Endothelium-Dependent Relaxation by Acetylcholine Is Impaired in Hypertriglyceridemic Humans With Normal Levels of Plasma LDL Cholesterol

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OBJECTIVES
Patients with high triglyceride (of which very low density lipoproteins [VLDL] are the main carriers), but with normal low density lipoprotein (LDL) cholesterol levels, were examined for in vivo endothelium function status.

BACKGROUND
Very low density lipoproteins inhibit endothelium-dependent, but not -independent, vasorelaxation in vitro.

METHODS
Three groups were studied: 1) healthy volunteers (n = 10; triglyceride 1.24 ± 0.14 mmol/liter, LDL cholesterol 2.99 ± 0.24 mmol/liter); 2) hypertriglyceridemic (n = 11; triglyceride 6.97 ± 1.19 mmol/liter,* LDL cholesterol 2.17 ± 0.2 mmol/liter, *p < 0.05); and 3) hypercholesterolemic (n = 10; triglyceride 2.25 ± 0.29 mmol/liter,* LDL cholesterol 5.61 ± 0.54 mmol/liter; *p < 0.05) patients. Vasoactive responses to acetylcholine, sodium nitroprusside, noradrenaline, NG-monomethyl-L-arginine and postischemic hyperemia were determined using forearm venous occlusion plethysmography.

RESULTS
Responses to acetylcholine (37 µg/min) were significantly dampened both in hypercholesterolemic (% increase in forearm blood flow: 268.2 ± 62) and hypertriglyceridemic patients (232.6 ± 45.2) when compared with controls (547.8 ± 108.9; ANOVA p < 0.05). Responses to sodium nitroprusside (at 1.6 µg/min: controls vs. hypercholesterolemics vs. hypertriglyceridemic: 168.7 ± 25.1 vs. 140.6 ± 38.9 vs. 178.5 ± 54.5% increase), noradrenaline, N⁴-monomethyl-L-arginine and postischemic hyperemic responses were not different among the groups examined.

CONCLUSIONS
Acetylcholine responses are impaired in patients with pathophysiologic levels of plasma triglycerides but normal plasma levels of LDL cholesterol. The impairment observed was comparable to that obtained in hypercholesterolemic patients. We conclude that impaired responses to acetylcholine normally associated with hypercholesterolemia also occur in hypertriglyceridemia. These findings identify a potential mechanism by which high plasma triglyceride levels may be atherogenic independent of LDL cholesterol levels. (J Am Coll Cardiol 1999;33:805–12) © 1999 by the American College of Cardiology

Hypercholesterolemia is associated with impaired endothelium-dependent dilatation even in the absence of atherosclerotic plaques (1). This has been evidenced in humans by a reduction in responses to the muscarinic agonist acetylcholine in resistance arteries of the upper limbs (vessels not prone to the development of atherosclerotic lesions) with preserved responses to the non-endothelium-dependent nitrovasodilator sodium nitroprusside (2,3). This finding has also been described in angiographically normal coronary arteries of patients with hypercholesterolemia (4). The way in which the impairment occurs is unclear, and it is likely that there is contribution from more than one mechanism. Low density lipoproteins (LDL) isolated from human plasma alter vasomotion through inhibition of endothelium-dependent relaxation (5,6). In most ex vivo studies, oxidized LDL appears to play a major role (7) and has been postulated to impair signal transduction between endothelial cell surface receptors and nitric oxide production (8). This has also been shown to be true of nonmodified LDL (9). Other possibilities include the inhibition of nitric oxide synthase expression (10) and the inactivation of nitric oxide released from the endothelium (11). That elevated levels of LDL cholesterol impair endothelium-dependent relaxation is evident from a number of studies. Thus, a normalized response to acetylcholine
occurs following reduction of plasma LDL levels using lipid-lowering therapy (3,12,13). Even a single session of LDL apheresis (resulting in the reduction of total LDL and oxidized LDL) improves endothelium function, assessed as an increased vasorelaxation response to acetylcholine as well as an enhanced production of metabolites of nitric oxide during acetylcholine infusion (14). In addition, the degree of impairment of acetylcholine-induced vasomotor response appears to be related to the LDL cholesterol level after therapy (12).

We reported recently that very low density lipoproteins (VLDL) isolated from human plasma inhibit vasorelaxation mediated by endothelium-dependent but not independent dilators in isolated rat aortic rings (6). As an extension to these in vitro studies, patients with high triglyceride levels (of which VLDLs are the main carriers), but with normal LDL cholesterol levels, were examined for in vivo endothelium function status. Although an association between plasma triglyceride levels and coronary heart disease has been postulated (15,16), the role of triglyceride-rich lipoproteins per se in the etiology of coronary arteroma remains uncertain. Because an impaired response to nitric oxide-generated vasorelaxation may predispose to coronary vasospasm (17) and also contribute to the atherogenic process by promoting abnormal interactions between the vascular wall and platelets, neutrophils and macrophages (18,19), the current study lends insight into potential mechanisms for such a role.

METHODS

Three study groups were recruited: normal volunteers (n = 10; all men) by advertisement, and hypercholesterolemic (n = 10; 1 woman) and hypertriglyceridemic (n = 11; 1 woman) subjects from the Lipid Clinic at the Heart Centre, Alfred Hospital, Melbourne, Australia. All volunteers had normal findings on physical examination and no abnormalities on routine hematology and biochemical blood analyses, with the exception of defined plasma lipid changes. Subjects were classified as control subjects: triglyceride levels <2.00 mmol/liter and LDL cholesterol <3.50 mmol/liter; hypercholesterolemic subjects: triglyceride levels <2.50 mmol/liter and LDL cholesterol >5.00 mmol/liter; hypercholes-
terolemic subjects: triglyceride levels >6.5 mmol/liter and LDL cholesterol <3.5 mmol/liter. Exclusion criteria included: hypertension, cigarette smoking within 6 months, medication or vitamin supplements or a history of heart disease, diabetes or any other major illness. Hypercholesterolemic subjects taking lipid-lowering therapy (n = 2) were asked to cease therapy for a minimum of two weeks prior to the day of study. None of the hypertriglyceridemic subjects were taking lipid-lowering therapy. All hypertriglyceridemic patients had a fasting glucose of less than 6 mmol/liter (5.15 ± 0.23 mmol/liter) at the time of study. The exper-
imental protocol was approved by the Alfred Group of Hospitals Ethics Committee, and written informed consent was obtained from all subjects, who were also aware of their right to withdraw from the study at any time of their choice.

Experimental protocol. Subjects were studied after an overnight fast. Under strict aseptic conditions and local anesthesia (lignocaine 1%) the nondominant brachial artery was cannulated (3F, 5-cm catheter; Cook, Australia) for intra-arterial pressure recordings and drug infusions. A 20-ml blood sample was withdrawn for subsequent lipoprotein analysis. Arterial blood pressure was recorded with an AE 840 physiologic pressure transducer throughout the course of each experiment. Forearm blood flow was measured with venous occlusion plethysmography with a double sealed, alloy-filled (gallium and indium), double-strand strain gauge (Medasonic, Mountain View, California), with venous occlusion at 40 to 50 mm Hg for 10 out of every 20 s. Hand blood flow was occluded by a wrist cuff inflated to 200 mm Hg at the time of blood flow measurement.

To examine vascular reactivity after ischemia, forearm blood flow was occluded proximally on the upper arm at 200 mm Hg for 5 min. Forearm blood flow postsischemia was measured every 20 s for 2 min or until a plateau in blood flow was attained. In addition, blood flow responses were examined after discrete sequential infusions of acetylcholine (ACh: 9.25, 18.5 and 37 µg/min), sodium nitroprusside (SNP: 0.4, 0.8 and 1.6 µg/min), norepinephrine (NE: 25, 50 and 100 ng/min) and N⁶-monomethyl-L-arginine (l-NMMA; 4 µmol/min). Each drug was infused at 2 ml/min over a maximum of 7 min or until the response over three flow measurements reached a plateau (with the exception of l-NMMA, usually by 3 min). Rest periods of 5 min between concentrations and 10 min between drugs were observed. Physiologic saline (0.9%) was infused during rest periods. Basal blood flow (the mean of three flow measurements) was obtained just before each intervention.

Resting blood pressure was recorded 30 min after the cannulation procedure, before the start of flow measurements. To account for between-subject basal variation of forearm blood flow (20), results were expressed as a percentage change compared with the basal forearm blood flow present immediately prior to each infusion or to ischemia.

Lipoprotein analysis. Plasma lipoproteins were isolated by a combination of ultracentrifugation and selective precipitation. Both VLDL and chylomicrons were isolated as a floating fraction at a density of 1.006 after being spun for a minimum of 16 h at 40,000 rpm at 20°C in quickseal polyallomer ultracentrifuge tubes (Beckman 34619). Tubes were sliced 15 mm from the top; the bottom fraction was treated with heparin/Mn⁴⁺ to precipitate LDL, leaving high density lipoprotein (HDL) in solution. Lipid measurements were made of whole plasma, top fraction (VLDL) and bottom fraction (LDL and HDL) and of the heparinized supernatant (HDL). The LDL parameters were calculated by subtracting the supernatant from the bottom fraction value. Analytical kits from Boehringer Mannheim were used for measurement of total cholesterol (237574),
free cholesterol (310329), triglyceride without free glycerol (450032), phospholipid (691844), apolipoprotein A1 (1378686) and apolipoprotein B (1378694). Calibrators (759350; Precipath 781827) and normal (Precinorm 1285874) control sera were also from Boehringer Mannheim. Kits for lipoprotein(a) were from Incstar (Atlantic Antibodies ATA 86084).

**LDL particle sizes.** Individual plasma samples were frozen at −80°C. The LDL was isolated by a two-step gradient ultracentrifugation method (21); LDL particle diameters were determined on 3% to 13% nondenaturing gradient gels (Gradiapore GS313). An LDL sample was loaded onto a gradient gel, adjacent to which was loaded a lane with Polystyrene Latex Microspheres 28-nm diameter (Duke 5003A) and a lane of High Molecular Weight standards (Pharmacia 17-0445-01). Electrophoresis was carried out in nondenaturing running buffer for 16 h at 190 V with circulation at 10°C; gels were fixed with 10% sulphosalicylic acid for 1 h, stained with 0.04% PAGE Blue G90 (E lectran 44248) for 3 h and destained with 5% acetic acid overnight or until the background was clear. The LDL peak sizes were estimated against a standard curve created from the markers of known diameter: latex 28 nm, thyroglobulin 17 nm, ferritin 12.2 nm, and catalase 10.4 nm. We have previously estimated against a standard curve created from the markers of known diameter: latex 28 nm, thyroglobulin 17 nm, ferritin 12.2 nm, and catalase 10.4 nm. We have previously demonstrated that there is no significant difference between fresh and frozen LDL prepared from aliquots stored at −80°C for up to 3 months (22).

The LDL particle sizes were quantified for each peak in every individual subject. As noted previously (22) some subjects had more than one LDL peak. In these cases the diameter of each peak was determined as well as the percentage contribution of each LDL peak to the total area under all peaks. The weighted average LDL diameter was then determined for each subject. There was, however, no significant difference between the peak of the major LDL sphere and the weighted LDL diameter, and virtually identical results were obtained in all statistical analyses whether the diameter of the major peak or the weighted average was used. Thus, the data shown pertain to the LDL diameter of the major peak.

**Data analysis.** Data are presented as mean ± standard error mean (SEM). Comparison of anthropometric, hemo-

dynamic and lipid data between groups was performed by analysis of variance (ANOVA) followed by the Student unpaired t test, using SigmaStat (Version 2.03) Statistical Software (Jandel Scientific, San Rafael, California), which analyzes data for normality prior to performing parametric analysis. Nonparametric data were examined by a Mann-Whitney Rank Sum test. Responses to various agonists were performed using two-way repeated measures ANOVA (Jandel Scientific, San Rafael, California) followed where significant by a Student-Newman-Keuls test. The level of statistical significance employed was p < 0.05.

**RESULTS**

**Anthropometric data.** Control subjects did not differ from either the hypercholesterolemic or hypertriglyceridemic groups in age or in either systolic or diastolic blood pressure. Body mass index (BMI) was significantly higher in the hypertriglyceridemic subjects when compared with control subjects. There was no difference in BMI between control and hypercholesterolemic subjects (see Table 1).

**Plasma lipid and lipoprotein fractions.** Plasma lipids and lipoprotein composition are shown in Tables 2 and 3. Hypercholesterolemic subjects were characterized by elevated LDL cholesterol, and hypertriglyceridemic subjects by elevated VLDL triglyceride concentrations. Plasma phospholipids were elevated in both hyperlipidemic groups compared with controls. Moreover, VLDL phospholipids were elevated in both hyperlipidemic groups, but LDL phospholipids were elevated only in the hypercholesterolemic group compared with control subjects. The LDL particles were significantly smaller in both hyperlipidemic groups when compared with the control group.

**Forearm blood flow.** Basal forearm blood flow at rest was not different among the three groups (ml/100 ml/min: control vs. hypercholesterolemic vs. hypertriglyceridemic: 2.91 ± 0.55 vs. 2.80 ± 0.40 vs. 1.71 ± 0.15; one-way ANOVA; p = NS). Vasodilatory responses to acetylcholine were dose-dependent in the three groups. These responses were significantly dampened both in patients with high triglycerides and in patients with high cholesterol levels.
when compared to controls regardless of whether the data were expressed as absolute forearm blood flow or as a % increase in forearm blood flow (Fig. 1). The endothelium-independent agonist sodium nitroprusside also induced dose-dependent vasodilator responses. Although these responses were significantly dampened in patients with high triglyceride levels when expressed as absolute forearm blood flow, they did not differ when corrected for differences in basal flow (Fig. 2).

Similarly, postischemic hyperemic responses were significantly dampened in patients with hypertriglyceridemia when expressed as absolute forearm blood flow but did not differ when corrected for differences in preischemic (basal) forearm blood flows (Fig. 3). Neither responses to the vasoconstrictor noradrenaline nor responses to the NO synthase inhibitor L-NMMA differed among the three groups studied, regardless of whether the data were expressed as absolute flow or when corrected for basal flow (Fig. 4).

**DISCUSSION**

We have demonstrated, for the first time, impaired endothelium-dependent relaxation to acetylcholine in subjects with elevated plasma triglyceride but normal LDL cholesterol levels. This impairment was comparable to that observed in patients with high cholesterol levels, and we conclude that the dampened response to this agonist normally associated with high plasma levels of cholesterol is not specific to this lipid but is also true of another form of hyperlipidemia. We conclude that a dampened response to

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Control Subjects</th>
<th>HTg Subjects</th>
<th>HCh Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/liter)</td>
<td>4.55 ± 0.29</td>
<td>5.23 ± 0.36</td>
<td>7.16 ± 0.41**</td>
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<td>Free cholesterol (mmol/liter)</td>
<td>0.99 ± 0.09</td>
<td>1.51 ± 0.13*</td>
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<td>Esterified cholesterol (mmol/liter)</td>
<td>3.48 ± 0.27</td>
<td>4.00 ± 0.35</td>
<td>5.86 ± 0.45*</td>
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<td>Triglycerides (mmol/liter)</td>
<td>1.24 ± 0.14</td>
<td>6.97 ± 1.19*</td>
<td>2.24 ± 0.33*</td>
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<tr>
<td>Phospholipids (mmol/liter)</td>
<td>2.40 ± 0.13</td>
<td>3.30 ± 0.38*</td>
<td>3.32 ± 0.18*</td>
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<tr>
<td>Apolipoprotein A1 (mg/dl)</td>
<td>127.15 ± 6.67</td>
<td>105.8 ± 5.77</td>
<td>110.5 ± 7.05</td>
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<tr>
<td>Apolipoprotein B (mg/dl)</td>
<td>92.88 ± 7.48</td>
<td>83.5 ± 10.77</td>
<td>157.6 ± 20.68*</td>
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<tr>
<td>Lipoprotein (a) (mg/dl)</td>
<td>15 (9.04, 37.1)†</td>
<td>11.2 (5.0, 18.0)†</td>
<td>25.5 (15.32, 27.4)†</td>
</tr>
</tbody>
</table>

*p < 0.05 values in comparison with controls. † Median (range) shown as data are normally distributed.

<table>
<thead>
<tr>
<th>Lipoprotein Fractions (mmol/liter)</th>
<th>Control Subjects</th>
<th>HTg Subjects</th>
<th>HCh Subjects</th>
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<tr>
<td>Very low density lipoprotein</td>
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<tr>
<td>Total cholesterol</td>
<td>0.32 ± 0.05</td>
<td>2.13 ± 0.26*</td>
<td>0.84 ± 0.16*</td>
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<td>Free cholesterol</td>
<td>0.10 ± 0.01</td>
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<td>Esterified cholesterol</td>
<td>0.22 ± 0.05</td>
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<td>Triglycerides</td>
<td>0.87 ± 0.33</td>
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<td>1.42 ± 0.26*</td>
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<td>Phospholipids</td>
<td>0.19 ± 0.03</td>
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<td>0.49 ± 0.08*</td>
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<td>Low density lipoprotein</td>
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<tr>
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<td>5.17 ± 0.34*</td>
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<td>1.94 ± 0.20</td>
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<td>1.01 ± 0.12</td>
<td>1.81 ± 0.14*</td>
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<td>Particle size (µm)</td>
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<td>23.90 ± 0.20*</td>
<td>24.30 ± 0.15*</td>
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<tr>
<td>High density lipoprotein</td>
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<tr>
<td>Total cholesterol</td>
<td>1.07 ± 0.12</td>
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<td>0.97 ± 0.07</td>
</tr>
<tr>
<td>Free cholesterol</td>
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<td>0.09 ± 0.01</td>
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<tr>
<td>Esterified cholesterol</td>
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<td>0.72 ± 0.06</td>
<td>0.83 ± 0.07</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.14 ± 0.01</td>
<td>0.55 ± 0.27*</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>1.06 ± 0.08</td>
<td>0.83 ± 0.06*</td>
<td>0.96 ± 0.09</td>
</tr>
</tbody>
</table>

*p < 0.05 values in comparison with controls.
Acetylcholine occurs with high plasma triglyceride levels independently of LDL cholesterol levels.

Inclusion of the small number of women. The inclusion of the two women into our study was reexamined following reports of sex differences on endothelium function (23). Both the women studied were postmenopausal and not on hormone replacement therapy. Nevertheless, the entire data set was analyzed excluding the contributions of both women. As the findings were similar with or without their inclusion, we opted to include the total data set to allow for the larger study groups.

Basal forearm blood flow: Effect of vasodilatory responses. Further to the finding of a dampened response to acetylcholine, we observed a tendency for patients with hypertriglyceridemia to have a depressed basal forearm blood flow, suggesting a basal vasoconstrictive state (1.71 ± 0.15 ml/100 ml/min in hypertriglyceridemics vs. 2.91 ± 0.55 in controls; p = NS on ANOVA), which was not observed in patients with hypercholesterolemia (2.80 ± 0.40 ml/100 ml/min). Starting from this lower basal flow level, responses to both the direct-acting smooth muscle vasodilator, sodium nitroprusside, and to postischemic vasodilatation were significantly dampened in hypertriglyceridemics, implicating a more general vasoconstrictive profile in these patients. In contrast to responses to acetylcholine, however, the dampened effects to both these stimulants were no longer evident when responses were corrected for basal forearm blood flow. The null effect of hyperlipidemia on responses to sodium nitroprusside would suggest that the impairment is at the level of the endothelium rather than smooth muscle cell. Further, as postischemic vasodilatation has been shown in part to be due to endothelial release of

Figure 1. Dose-dependent vasodilatory responses to acetylcholine in controls and in patients with hypercholesterolemia and hypertriglyceridemia. **Top panel** shows responses expressed as absolute forearm blood flow; **bottom panel** as % increase in basal forearm blood flow. Analysis was by two-way repeated measures (RM) ANOVA followed by Student-Newman-Keuls test (*p < 0.05 when compared to control point). Results are expressed as mean ± standard error of the mean.

Figure 2. Dose-dependent vasodilatory responses to sodium nitroprusside in controls and in patients with hypercholesterolemia and hypertriglyceridemia. **Top panel** shows responses expressed as absolute forearm blood flow; **bottom panel** as % increase in basal forearm blood flow. Analysis was by two-way repeated measures (RM) ANOVA followed by Student-Newman-Keuls test (*p < 0.05 when compared to control point). Results are expressed as mean ± standard error of the mean.
nitric oxide (24) and because these responses were not altered in either group, we suggest that this is further evidence supporting the hypothesis (25) that endothelium-dependent impairment associated with hyperlipidemia may, in the early stages, be restricted to pertussis-toxin-sensitive stimulants such as acetylcholine, leaving pertussis-toxin-insensitive stimulation, such as shear stress (26), unaffected.

Basal forearm blood flow: Effect of vasoconstrictory responses. Regulation of basal vascular tone is partially regulated by nitric oxide. Inhibition of nitric oxide production by infusion of L-NMMA, an inhibitor of nitric oxide synthase, provides an index of basal nitric oxide production. We report an equal depression of forearm blood flow following L-NMMA infusion in the three subject groups examined, confirming previous reports of unaltered basal nitric oxide production in hypercholesterolemic subjects (13). In addition, the current results demonstrate no effect of elevated triglycerides on basal nitric oxide production. Further evidence supporting unchanged basal nitric oxide production in the presence of hyperlipidemia was the similar contractile responses to noradrenaline in all three groups. Other studies examining other contractile agonists, such as phenylephrine, have also reported no difference between control and hypercholesterolemic subjects (27).

Possible mechanisms of action. Previous human (12) studies have reported on an inverse relationship between the level of total and LDL cholesterol and impaired endothelium-dependent relaxation. We have now shown that subjects with elevated triglyceride but normal LDL cholesterol levels also have impaired endothelium-dependent relaxation. We can discount undiagnosed diahe-
tes as a possible cause for the impairment in the hypertriglyceridemic subjects as all the patients studied had a fasting glucose level of less than 6.0 mmol/liter at the time of study.

The finding that both an elevated LDL cholesterol and an elevation of triglyceride-enriched lipoproteins can impair endothelium-dependent vasodilation may imply that there is more than one mechanism leading to impairment or there may be other features common to these patient groups that are responsible. Some studies have suggested that LDL oxidation amplifies the inhibitory effect of LDL on endothelium-dependent relaxation (7). In addition, small dense particles, which may themselves be prone to oxidation, are particularly responsible (28). Because small dense LDL particles are a feature of both hypercholesterolemia and hypertriglyceridemia, we speculate that this may be a potential common pathway causing impaired endothelium-dependent vasodilation.

The current results are consistent with those of Vogel and colleagues (29), who demonstrated that mean changes in postprandial flow-mediated vasoactivity at 2, 3 and 4 h correlate significantly with changes in serum triglyceride levels. Conversely, our findings of impaired endothelium function in hypertriglyceridemics are in direct contrast to those reported by Chowienczyk et al. (30) where patients with severe hypertriglyceridemia, but normal LDL cholesterol levels, exhibited normal responses to ACh. A potential difference between the two studies is the significantly higher BMI in the subjects of the current study compared with the controls. Although univariate analysis revealed no correlation between BMI and maximum response obtained to ACh (data not shown), the significantly higher BMI may be indicative that the current patient group had insulin-resistance syndrome, a possibility that was not examined.

Conclusions. In conclusion, we have found that endothelium-dependent relaxation mediated by acetylcholine is impaired both in subjects with hypertriglyceridemia and in subjects with hypercholesterolemia. The design of this study precludes us from determining the mechanism by which elevated lipids impair endothelium-dependent relaxation, but it suggests that the endothelial impairment normally associated with high plasma levels of LDL cholesterol is not specific to these lipids but is also true of high triglyceride levels. These findings identify a potential mechanism by which high plasma triglyceride levels may be atherogenic-independent of LDL cholesterol levels.

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