Systemic Acyl-CoA:Cholesterol Acyltransferase Inhibition Reduces Inflammation and Improves Vascular Function in Hypercholesterolemia

Rajesh K. Kharbanda, PhD, MRCP; Sharon Wallace, BA; Benjamin Walton, MRCP; Ann Donald, AVS; Jennifer M. Cross, PhD, MRCP; John Deanfield, MA, FRCP

Background—Circulating lipids may initiate and progress atherosclerosis by causing vascular inflammation. Monocytes and tissue macrophages are involved and regulate lipid metabolism in the vascular wall through acetylation of cholesterol by acyl-CoA:cholesterol acyltransferase (ACAT). ACAT inhibition reduces atherosclerosis in animal models by mechanisms that may be independent of their effects on circulating lipids. Because endothelial dysfunction is an important factor in atherosclerosis, we tested the hypothesis that systemic ACAT inhibition would improve endothelial function in hypercholesterolemic humans and assessed its effects on circulating lipids and markers of systemic inflammation.

Methods and Results—We studied 21 hypercholesterolemic subjects in a double-blind, randomized-crossover, placebo-controlled trial with assessments of circulating lipids, markers of inflammation, resistance-vessel endothelial function (with venous occlusion plethysmography), and conduit-vessel vasoreactivity (brachial artery flow-mediated dilation at baseline and after placebo or treatment with avasimibe 750 mg QDS for 8 weeks. There was a small change in total cholesterol with treatment (326±22 mg/dL, P=0.04). Circulating tumor necrosis factor-α was significantly reduced (4.0±0.3 to 3.6±0.2 pg/mL, P=0.02); resistance vessel responses to acetylcholine, bradykinin, and verapamil were significantly enhanced; and responses to nitroglycerin and conduit-vessel vasoreactivity were unchanged after ACAT inhibition.

Conclusions—Systemic ACAT inhibition reduces circulating tumor necrosis factor-α levels in hypercholesterolemic subjects and improves resistance-vessel endothelial function, with small effects on circulating cholesterol. This may be a novel therapeutic strategy to target vascular inflammation and endothelial dysfunction in atherosclerosis. (Circulation. 2005;111:804-807.)

Key Words: endothelium ■ hypercholesterolemia ■ inflammation

Atherosclerosis is now recognized as an inflammatory disease. Circulating lipids may initiate or perpetuate vascular inflammation in several ways: They may be directly toxic to the endothelium, cause endothelial dysfunction, and initiate the cascade of events leading to atherosclerosis by activating inflammatory mechanisms. An early abnormality in atherosclerosis is monocyte adhesion to the endothelium, migration into the intima, and transformation into foam cells. These cells become distended with toxic lipids and die, releasing their contents into the lipid core. Local vascular inflammation ensues, with the production of a range of proinflammatory cytokines; these circulating cytokines may cause further endothelial dysfunction and amplify the process of atherosclerosis.

Most therapies have targeted LDL cholesterol in the circulation, and dramatic clinical benefits have been demonstrated with HMG-CoA reductase inhibitors. Another potential therapeutic approach is the modification of cholesterol uptake and esterification by macrophages, which are located in the atherosclerotic plaque. The enzyme acyl-CoA:cholesterol acyltransferase (ACAT) catalyzes the formation of cholesterol/fatty acyl-CoA esters. In hepatocytes and enterocytes, it regulates dietary uptake of cholesterol. In animal models, ACAT inhibitors reduce atherosclerosis by reducing intestinal cholesterol absorption, lowering circulating plasma levels, and inhibiting foam cell formation in the vessel wall.

It is possible that ACAT inhibitors may also improve endothelial function and modulate the inflammatory component of atherosclerosis, but this has not been studied in human atherosclerosis. We tested the hypothesis that treatment of young hypercholesterolemic patients with the ACAT inhibitor avasimibe would result in improved endothelial function and reduction in inflammation.
Methods

Study Design
We performed a single-center, randomized, double-blinded cross-over trial. The University College London Hospitals Research Ethics Committee approved the protocol, and all participants gave written informed consent.

Subjects
Eligible participants were men and women (who were not of childbearing potential) with hypercholesterolemia (LDL cholesterol ≥160 mg/dL or ≥4.14 mmol/L at baseline). Patients were excluded if they met one or more of the following criteria: age <18 or >70 years; a history of any of cardiac or cerebrovascular disease; uncontrolled hypertension (defined as systolic blood pressure >160 mm Hg or diastolic blood pressure >100 mm Hg); abnormal laboratory findings as measured during the screening or baseline visits (renal dysfunction, nephrotic syndrome, or azotemia with a level of serum creatinine ≥3 mg/dL or 265 μmol/L, active liver disease, liver dysfunction with aspartate aminotransferase or alanine aminotransferase ≥2× upper limit of normal; or creatine phosphokinase levels >5× upper limit of normal); use of any of the following: hormonal contraceptives, L-arginine supplements, dipyridamole, cytochrome P450 3A4 substrates or inhibitors, lipid-regulating drugs, or ACE inhibitors and angiotensin II receptor inhibitors; failure to reach ≥80% compliance to study medication regimen during the placebo-baseline phase; concurrent participation in another clinical study; or significant abnormalities that the investigators believed might compromise the patient’s safety or successful participation in the study.

Protocol

Screening
Subjects were asked to withdraw lipid-lowering therapy for 8 weeks before study 1, at which they underwent a physical examination, ECG, and measurement of vascular function, renal and hepatic function, plasma lipids, and inflammatory mediators as described below. Eligible subjects were randomized to receive 750 mg of avasimibe or placebo in 4 doses per day. Eight weeks after randomization, subjects were restudied and crossed over to receive the alternative treatment for a further 8 weeks. At the end of this period, they underwent further study with an identical protocol. All clinical studies were performed in a temperature-controlled laboratory (24°C to 26°C).

Conduit-Vessel Function
Brachial artery diameter in the dominant arm was measured with high-resolution vascular ultrasound as described previously. Flow-mediated dilation (FMD) was defined as the maximum percentage increase in vessel diameter during reactive hyperemia and nitroglycerin (NTG)–mediated dilation as the maximum percentage increase in vessel diameter after 25 μg of sublingual NTG.

Resistance-Vessel Function
Strain-gauge plethysmography was used to measure forearm blood flow (FBF) in both arms as described previously. We measured FBF responses to infusions of 4 vasodilator drugs: bradykinin (BK) 20, 40, and 80 pmol/min, each dose for 3 minutes (Clinalfa AG); acetylcholine (ACH) 25, 50, and 100 nmol/min, each dose for 3 minutes (Sigma Chemical Co); NTG 4, 8, and 16 nmol/min, each dose for 3 minutes (David Bull Laboratories); and verapamil 20, 40, and 80 nmol/min, each dose for 3 minutes (Knoll Ltd). The order of drug infusions was varied between studies, but verapamil was always infused last. The ratio of flow in the infused/noninfused (control) arm was calculated for each measurement period. Vasodilator responses were expressed as the percentage increase in the ratio of FBF (infused/noninfused arm) relative to the immediately preceding baseline flow.

Biochemistry and Inflammatory Markers
Blood for biochemistry, hematology, lipid profile, and inflammatory markers was drawn after a 12-hour fast and sent to a reference laboratory for analysis in a blinded manner.

Statistical Analysis
The sample size was based on a review of published literature investigating the effects of HMG-CoA reductase inhibitors on endothelial function. We calculated that to demonstrate a 30% increase in FMD with a power of 80% at α=5% would require 20 subjects. Results are expressed as mean (SEM) unless otherwise stated. FMD, NTG dilation, inflammatory indices, and cytokine levels during the placebo phase were compared with those during the active phase by paired t test. For resistance-vessel studies, dose-FBF response curves were constructed and comparisons made by 2-way repeated-measures ANOVA between the placebo and active phase. P<0.05 was considered statistically significant.

Results

A total of 24 subjects were recruited: 17 men (mean age 43±2.2 years) and 7 women (mean age 42±4.8 years) from the University College London lipid clinic. Of these, 22 (92%) were randomized to treatment. Two patients were not randomized because they did not meet eligibility criteria after the baseline investigations (cholesterol <4.1 mmol/L for both). One patient withdrew because of potential pregnancy. Twenty-one subjects (15 men [mean age 43±2.56 years] and 6 women [mean age 42±4.8 years] therefore completed the study. There were no serious adverse effects reported.

Twenty subjects completed the conduit-vessel studies, but one study was technically inadequate and was excluded. All 21 patients consented to resistance-vessel studies; in 2 subjects, the brachial artery could not be cannulated at baseline, and in another 2 subjects, the cannulation failed after the first treatment phase. In a further 4 subjects, cannulation failed at the final visit. Thirteen subjects (mean age 46.4 years, 11 men) completed resistance-vessel studies with ACh, BK, and NTG, and 10 subjects completed resistance-vessel studies with verapamil (intra-arterial needle displaced).

Vascular Function
Baseline blood pressure, FBF, vessel size, and heart rate were unchanged during the 3 phases of the study (Table).

Conduit-Vessel Function
Flow Stimulus
Baseline blood flow velocity (VTI) was unchanged during the study. Peak reactive hyperemia and area under the curve for reactive hyperemia in the 120 seconds after cuff release were similar at all studies (Table).

Dilatation
Peak FMD was higher after 8 weeks of ACAT inhibition, although this failed to reach statistical significance (6.6±0.5% versus 8.0±0.9%, P=0.09). There was no difference in the response to NTG (13.9±1.0% versus 12.5±1.0% after treatment, P=0.29).

Resistance-Vessel Function
There was a dose-dependent increase in FBF in response to ACh, BK, NTG, and verapamil. The FBF response to ACh and BK was significantly greater after 8 weeks of treatment...
with the ACAT inhibitor compared with treatment with placebo ($P = 0.01$ and $P = 0.001$, respectively; Figure, A and B), whereas the response to NTG was unchanged ($P = 0.06$; Figure, C). There was a significantly greater response to verapamil after treatment with the ACAT inhibitor than with placebo ($P = 0.04$; Figure, D). There were no differences between FBF responses during the placebo phase compared with baseline for any of these agents (data not shown).

**Biochemistry, Lipid Profiles, and Inflammatory Markers**

There was a reduction in total cholesterol between the placebo and treatment phases (from 326 ± 25 to 311 ± 22 mg/dL, $P = 0.04$). There were no changes in triglycerides or LDL subfractions between the placebo and treatment phase (Table).

Tumor necrosis factor-α (TNF-α) was reduced from 4.0 ± 0.3 pg/mL during the placebo phase to 3.6 ± 0.2 pg/mL ($P = 0.02$, paired $t$ test) after administration of ACAT. C-reactive protein, fibrinogen, E-selectin, interleukin-6, and transforming growth factor-β did not differ between the active and placebo phases (Table).

**Discussion**

This is the first human study to demonstrate that short-term systemic ACAT inhibition improves endothelial function of the resistance vessels, reduces circulating TNF-α levels, and is associated with a small reduction in circulating cholesterol in hypercholesterolemic humans. These data provide novel information about the role of ACAT in the vascular biology of human atherosclerosis and suggest that ACAT inhibition may be a strategy to improve endothelial function and inflammation in hypercholesterolemia.

ACAT inhibition reduces early atherosclerosis and causes regression of established atheroma in animal models. The published literature has investigated the efficacy and short-term safety of avasimibe in patients with combined hyperlipidemia and familial hypercholesterolemia. At the maximal studied dose of 750 mg QDS in familial hypercholesterolemic subjects, avasimibe was not effective in reducing cholesterol as monotherapy. In the present study, there was a small (4.9%) reduction in total cholesterol but no changes in other lipid measures.

There are no data on the anatomic or functional effects of these agents in humans. Endothelial dysfunction in both peripheral resistance and conduit arteries has been demon-

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IL-6 indicates interleukin-6; TGF-β, transforming growth factor-β; CRP, C-reactive protein; and AUC RH, area under the curve for reactive hyperemia. Sample sizes are in parentheses.

FBF responses to (A) ACh (n=13), (B) BK (n=13), (C) NTG (GTN; n=13), and (D) verapamil (Ver; n=10) after 8-week placebo phase (—heavy line) and 8-week treatment phase (—). Values represent mean ± SEM. Comparison by repeated-measures ANOVA of drug dose/response interaction by group.
strated in patients with conventional cardiovascular risk factors, and it predicts the future occurrence of cardiovascular events.9 The present data show significant improvement in resistance-vessel responses to the endothelium-dependent agonists ACh and BK and an intriguing enhanced response to verapamil after ACAT inhibition. The responses to NTG were unchanged (even when areas under the curve were compared). In the conduit vessel, ACAT inhibition caused a small increase in FMD, which did not reach statistical significance (P=0.09), without affecting the response to NTG; however, when normalized as a ratio of FMD to NTG-mediated dilation, there was a significant increase from 0.5 to 0.7 (P=0.03).

Although responses to intra-arterial verapamil can be variable, the present data are consistent with the hypothesis that ACAT inhibition may itself improve smooth muscle responses to calcium channel blockade, because the response to verapamil was increased. Indeed, cellular cholesterol is an important component of the intracellular signaling pathways, and in experimental models, altering cholesterol may alter smooth muscle responses to various agonists; these effects may in part be explained by changes in calcium flux.10 This interesting observation would need to be confirmed in larger studies but implies that avasimibe might augment the hypertensive effects of calcium channel blockers.

The mechanism by which ACAT inhibition improves vascular function is unknown and was not the specific objective of the present study. The present clinical data show that avasimibe 750 mg QDS does not change baseline blood pressure, LDL cholesterol, or triglyceride levels but induces a small reduction in total cholesterol. Macrophages are an important source of inflammatory cytokines, and the associated reduction in circulating TNF-α raises the possibility of a novel therapeutic strategy to target vascular inflammation, especially because TNF-α levels and matrix metalloproteinase expression in atherosclerotic plaques are important source of inflammatory cytokines, and the associated reduction in circulating TNF-α raises the possibility of a novel therapeutic strategy to target vascular inflammation, especially because TNF-α levels and matrix metalloproteinase expression in atherosclerotic plaques are associated with long-term outcome in atherosclerosis.11 Although at present there is no direct evidence that modulation of TNF-α levels is of any therapeutic value, the magnitude of difference achieved in this short period is similar to that seen between the 75th and 90th centiles in a high-risk postinfarction population. Furthermore, in this study by Ridker et al,11 the predictive value of TNF-α was independent of other inflammatory cytokines. Infusion of TNF-α itself causes acute arterial endothelial dysfunction in humans, and it would be important to investigate whether modulation of TNF-α is the reason for improved endothelial function after treatment by ACAT inhibitors.11 In animal models, ACAT inhibition is also associated with a reduction in monocyte-macrophages and metalloproteinase expression.13

The present data provide novel information about the functional effects of ACAT inhibition in humans and demonstrate improvement in the vascular phenotype. Furthermore, this intervention reduces circulating TNF-α levels and provides a rationale for larger studies to investigate its role in modifying the inflammatory component in atherosclerosis and modulating the initiation, progression, and clinical complications of this disease.

Potential Limitations
This present study is not a clinical end-point study. We have used resistance-vessel and conduit-artery endothelial function to investigate a potential mechanism by which ACAT inhibition may achieve biological effects in humans. We have also shown a selective reduction in circulating TNF-α levels, without changes in other cytokines. The dropout rate reduced the power of the present study to demonstrate improvement in conduit-vessel function. Larger studies would have more power to define this and other changes. The surrogate markers that we have used are well-established research tools associated with clinical end points, and the present data provide mechanistic support for this novel intervention to target atherosclerosis.

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References
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