acts merely as a surrogate marker of long-time hypertriglyceridemia, and the remnants of triglyceride-rich lipoproteins may actually be more important in the development of atherosclerosis.36 However, several experimental studies support the view that small, dense LDL particles are directly atherogenic. They are more easily oxidized6 and have a greater binding affinity to arterial wall proteoglycans9 and receptor-independent cell-surface binding sites37 than larger LDL subspecies. Small, dense LDL particles have diminished binding affinity to LDL receptors, which may be a result of conformational changes in apoprotein B.7,8 Which of these mechanisms, if any, are important in the development of atherosclerosis remains to be determined. The current data demonstrate that LDL particle size is significantly related to in vivo endothelial function in humans. If the hypothesis that endothelial dysfunction precedes atherosclerosis is confirmed, these data strengthen the concept of atherogenicity of small LDL particles in vivo.

Three recent studies have addressed the question of whether hypertriglyceridemia is associated with endothelial dysfunction in otherwise healthy subjects. In 2 studies, the method to determine endothelial function was similar to that

| TABLE 2. Correlations Between Endothelial Function, Insulin Sensitivity, and Physical and Biochemical Parameters |
|---------------------------------------------------------------|---------------------------------------------------------------|------------------|
| ACh-Induced Increase in Blood Flow | LDL Peak Particle Size | M |
| r | P | r | P | r | P |
| LDL peak particle size | 0.45 | 0.01 | ... | ... | 0.30 | 0.09 |
| M | 0.21 | 0.25 | 0.30 | 0.09 | ... | ... |
| LDL cholesterol | 0.22 | 0.22 | -0.11 | 0.55 | -0.22 | 0.21 |
| HDL cholesterol | 0.31 | 0.09 | 0.38 | 0.03 | 0.41 | 0.02 |
| Total triglycerides | -0.19 | 0.30 | -0.44 | 0.01 | -0.77 | <0.001 |
| Age | 0.22 | 0.22 | 0.35 | 0.05 | 0.12 | 0.49 |
| Body mass index | -0.21 | 0.24 | -0.09 | 0.62 | -0.41 | 0.02 |
| Systolic blood pressure | 0.28 | 0.12 | 0.12 | 0.48 | -0.07 | 0.32 |
| Diastolic blood pressure | 0.08 | 0.97 | -0.04 | 0.80 | -0.18 | 0.21 |

Pearson’s correlation coefficients for total group of subjects (n = 34). Triglyceride and concentration and mean ACh-induced blood flow increase are log_{10}-transformed.
used in the current study, whereas in the third study, endothelial function was measured from the percent of flow-mediated dilatation of the brachial artery with high-resolution ultrasound. Lewis et al compared obese hypertriglyceridemic (serum triglycerides 7.0 mmol/L) subjects with lean normotriglyceridemic subjects, Chowienczyk et al studied subjects with lipoprotein lipase deficiency resulting in chylomicronemia and total triglycerides of \( \approx 20 \) mmol/L, and Schnell et al compared subjects with mean serum triglycerides of 4.2 mmol/L with subjects with hypercholesterolemia and normolipemic control subjects. The results were contradictory. Endothelial function was impaired in the study comparing obese and nonobese subjects but normal in the 2 other studies. In the latter 2 studies, the methods were different, suggesting that they were not responsible. In the present study, triglycerides were also not correlated with endothelial function (\( r = -0.19 \), Table 2), whereas LDL size was correlated (\( r = 0.45 \)). The present data thus are consistent with experimental studies, which suggest that LDL size is a more immediate determinant of endothelial function than serum triglycerides.

A limitation of our study is the relatively small number of subjects studied. Although we did not find statistically significant associations between serum triglycerides, HDL cholesterol, or insulin resistance and endothelial function, such could be found in a larger cohort. The collinearity of some of the variables also limits the reliability of the multivariate analysis. Therefore, these data should be interpreted with caution and reproduced. Also, it should be determined whether an increase in LDL size by hypolipidemic drugs or other therapies enhances endothelial function independent of LDL cholesterol.

We conclude that men with small LDL particles, mildly increased serum triglycerides, low HDL cholesterol, and normal LDL cholesterol have impaired endothelium-dependent vasodilation compared with men with similar age, body mass index, and LDL cholesterol concentrations. The degree of endothelial dysfunction is significantly correlated with LDL particle size rather than LDL or HDL cholesterol or triglyceride concentrations. These data are consistent with those demonstrating small LDL size to be a predictor of cardiovascular events and to be more closely mechanistically related to the vascular dysfunction in the vessel wall than the serum triglyceride concentration.

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