Association of plasma ceramides and sphingomyelin with VLDL apoB-100 fractional catabolic rate before and after rosuvastatin treatment.

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ABSTRACT (160)

Introduction: To examine post-hoc associations between plasma sphingolipids and lipoprotein kinetics in men with the metabolic syndrome following rosuvastatin treatment.

Materials and Methods: Plasma sphingolipid profiling, determined by tandem mass spectrometry, was performed in a randomized, double-blind, triple-crossover trial (N=12) of 5-wk treatment periods with placebo or rosuvastatin (10 or 40 mg/day) with 2-wk wash-outs between treatments.

Results and Discussion: Baseline plasma ceramides were associated with VLDL apoB-100 concentration (r=0.58, P<0.05) and inversely with VLDL apoB-100 FCR (r=-0.67, P=0.02). Post-treatment changes with rosuvastatin (40 mg/day) in plasma ceramides were inversely associated with VLDL apoB-100 FCR (r=-0.62, P=0.03) independent of changes in plasma triglycerides, cholesterol, and LDL-cholesterol. By contrast, baseline and post-rosuvastatin treatment plasma sphingomyelin levels were not associated with apoB-100 kinetics. Plasma ceramides and sphingomyelin were not associated with the kinetics or concentrations of HDL apoA-I and LDL apoB. In the metabolic syndrome, the ability of rosuvastatin to increase VLDL apoB-100 FCR may reflect ceramide-specific mechanistic actions and/or sphingolipid exchange.
INTRODUCTION

The metabolic syndrome is characterized by central adiposity, insulin resistance, and atherogenic dyslipidemia contributing to an increased risk in cardiovascular disease (CVD) mortality (1). Statins, HMG-CoA reductase inhibitors, are effective at reducing plasma LDL-cholesterol and apoB concentrations, and are used to lower CVD risk in the metabolic syndrome (2). Recent studies have shown that statins may also exert pleiotropic effects that extend to other lipid classes such as sphingolipids (3,4).

Statins also lower plasma ceramide and sphingomyelin concentrations (4-6). We, and others, have previously shown that statins increase the catabolism of apoB-containing lipoproteins in the metabolic syndrome (7,8). In this study, we extend on our previous findings by exploring associations between the plasma sphingolipidome and lipoprotein kinetics in patients with the metabolic syndrome. Sphingolipidenrichment in lipoproteins impairs apoB catabolism, and because statins increase the catabolism of apoB, we hypothesized that the lowering of plasma sphingolipids with rosuvastatin would be associated with an increased fractional catabolism of apoB.
MATERIALS AND METHODS

Subjects

Twelve men with the metabolic syndrome (National Cholesterol Education Program Adult Treatment Panel III criteria (9), and with HDL cholesterol ≤1.2 mmol/L were recruited for a study of the effects of rosvuastatin on lipoprotein kinetics (15). Participants provided informed written consent, and the study was approved by the Ethics Committee of the Royal Perth Hospital.

Study design

This was a randomized, double-blind, placebo-controlled, three-way cross-over trial. Eligible subjects entered a 4-week weight maintenance, placebo run-in period followed by randomization to a 5-week treatment period of either 10 or 40 mg rosvuastatin or matching placebo taken orally at night, with cross-over to two further 5-week treatment periods interspersed by 2-week wash-outs.

Apolipoprotein kinetics

VLDL, IDL, and LDL apoB, and HDL apo A-I kinetics were determined by stable isotopy, GCMS, and compartment modelling as described previously (8). For details of the clinical and lipoprotein metabolic protocols see the Supplementary Materials and Methods.

Plasma sphingolipidomics

Lipid profiling was performed by liquid chromatography, electrospray ionisation-tandem mass spectrometry (LC ESI-MS/MS) as described previously (10). For details see the Supplemental Materials and Methods.

Statistical analyses
Skewed variables were logarithmically transformed where appropriate. Data at the end of the three treatment periods were compared using a mixed-effects model (SAS Proc Mixed, SAS Institute), which also tested and excluded carryover and time-dependent effects. The $P$-values are reported, with statistical significance set at the 5% level, following adjustment for multiple comparisons using the Tukey-Kramer method. Univariate associations between the plasma lipidome and lipoprotein kinetics were examined on both doses of rosuvastatin using Spearman's Rho and adjustments for plasma lipids were made using partial correlation.
RESULTS

Clinical and biochemical characteristics

The 12 male subjects recruited were (mean±SD) middle-aged (48.6±8.5 y), centrally-obese (BMI 33.8±5.1 kg/m², waist 114.0±12.5 cm), normotensive (systolic 131.9±8.4, diastolic 79.6±6.6 mmHg), dyslipidemic (cholesterol 5.52±0.88, triglycerides 2.47±1.12, HDL-cholesterol 0.94±0.15 mmol/L), normoglycemic (5.43±0.66 mmol/L) and insulin resistant (HOMA score 3.75±1.77). Treatment with rosvastatin was carried out with no significant changes in body weight, blood pressure, insulin, glucose, fatty acids, HOMA score, and nutrient intake. Both rosvastatin 10 and 40 mg were well tolerated.

Plasma concentrations of lipids, sphingolipids and phospholipids, lipoproteins, apolipoproteins, indices of cholesterol metabolism, and cholesterol ester transfer protein activity on placebo, 10 mg/day rosvastatin (R10) and 40 mg/day rosvastatin (R40) have been reported previously (6,8,11). In brief, rosvastatin lowered apoB-100 and apoC-III concentration chiefly by increasing its fractional catabolism with no significant effect on its production rate (8). In the present study, we report in the same cohort of subjects, an inverse and independent association between plasma ceramides and VLDL apoB-100 FCR at baseline and with high-dose rosvastatin.

Baseline associations: Plasma sphingolipidome and apoB-100 kinetics

Baseline total plasma ceramide was inversely associated with VLDL apoB-100 FCR (r = -0.67 p = 0.017) (Figure 1) and to a lesser extent, IDL apoB-100 FCR (r=-0.54 p=0.07), and directly associated with VLDL apoB-100 concentration (r=0.58 p=0.048). We found that four of the six ceramide species with longer chain fatty acids including C20:0 (r=-0.67 p=0.02), C22:0 (r=-0.73 p=0.007), C24:1 (r=-0.71 p=0.009), and C24:0 (r=-0.65 p=0.02) were significantly and inversely associated with VLDL apoB-100 FCR. These associations, however, were no longer significant after adjustment for plasma triglycerides (partial r=0.01, p=0.99) or apoC-III (partial r=-0.08, p=0.81). The shorter chain length ceramide species showed a similar trend with VLDL apoB-100 FCR, but did not reach statistical significance (C16:0 r=-0.55 p=0.06 and C18:0 r=-0.52 p=0.08). Similar results were observed with plasma GM₃ gangliosides and
VLDL apoB-100 FCR ($r=-0.59$, $p=0.04$). No significant baseline associations were found with plasma ceramide and VLDL apoB-100 PR. No significant associations between baseline plasma dihydroceramides, monohexosylceramides, dihexosylceramides, trihexosylceramides, and sphingomyelin and apoB-100 kinetics were found.

**Post-treatment associations: Plasma sphingolipidome and apoB-100 kinetics with R40**

There were no significant associations between changes in plasma sphingolipids and apoB-100 kinetics with R10 treatment compared with baseline. However, with R40 the increase in VLDL apoB-100 FCR was associated with lower plasma ceramide concentration ($r=-0.62$, $p=0.03$) (Figure 2) and its subspecies containing the following fatty acid moieties: C18:0 ($r=-0.62$, $p=0.03$), C20:0 ($r=-0.63$, $p=0.03$), C22:0 ($r=-0.69$, $p=0.01$), and C24:1 ($r=-0.59$, $p=0.04$). The association between VLDL apoB-100 FCR and plasma ceramides remained significant after adjustment for plasma triglycerides (partial $r=-0.73$, $p=0.01$), total cholesterol (partial $r=-0.72$, $p=0.01$), and LDL-cholesterol (partial $r=-0.73$, $p=0.01$); however, adjustment for apoC-III attenuated the association (partial $r=-0.53$, $p=0.09$). Although the change in VLDL apoB-100 concentration was not significantly associated with the change in total plasma ceramide concentration, it was directly associated with the change in plasma ceramides C18:0 ($r=0.60$, $p=0.04$) and C20:0 ($r=0.59$, $p=0.045$). The reduction in plasma sphingosine was associated with a reduction in IDL apoB-100 concentration ($r=0.74$, $p=0.006$). No significant post-treatment associations were found with VLDL apoB-100 PR and plasma ceramide. Furthermore, no significant associations were found between changes in LDL apoB-100 kinetics and the plasma sphingolipidome.

**Post-treatment associations: Plasma sphingolipidome and HDL kinetics with R40**

No significant associations were observed between plasma sphingolipids and apoA-I kinetic parameters at baseline or with R10 and R40.
**DISCUSSION**

*Principal finding*

Our principal findings were that total plasma and individual ceramide concentrations were directly associated with baseline VLDL apoB-100 concentration. Furthermore, the change in ceramide concentration resulting from high-dose rosuvastatin treatment was inversely associated with VLDL apoB-100 FCR, in men with the metabolic syndrome. This association was independent of changes in plasma triglycerides, total- and LDL-cholesterol. This association, however, was not observed with plasma sphingomyelin nor with treatment using low dose rosuvastatin. Given that sphingomyelin and ceramide may undergo inter-conversion, a process facilitated by sphingomyelinase and sphingomyelin synthase, the lack of an association between plasma sphingomyelin and VLDL fractional catabolism was surprising. We postulate that this may reflect a greater contribution of ceramide in the regulation of VLDL catabolism compared with other sphingolipids. Differences between low and high dose rosuvastatin suggests that dose effect may also be a contributing factor with a higher dose required to elicit ceramide effects on VLDL particle clearance. To our knowledge, this is the first study to examine associations between ceramides and the kinetics of apoB-100-containing lipoproteins, and to report on statin-induced changes in plasma ceramides and lipoprotein kinetics in humans.

*Potential Mechanisms*

Sphingolipids are packaged into primordial VLDL apoB particles in the liver, though they are not prerequisites for VLDL secretion (12), and are associated with lipoproteins in circulation. VLDL-lipids (glycerolipids and sphingolipids) can undergo enzymatic hydrolysis, conversion to lower or higher order lipid species, and/or can be exchanged with high-density lipoproteins. These processes facilitate the conversion of VLDL to IDL and LDL through the delipidation cascade. ApoB-containing lipoprotein clearance from circulation is achieved primarily by hepatic receptors (13).
Elevated sphingolipid enrichment of lipoproteins is associated with dysregulated lipoprotein metabolism, in particular overproduction and delayed catabolism of apoB-containing lipoproteins, attenuated LCAT activity, and increased resistance to LPL-mediated lipolysis. Furthermore, increased sphingolipid enrichment increases the susceptibility to arterial sphingomyelinase and promotes lipoprotein aggregation and arterial foam cell formation (14-19). Our finding of an association between plasma ceramide and impaired VLDL apoB catabolism is consistent with the aforementioned mechanisms in men with the metabolic syndrome.

The mechanisms by which statins modulate sphingolipid metabolism are unknown. Rosuvastatin may decrease hepatic ceramide incorporation onto VLDL particles prior to secretion, making the particles more amenable to LPL-mediated lipolysis and receptor-mediated clearance, and together with statin-induced upregulation of LDL receptor expression may account for the association between plasma ceramides and VLDL apoB catabolism. Rosuvastatin also lowers plasma apoC-III, which promotes LPL-mediated hydrolysis and hepatic uptake of remnant lipoproteins (20). That the association between plasma ceramide and VLDL apoB fractional catabolism was attenuated by plasma apoC-III suggests that the mechanistic drivers may be partly mediated by apoC-III, though this warrants further investigation.

Adiponectin has recently been shown to lower ceramide levels by altering ceramidase activity via adiponectin receptors and may be independent of AMPK (21), though this warrants further investigation. We have previously reported an association between low plasma adiponectin and impaired VLDL apoB fractional catabolism in a cohort of men with wide ranging adiposity (22). Rosuvastatin may lower plasma ceramide, in part, by modulating adiponectin effects on hepatic ceramidase activity prior to VLDL synthesis and secretion, thus producing a particle that is amenable to hydrolysis and clearance. Because ceramide impairs insulin signalling in different tissues, it is plausible that changes to plasma ceramide with rosuvastatin may improve peripheral insulin signalling to facilitate lipoprotein clearance without changes to insulin resistance. In addition, whether statins contribute to the adiponectin-mediated effects
on plasma ceramides warrants further investigation. Lastly, because our study did not include the kinetics of VLDL\textsubscript{1} and VLDL\textsubscript{2} subfractions, which have distinct metabolic characteristics, the associations observed in this study may differ with VLDL subspecies.

Conclusions and implications

Elevated plasma ceramide and sphingomyelin concentrations are associated with atherogenesis, dyslipoproteinemia, insulin resistance, and type-2 diabetes. Clinical trials have demonstrated that statins reduce CVD morbidity and mortality, but a significant residual CVD risk remains. We show that plasma ceramide is related to the fractional catabolism of VLDL apoB in men with the metabolic syndrome. In light of previously reported associations between plasma ceramide and CVD, this may have potential implications for reducing the development of atherogenesis and could offer new therapeutic avenues beyond LDL-cholesterol lowering. Because age and gender can influence the plasma lipidome, further studies in larger cohorts using standardized extraction protocols are required to corroborate our findings.
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REFERENCES


Figure 1. Correlation between total plasma concentration of ceramides and VLDL apoB-100 FCR before treatment with rosvastatin in men with the metabolic syndrome. Plasma ceramides are carried by apoB-containing lipoproteins and high-density lipoproteins in circulation with varying composition.
Figure 2. Correlation between the percent change in plasma concentration of ceramides and percent change in VLDL apoB-100 FCR after treatment with 40 mg/day rosuvastatin. The correlation remained significant after adjustments for the reductions in plasma triglyceride (partial $r=-0.73$ $P=0.01$), cholesterol (partial $r=-0.72$ $P=0.01$), and LDL-cholesterol (partial $r=-0.73$, $P=0.01$) concentrations.