Arteriosclerosis, Thrombosis, and Vascular Biology

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Objective—The objective of this study was to investigate the effects of lowering plasma triglycerides (TGs) on endothelial function and gain insight into the role played by free fatty acids (FFAs) in hypertriglyceridemia-associated vascular dysfunction.

Methods and Results—Eleven hypertriglyceridemic subjects without coronary artery disease, diabetes, elevated low-density lipoprotein cholesterol, tobacco use, or hypertension were studied using a randomized, double-blinded, crossover design (fenofibrate and placebo, 14 days). After each regimen, forearm blood flow was assessed by plethysmography in response to arterial acetylcholine, nitroprusside, and verapamil infusion. Hourly plasma TGs, FFA, glucose, and insulin were measured during a 24-hour feeding cycle to characterize the metabolic environment. Changes in plasma FFA after intravenous heparin were used to estimate typical FFA accumulation in the luminal endothelial microenvironment. Fenofibrate lowered plasma TG ($P<0.001$), total cholesterol ($P<0.01$), and apolipoprotein B ($P<0.01$) without altering high-density lipoprotein or low-density lipoprotein cholesterol concentrations. Forearm blood flow in response to acetylcholine ($P<0.0001$), nitroprusside ($P<0.001$), and verapamil ($P<0.0001$) improved after fenofibrate. Fenofibrate lowered 24-hour ($P<0.0001$) and post-heparin ($P<0.001$) TG and tended to lower 24-hour ($P=0.054$) and post-heparin ($P=0.028$) FFA.

Conclusions—Vascular smooth muscle function significantly improves after lowering plasma TG without changes in confounding lipoproteins or insulin resistance. The data raise additional questions regarding the role of FFA in hypertriglyceridemia-associated vascular dysfunction. (*Arterioscler Thromb Vasc Biol*. 2003;23:307-313.)

Key Words: endothelial function ∎ postprandial lipemia ∎ free fatty acids ∎ lipoprotein lipase ∎ metabolic syndrome

Elevated plasma triglycerides (TGs) have been shown to be an independent risk factor for coronary artery disease (CAD)[1] and are gaining acceptance as a therapeutic target in CAD prevention.[2] Several potential mechanisms have been proposed to explain how TG may promote atherogenesis, including enhancement of oxidative stress, reduction of high-density lipoprotein (HDL) cholesterol, influence on low-density lipoprotein (LDL) size distribution, induction of cell adhesion molecule expression, direct effects of TG-rich lipoproteins (TRLs) on the vessel wall, and effects of fatty acids, which are often elevated in parallel with TG, on the endothelium.[3]

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Of these mechanisms, the role of fatty acids is intriguing in light of associations between lipoprotein lipase (LPL) and atherosclerosis. LPL, an enzyme located at the capillary endothelial surface of various tissues, hydrolyzes lipoprotein TG to make fatty acids available to cells.[4] Not synthesized by endothelial cells, LPL present at the endothelial surface of large vessels is thought to originate from smooth muscle cells and macrophages in the arterial wall.[5] Studies in animal models have demonstrated an association between macrophage LPL expression and susceptibility to atherosclerosis.[6] Rodents deficient in macrophage LPL are relatively protected from atherosclerosis.[7–9] Furthermore, LPL expression is increased on the surface of human atherosclerotic plaques.[10] Thus, vessel wall LPL is relevant in atherogenesis, possibly related to free fatty acid (FFA) release from TRL metabolism at the endothelial surface of arteries.

Because of its relationship with atherosclerosis, endothelial dysfunction provides a useful surrogate end point to study risk factors for vascular disease.[11] Hypertriglyceridemia (HTG) induced by lipid infusion or fatty meal consumption has been shown to cause endothelial dysfunction in healthy subjects,[12–15] although some reports have failed to show such an effect.[16–18] Endothelial dysfunction has also been demon-

strated in otherwise healthy patients with chronic HTG,[19] but this finding has also been inconsistent.[20]

Interestingly, subjects with severe HTG attributable to LPL deficiency have preserved endothelial function.[21] This finding raises the possibility that TG hydrolysis products are involved in HTG-associated endothelial dysfunction. In support of this hypothesis, fatty acids[22,23] and TRL remnants[24] have been shown to be toxic to endothelial cells in vitro. Furthermore, acute elevations in FFA levels from both endogenous and exogenous sources cause endothelial dysfunction in healthy subjects.[25,26] However, a relationship between circulating FFA and endothelial function in chronic HTG has yet to be examined.

Studies comprehensively evaluating vascular function in chronic HTG, especially in the absence of confounding variables such as diabetes and elevated LDL cholesterol, are lacking. By lowering plasma TG without altering HDL and LDL cholesterol levels, we assessed the effects of plasma TG on endothelial function. By examining the impact of lowering plasma TG on plasma FFA levels, we also sought to gain insight into the role of FFA in the pathogenesis of HTG-associated endothelial dysfunction.

Methods

Subjects

Twelve men and postmenopausal women aged ≤65 years with a history of HTG and normal LDL cholesterol were recruited from University of Colorado affiliated hospitals. Exclusion criteria included known vascular disease, angina, diabetes mellitus, hypertension, tobacco use within the last 5 years, excessive alcohol consumption (>10 drinks per week), and current use of estrogens, vitamins E or C, warfarin, or vasoactive or lipid-lowering medications.

All subjects underwent a screening history and physical examination with measurement of fasting lipid profile and glucose. Subjects all had fasting TG between 200 and 750 mg/dL, LDL cholesterol ≤160 mg/dL, glucose ≤112 mg/dL, and no cardiac ischemia by exercise treadmill test (Standard Bruce Protocol). One female subject had an equivocal exercise treadmill test but no detectable coronary calcification by electron beam CT scan. Subjects with reproducibly elevated blood pressure (≥140/90 mm Hg) were excluded. For analyses, blood pressures reported are mean values for each subject over 3 separate study visits. One subject withdrew before data collection because of scheduling difficulties. Each subject gave informed consent and was paid for participation. Studies were carried out on the Adult GCRC at the University of Colorado in accordance with institutional guidelines and were approved by the Colorado Multiple Institutional Review and Western Institutional Review Boards.

Study Design

Subjects were randomized to take fenofibrate (201 mg daily, Abbott Laboratories) or placebo during phase one of the study. Subjects and investigators were blinded to medications throughout the protocol. Subjects received dietician counseling to comply with a step 2 AHA diet. After 14 days of the phase-one medication, fasting blood was obtained for lipid profile, apolipoprotein B (apoB), and fibrinogen determination. Each subject then underwent vascular studies (described below). Three days later, inpatient 24-hour feeding and post-heparin studies (see below) were performed; subjects remained on medication through the completion of these inpatient studies.

After these studies, subjects stopped medication for a variable period of time (median, 18 days; range, 4 to 54 days) until the next phase could be scheduled; after this period, the phase-two medication was initiated. Vascular, 24-hour feeding and post-heparin studies were then repeated as in phase one. Thus, each subject had

stopped the phase-one medication a minimum of 18 days before beginning the phase-two studies.

Vascular Studies

Alcohol, caffeine, and exercise were prohibited within 72 hours of vascular studies, and all subjects took salicylic acid (325 mg daily) for 5 days before each study to minimize potential circulating prostanoids that could affect vascular tone.[27] Studies were conducted after a 12-hour fast in a quiet, temperature-controlled room.

Forearm blood flow (FBF) was measured using venous-occlusion strain-gauge plethysmography (D.E. Hokanson) as previously described.[28] Under sterile conditions, a catheter was inserted into the brachial artery of the nondominant arm for vasodilator administration. Subjects rested a minimum of 30 minutes after catheter placement until consistent resting FBF was achieved. Acetylcholine chloride (CIBAVision) was infused at rates of 0.3, 1.0, 3.0, and 10.0 μg/min to assess endothelial-dependent vasodilation. Sodium nitroprusside (Abbott) was infused at rates of 0.3, 1.0, 3.0, and 10.0 μg/min to assess endothelial-independent, NO-mediated vasodilation. To evaluate vascular smooth muscle integrity, the NO-independent vasodilator verapamil (Abbott) was infused at rates of 10, 30, 100, and 300 μg/min. Each dose was infused for 5 minutes, and sufficient time (≈20 minutes) was allowed for FBF to return to resting levels between drug infusions. To avoid an order effect, the sequence of administration of acetylcholine and nitroprusside was randomized; however, verapamil was always infused last because of its prolonged half-life. Flow was recorded 4 times each minute at rest and throughout each drug infusion protocol. The mean value of FBF during the last minute of rest (dose=0) and each drug dose is reported.

Twenty-Four-Hour Feeding Studies

Subjects were admitted to the GCRC 3 days after the vascular study following a 12-hour fast. All meals were provided to subjects during this 3-day interlude so that each entered with similar caloric and macronutrient consumption.

At 8:00 AM (time=0), fasting blood was sampled and subjects were given the first of three step 2 AHA meals. The second and third meals were provided at 12:00 PM (t=4 hours) and 5:00 PM (t=9 hours), respectively. Total daily calories, calculated by the Harris-Benedict equation,[29] were distributed between the meals (30% first, 30% second, 40% third meal). Subjects completed each meal within 20 minutes and were prohibited from consuming additional calories. After the fasting blood draw, samples were obtained hourly for 24 hours with additional sampling 30 minutes after the initiation of each meal. Samples were analyzed for TG, FFA, glucose, and insulin.

Post-Heparin Studies

At the end of the 24-hour feeding study, subjects had been fasting 14 hours. Fasting TG and FFA were drawn (time=0″), and subjects were given an intravenous bolus of heparin (100 U/kg). At t=15″, blood was sampled for measurement of TG, FFA, and LPL activity. Subsequent measurements of TG and FFA were made at t=30″, 60″, 120″, and 180″. After heparin, all samples for FFA were collected in prechilled tubes containing 25 μL tetrahydrolipstatin solution (1 mg/mL ethanol) to prevent ex vivo lipolysis.[30] Tetrahydrolipstatin was kindly provided by Dr M.K. Meier and P. Weber, F. Hoffman-La Roche Ltd, Basel, Switzerland.

Biochemical Measurements

Fasting TG, total cholesterol, and HDL cholesterol were measured via enzymatic kits (Roche Diagnostic) on a Hitachi 717 automated chemistry analyzer. LDL cholesterol was calculated from plasma total and HDL cholesterol measurement after ultracentrifugation removal of very-low-density lipoprotein (VLDL) fractions. Fasting glucose was measured using a hexokinase colorimetric assay (Roche). ApoB was determined by nephelometry assay (Behring) on a Behring 100 analyzer. Fibrinogen was measured by enzymatic clotting assay (American Bioproducts) on an STA analyzer (Diagnostica Stago).

Baseline Characteristics of the Study Population

	Mean±SD	Range
Age, y	44.1±13.9	19–59
Sex, male/female	8/3	—
BMI, kg/m^2	30.6±4.1	25.5–36.3
Waist circumference, cm	103±12	88.5–121.5
Male	102±13	88.5–121.5
Female	103±8	95.5–112
Systolic BP, mm Hg	124±10	111–139
Diastolic BP, mm Hg	76±6	67–85
Triglycerides, mg/dL	310±71	208–416
Total cholesterol, mg/dL	222±32	179–272
HDL cholesterol, mg/dL	40±8	25–55
Male	39±8	25–49
Female	43±10	36–55
LDL cholesterol, mg/dL	121±26	77–160
Fasting glucose, mg/dL	97±12	74–112

BMI indicates body mass index; BP, blood pressure.

For 24-hour feeding and post-heparin studies, all FFA (Wako), TG (Roche), and glucose (Roche) measurements were made by colorimetric assay on a COBAS Mira CC+ analyzer (Roche). Insulin was measured by radioimmunoassay (Pharmacia). LPL activity was assayed as previously described.[31] Insulin sensitivity index (QUICKI)[32] was calculated using the fasting glucose and insulin levels (time=0) from the 24-hour feeding study.

Statistical Analyses

Analyses were performed using the statistical analysis software SAS (version 8.2). To correct for comparison of multiple end points, we used a significance level of $P=0.01$. Results are presented as mean±SEM, except where otherwise noted.

To investigate outcomes of the above studies, we used mixed models with fixed effect terms for period (indicating which treatment was given first), treatment (fenofibrate or placebo), dose (of vasodilator), or time (where applicable) and the interaction of dose (or time) and treatment. Subjects were included as random effects to account for within-subject correlation. If period was not significant, it was excluded from the model. When the interaction term was not significant, we used an additive model and tested dose (or time) and treatment. To test the influence of subject age on changes in FBF, a separate term for age was added to the model and tested for significance. Additional data (fasting lipids, apoB, LPL activity, fibrinogen, and QUICKI) were tested using paired two-tailed *t* tests. The repeated measures nature of the study design was accounted for by using the covariance structures available in *SAS PROC MIXED*. Because nonindependence of observations violates the assumptions of ordinary regression and ANOVA, mixed models are more appropriate for these analyses.[33]

In retrospect, we compared subgroups that did (n=5) and did not (n=6) meet published criteria for the diagnosis of the metabolic syndrome.[2] Baseline comparisons between these subgroups were made with unpaired *t* tests. To assess the effect of the metabolic syndrome on FBF, an additional term for metabolic syndrome was added to the previous FBF models and tested for significance.

Results

Baseline characteristics of the 11 subjects are presented in the Table. On average, these subjects were moderately hypertriglyceridemic and modestly hypercholesterolemic but had normal HDL and LDL cholesterol levels. All subjects were
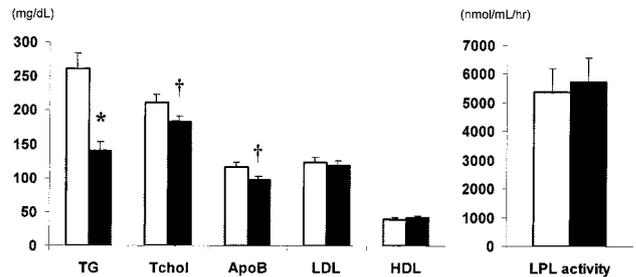

Figure 1. Fasting lipids and post-heparin LPL activities for the study population after placebo (open bars) and fenofibrate (solid bars) phases. *$P<0.001$, †$P<0.01$.

overweight (body mass index >25.0 kg/m^2), but none were severely obese (body mass index >40.0 kg/m^2).

Effects of fenofibrate on fasting lipid parameters and apoB are shown in Figure 1. Fasting TGs decreased by 45% after fenofibrate (261±74 versus 140±45 mg/dL; $P=0.0002$). Total cholesterol (212±38 versus 183±29 mg/dL; $P=0.0047$) and apoB (115±25 versus 97±19 mg/dL; $P=0.0013$) also decreased after fenofibrate. No significant changes were observed in LDL cholesterol (122±29 versus 118±23 mg/dL; $P=0.61$) or HDL (39±9 versus 42±11 mg/dL; $P=0.17$), LPL activity (5363±2632 versus 5741±2632 nmol/mL per hour; $P=0.96$), or fibrinogen (324±62 versus 324±56 mg/dL; $P=0.98$) between placebo and fenofibrate treatments.

FBF increased in a dose-dependent manner with each vasodilator (Figure 2). In response to acetylcholine, FBF was significantly increased after fenofibrate compared with placebo ($P<0.0001$, Figure 2A), and an interaction between treatment and dose was present ($P=0.02$). FBF in response to nitroprusside ($P=0.0003$, Figure 2B) and verapamil ($P<0.0001$, Figure 2C) also increased after fenofibrate. There was no significant period effect or age effect on vascular flow.

The relatively decreased fasting TG levels observed during fenofibrate treatment were sustained throughout the 24-hour feeding period ($P<0.0001$; Figure 3A). FFA levels undulated over this study period, with expected preprandial rises and postprandial falls (Figure 3B), consistent with insulin effects on adipose tissue. There was a nonsignificant trend toward lower FFA levels on fenofibrate during the 24-hour period ($P=0.054$).

Plasma glucose concentrations (Figure 4A) rose after each meal, as expected, but did not differ between fenofibrate and placebo treatments ($P=0.45$). Insulin levels showed similar postprandial rises (Figure 4B) and also did not differ between treatments ($P=0.63$). Accordingly, QUICKI did not differ between treatments (0.344±0.032 versus 0.353±0.024, placebo versus fenofibrate; $P=0.22$).

Plasma TG levels demonstrated an expected decline after intravenous heparin administration (Figure 5A). Post-heparin TG levels remained lower throughout the post-heparin sampling period after fenofibrate ($P=0.0002$). Plasma FFA levels rose sharply after heparin and began declining by 30 minutes (Figure 5B). Post-heparin FFA levels were marginally lower after fenofibrate ($P=0.028$). There were significant interac-
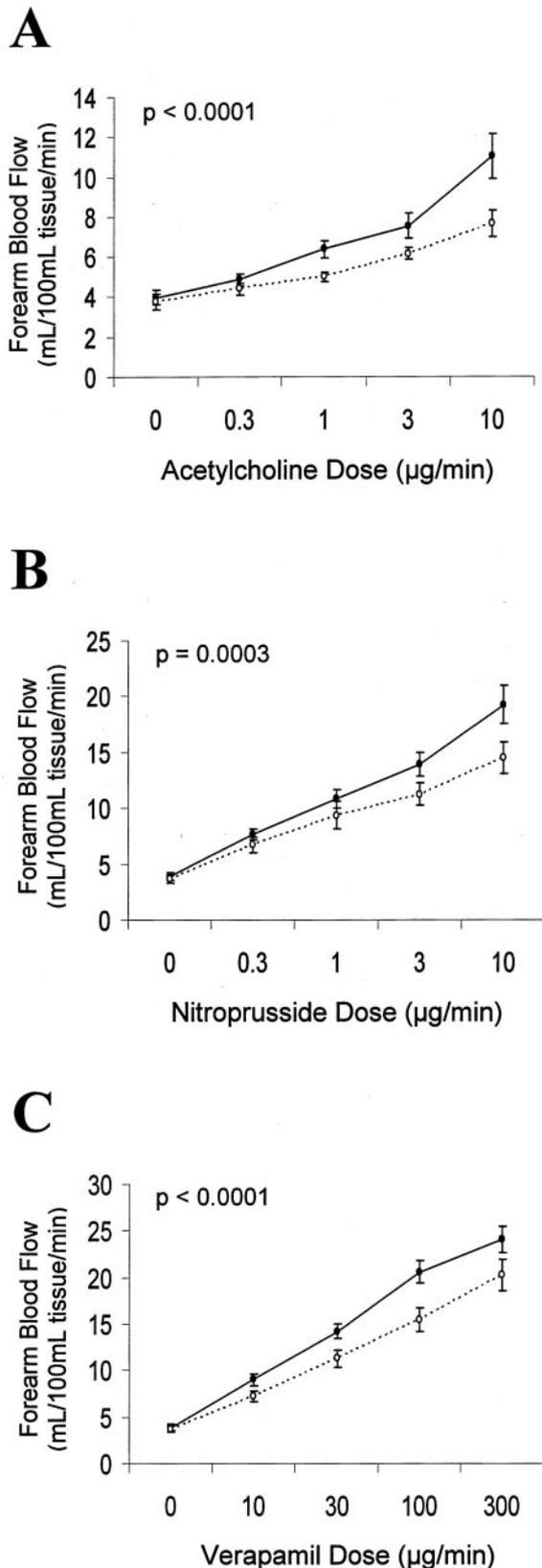
Figure 2. FBF after treatment with placebo (○) and fenofibrate (●) in response to increasing doses of acetylcholine (A; endothelium-dependent, NO-dependent stimulus), nitroprusside (B; endothelium-independent, NO-dependent stimulus), and verapamil (C; NO-independent stimulus).
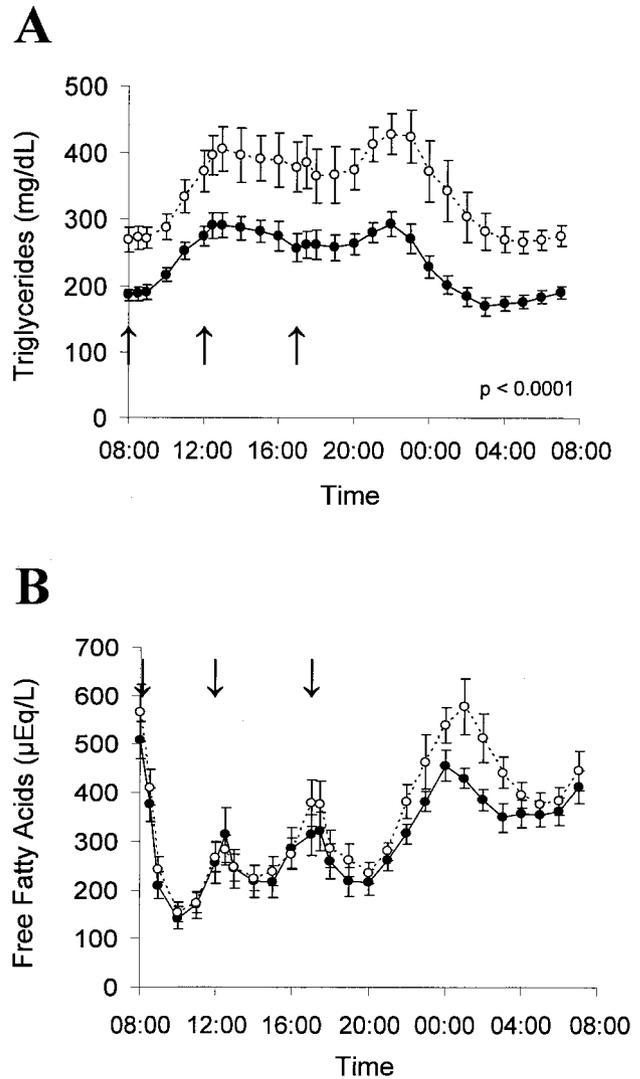

Figure 3. Hourly TG (A) and FFA (B) over a 24-hour feeding period. Measurements 30 minutes after the initiation of meals, denoted by the arrows, were also made. Data are shown after placebo (○) and fenofibrate (●) treatments.

tions between treatment and time for both post-heparin TG ($P=0.0005$) and FFA ($P=0.0006$).

No consistent associations were observed between changes in the above FFA, TG, glucose, insulin, fasting lipid, LPL activity, and QUICKI data and changes in FBF. Subjects retrospectively diagnosed with the metabolic syndrome (n=5; mean of 3.2 NCEP ATP III criteria) differed from those without (n=6; mean of 1.7 NCEP ATP III criteria) only in a tendency toward lower baseline HDL cholesterol levels (34±6 versus 45±6; mean±SD; $P=0.015$). However, the presence of the metabolic syndrome did not affect the observed differences in FBF between treatments.

Discussion

In the present study, we demonstrated significant improvements in vasodilator function after plasma TG lowering in patients with chronic hypertriglyceridemia. Furthermore, this improvement occurred in a setting in which potential con-
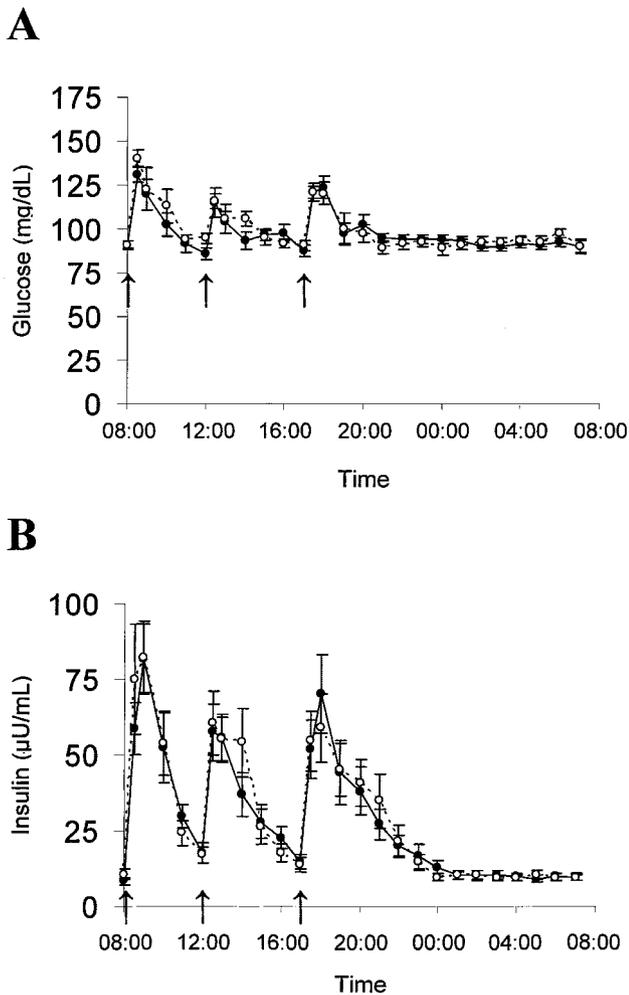
A

B

Figure 4. Hourly glucose (A) and insulin (B) levels over a 24-hour feeding period. Additional measurements 30 minutes after the initiation of meals, denoted by the arrows, were also made. Data are shown after placebo (○) and fenofibrate (●) treatments.

A

B

Figure 5. Plasma TGs (A) and FFA (B) after an intravenous heparin bolus (100 U/kg) given at time zero. Data are shown after placebo (○) and fenofibrate (●) treatment.

founding variables such as HDL cholesterol, LDL cholesterol, and insulin sensitivity did not change. Given the relationship between impaired vascular reactivity and the development of atherosclerosis, these results could have broad implications for the role of HTG in atherogenesis.

Interestingly, the impaired vasodilation in subjects on placebo was not confined to endothelial-dependent mechanisms. Dysfunction was also evident in response to the endothelial-independent vasodilators nitroprusside and verapamil. Because verapamil-mediated flow improved with fenofibrate, it is likely that vascular smooth muscle function was generally impaired during placebo treatment. Although additional defects in endothelial and NO signaling may have been present, it remains possible that the abnormalities we observed in these NO-mediated pathways resulted entirely from disrupted vascular smooth muscle function.

The present study is the first to demonstrate global vasodilator dysfunction in association with chronic HTG. Only a minority of the previous studies of vascular function in nondiabetic, chronic hypertriglyceridemic states examined
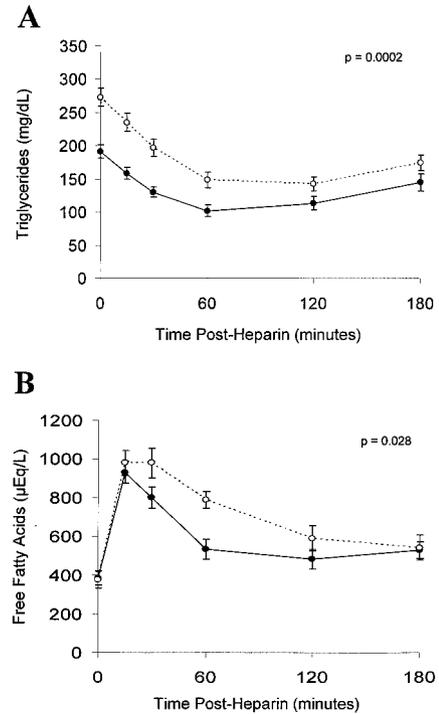
endothelial-independent mechanisms; those that did found abnormalities in endothelium-dependent pathways only.[16,19,34] In contrast, defects in both endothelium-dependent and -independent NO-mediated vasodilation have been previously reported in subjects with type 2 diabetes mellitus,[27,35] a disorder commonly associated with HTG attributable in part to TRL overproduction.[36] It is possible then that increased TRLs, remnants, or FFA flux in this disease state contribute to more global vascular dysfunction. Interestingly, a recent report examining ciprofibrate treatment on endothelial function in subjects with type 2 diabetes demonstrated improvement in endothelial-dependent vasodilation only.[37] However, these subjects had persistent fasting hyperglycemia, which is known to independently impair vascular reactivity.[38]

As in patients with type 2 diabetes mellitus, our subjects also had evidence of elevated circulating TRLs. The elevated plasma TG with relatively high apoB on average in our subjects during placebo treatment is a pattern consistent with familial combined hyperlipidemia. This disorder carries an increased risk of CAD,[39] possibly related to increased TRL production.[40] The decrease in total cholesterol and apoB, without change in LDL cholesterol, on fenofibrate suggests that fasting TGs were lowered largely by a decrease in circulating TRLs. Because the decreases in plasma TG, total cholesterol, and apoB reflected their typical proportions in VLDL particles, the amount of per-particle TG was probably only minimally altered. Furthermore, these fasting plasma TG reductions on fenofibrate were sustained throughout a typical 24-hour feeding period. Improved vascular smooth muscle function in association with decreased circulating TRLs is consistent with previous findings that VLDL hydrolysis and purified FFA are toxic to vascular smooth muscle cells.[41]

The short-term nature of the pharmacologic treatment in this study additionally highlights the observed reciprocal changes in vascular function and circulating TG levels, because it minimizes the impact of other potential long-term effects of fenofibrate. Changes in peroxisome proliferator-activated receptor (PPAR)-related enzyme expression have also been demonstrated with short-term fibrate treatment.[42] Because PPAR-α receptors are present on vascular smooth muscle cells,[43] direct effects of fenofibrate on the vessel wall could also help explain this improved vascular function.

A secondary aim of this study was to gain insight into the role of FFA in HTG-associated endothelial dysfunction. To do this, we examined daily circulating FFA and an estimate of FFA accumulation in the local endothelial environment of lipolysis sites. Because there is presently no reliable method to sample the endothelial microenvironment of large arteries in vivo, we used plasma FFA levels after a heparin bolus to indirectly assess local endothelial FFA accumulation.

Normally, endothelial-bound LPL hydrolyzes lipoprotein TG at the endothelial surface, producing FFA for cellular uptake. When released from the endothelial surface by heparin, LPL has greater exposure to circulating TG substrate, resulting in enhanced lipolysis and release of FFA in plasma.[44] This enhanced FFA release causes measurable changes in plasma FFA, which are eventually cleared through usual cellular uptake mechanisms. Post-heparin FFA levels then represent the net balance between plasma FFA release and clearance at each time point. Thus, post-heparin FFA levels provide a measurable, graphic representation of the interaction between a subject's own LPL and TG substrate pools. Assuming this interaction in plasma is proportional to that which occurs between the subject's membrane-bound LPL and TG substrate pools at the endothelial surface, this method could provide insight into differences in FFA accumulation at the endothelial surface. Although most of this lipolysis would normally occur in adipose and skeletal muscle capillary beds, such lipolysis and FFA accumulation at the surface of large vessels may be proportional based on relative LPL expression.

In general, circulating FFA and estimated endothelial FFA accumulation at sites of lipolysis tended to decrease with fenofibrate treatment. However, these effects were weak and failed to reach statistical significance. Furthermore, a lack of significant association between vascular function and plasma FFA levels raises some question as to the meaning of these trends with regard to vascular function. The lack of consistent correlations may have been attributable to insufficient statistical power for those analyses. Alternatively, effects of fenofibrate independent of lipid altering, such as PPAR-α activation, must be considered. Of note, gemfibrozil therapy in normotriglyceridemic subjects did not affect fasting or postprandial vascular function in a recent study.[45] Nevertheless, additional studies with greater statistical power and studies using fenofibrate in normolipidemic subjects could help clarify these relationships.

When this study was initiated, there were no published diagnostic criteria for the metabolic syndrome. Although selected primarily for HTG, it was evident retrospectively that a subset of our study population met these criteria. It could therefore be argued that this subgroup had a different CAD risk profile than the remaining subjects. Because the presence or absence of the metabolic syndrome did not significantly affect our results, fenofibrate may have similar effects on vascular function in HTG associated with the metabolic syndrome and HTG associated with other underlying mechanisms.

This finding, along with the observed improved vascular function without changes in insulin sensitivity, would seem to contrast those of Jonkers et al.[46] This group found that only the more-insulin-resistant subjects in their study population demonstrated endothelial dysfunction, whereas the less-insulin-resistant subjects did not, despite similar degrees of HTG between the subgroups. However, the much higher mean fasting TG levels in their subjects suggests that a significant proportion had fasting chylomicronemia. Because such subjects were excluded from the present study, it is likely that the population we examined had hypertriglyceridemia attributable to differing underlying pathophysiology. Additionally, the distinction between patients with and without metabolic syndrome in the present study should be closely examined. Although decreased HDL is an important marker of insulin resistance, these subgroups did not differ in any other relevant characteristic, including QUICKI and 24-hour insulin and glucose levels. It is therefore likely that our subgroups were relatively similar in regard to underlying insulin resistance, potentially minimizing any effect of this variable on vascular function improvement.

Chronic HTG is a common clinical entity, often resulting from overproduction of TRLs. We have provided evidence that pharmacologic intervention primarily affecting fasting and postprandial TG also positively impacts HTG-associated vascular function in the fasting state. Such treatment might also favorably impact circulating FFA levels and potential FFA accumulation in the endothelial microenvironment at intravascular sites of lipolysis. Additional studies targeted at understanding the specific effects of chronic HTG, as well as TG lipolysis products, on vascular function in vivo would be valuable to the understanding of TGs as a risk factor for CAD.

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