Serum sphingomyelin levels are related to the clearance of postprandial remnant-like particles

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Short title

Sphingomyelin and remnant-like particles

Acknowledgments

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1. Abbreviations used in this paper: RLP, remnant-like particles; BLp, apoB-containing lipoprotein particles; TRL, triglyceride-rich lipoprotein; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; SM, sphingomyelin; TG, triglyceride.
Abstract

It is known that sphingomyelin (SM) content is higher in apoB-containing particles (BLp) than in high-density lipoprotein, and that BLp levels, including chylomicrons and their remnant particles, are positively related to atherosclerosis. To evaluate the relationship between serum SM and postprandial remnant particle levels, we determined SM, triglyceride (TG), and cholesterol levels in serum and in remnant-like particles (RLP) before and 3, 5, 7, and 10 hours after a high-fat meal, in 31 healthy subjects. We found that serum SM, like serum TG, was increased to its maximum 3 hours after fat loading, and then gradually decreased to basal levels after 10 hours. More important, we determined that SM and TG levels in RLP were parallel. Serum SM was positively correlated with serum TG (p<0.001), RLP SM (p<0.001), RLP TG (p<0.001), and RLP cholesterol levels (p<0.001), respectively. It is our conclusion that serum SM is a marker for the clearance of RLP.
Introduction

Sphingomyelin (SM) is the most abundant sphingolipid in plasma membranes, organelle membranes, and lipoproteins. Abnormal SM metabolism has been associated with atherosclerosis and other diseases (1,2). It is well known that the SM content is much higher in apoB-containing lipoprotein particles (BLp) or triglyceride-rich lipoproteins (TRL) than in high-density lipoprotein (HDL) (3). Elevated plasma levels of BLp, including chylomicrons, very low-density lipoprotein (VLDL), and their remnants, as well as low-density lipoprotein (LDL), are acknowledged risk factors for cardiovascular disease (4,5).

It has been suggested that retention of BLp on the subendothelial matrix and subsequent aggregation triggers macrophage foam cell formation and atherogenesis (6). BLp aggregation in the vessel wall may result from enzymatic modification of BLp, induced by locally produced sphingomyelinase (7).

In a case-control study (279 cases and 277 controls), we have previously shown that plasma SM levels are an independent risk factor for coronary artery disease (CAD), and also demonstrated a significant correlation with remnant cholesterol levels (8). Based on these results, we hypothesized that SM could be a marker for the clearance of TRL remnants, and that enrichment of SM on the remnants could be atherogenic. In the present study we investigated: 1) postprandial serum SM and RLP SM levels after high-fat diet loading, and 2) a possible predictive value of serum SM for RLP metabolism. We found that SM is a clearance marker for RLP, and also that prolonging the metabolic rate of those particles might have atherogenic consequences.
Material and Methods

Study population

The group studied consisted of 31 subjects with no clinical or anamnestic evidence of atherosclerosis. All subjects were of German nationality, and were inhabitants of the Rhein-Main Area. The study was approved by the ethics committee of the University of Mainz. Participation was voluntary and each study subject gave written informed consent.

Laboratory methods

Blood samples were taken from all subjects under standardized conditions after an overnight period of fasting. After this first blood sample was taken, subjects ate, in a period of approximately 15 minutes, a meal high in fat, consisting of a total of 1,265 kcal/m² body surface area (105 g of fat, consisting of 52 g saturated fat and 300 mg cholesterol, with 48 g carbohydrates and 32 g protein). Subjects were instructed not to eat any other food until after the last blood sample. Further blood samples were taken after 3, 5, 7, and 10 hours. These were immediately centrifuged at 4,000 rpm for 10 minutes, and divided into aliquots. All aliquots for the detection of total cholesterol, HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), apolipoprotein A-I (apoA-I), apolipoprotein B (apoB), and triglyceride were refrigerated until analysis. Samples for the detection of SM and RLP were immediately frozen at –80°C until analysis.

Serum SM levels were measured as previously described (8). RLP cholesterol, RLP SM, and RLP TG levels were determined using a modification of an RLP assay system (Japan Immunoresearch Laboratories, Co., Ltd., Japan). Briefly, 20 µl serum was incubated with 400 µl gel containing apoB-100 and apoA-I antibodies, according to the kit guidelines.
The supernatant (approximately 350 µl) was separated by centrifugation at 14,000 rpm for 10 minutes. The RLP–containing supernatant was lyophilized until 100 µl of solution remained. Cholesterol, SM, and TG levels were then determined in the supernatant. The intra-assay coefficients of variation for all three assays were less than 10%. The RLP cholesterol values determined by this modified method were comparable to the kit guidelines.

The total choline-containing phospholipid in plasma was assayed by an enzymatic method (Wako Pure Chemical Industries Ltd). PC concentration was obtained by subtracting SM from total phospholipid concentration. Serum lipid levels were determined immediately (cholesterol, Roche Diagnostics, Germany; HDL-C, Rolf Greiner Biochemica, Germany; LDL-C, calculated according to the Friedewald formula; TG, Roche Diagnostics, Germany). ApoA-I and apoB concentrations were determined by immunoturbidimetric assays (Tina-quant, Roche Diagnostics, Germany).

Statistical analysis

Estimating the correlation between lipid values, which were repeatedly measured in different time points from the same subject, is outside the scope of standard correlation (i.e., Pearson r) and linear regression analysis, because the observations are not independent. Therefore, a general linear mixed model was used to perform repeated measures regression in predicting time-dependent lipid measures. From this model we could estimate correlation values. Here, we chose serum SM or serum TG as predictors for the dependent measure of other lipid parameters. Since a general linear mixed model does not produce a multivariate R term, an analog of R was estimated. This was done by calculating the correlation of the predicted model values and the actual values of RLP SM, RLP TG, and RLP cholesterol, respectively. All analyses were carried out using SPSS 11.0.
Results

RLP isolation was based on removal of apo A-I-containing particles (HDL) and most apo B-containing particles (LDL, nascent VLDL, and nascent chylomicrons), using an immunoseparation technique (Japan Immunoresearch Laboratories, Takasaki, Japan), as previously described (9). This has been shown to leave particles characteristic of the previously mentioned chylomicron and VLDL remnants in the unbound fraction (10).

We found that mean serum concentrations of TG, SM, RLP cholesterol, RLP TG, and RLP SM, were low at fasting. These levels were increased 3 hours after the high-fat meal, and then returned toward pre-treatment levels during the subsequent 7-hour study period (Figs. 1 and 2). The differences between fasting and 3-hour points were statistically significant in all parameters, as compared by ANOVA. The differences between fasting and 5-hour points remained significant for serum TG, serum SM, RLP TG, and RLP SM, respectively. The differences between fasting and 7-hour or 10-hour points remained significant for serum TG and serum SM (Figs. 1 and 2), respectively. As presented in Table 1, serum concentrations of LDL-C, HDL-C, phosphotidylcholine (PC), apoA-I, and apoB were nearly stable during the study period.

To evaluate further the predictive value of serum SM for other lipid parameters, we created a general linear mixed model. This model, implying the average correlation across time between SM and other parameters, indicated that serum SM has significant predictive value on serum TG (R = 0.552, p<0.001), RLP SM (R = 0.658, p<0.001), RLP TG (R = 0.535), and RLP cholesterol (R = 0.647, p<0.001), respectively (Table 2). We also used this model to correlate between serum TG and other variables, finding that serum TG also has significant predictive value on serum SM (R=0.521, p<0.001), RLP cholesterol (R=0.369, p<0.01), RLP SM (R=0.327, p<0.01), and RLP TG (R=0.96, P<0.001) (Table 3).
Discussion

In the present study, we have demonstrated for the first time that: 1) postprandial serum SM and RLP SM levels, like serum TG levels, have a bell-shaped curve with a peak at 3 hours; 2) serum SM levels have a statistically significant predictive value for serum TG, RLP SM, RLP TG, and RLP cholesterol, respectively; and 3) serum TG levels also have a statistically significant predictive value for serum SM, RLP SM, RLP TG, and RLP cholesterol, respectively. These results suggest that SM is one of the RLP clearance markers.

Recent data imply that impaired postprandial lipoprotein metabolism may contribute to, or be a marker for, the development and progression of atherosclerotic diseases (11). Patients with ischemic heart disease have delayed clearance of triglyceride-rich lipoproteins following consumption of a high-fat meal, compared with controls (11,12). Several lines of evidence suggest that such remnants are particularly atherogenic (13). RLP show a marked increase and remain high even 8 hours after fat loading, especially in patients with coronary artery disease or diabetes mellitus, indicating that the postprandial state persists almost the whole day in these patients (14). Immunohistochemical studies indicate colocalization of anti-apo B48 receptor antibody in human atherosclerotic lesion foam cells, implying that apo B48 receptor may contribute to foam cell formation and atherosclerosis (14).

Remnants are related to intima media thickness, an early marker for atherosclerosis and an important prospective marker for patients with CAD (15). Studies have also demonstrated the association of delayed postprandial fat clearance with abnormalities in the lipoprotein profile. Delayed clearance is seen in conjunction with hypertriglyceridemia, low levels of HDL cholesterol (primarily the anti-atherogenic HDL₂ subfraction), and the presence of small dense LDL, i.e., a pattern typical of insulin resistance (16). Patients carrying the apoe2 allele also have slower postprandial fat clearance (17). It is known that
plasma SM cannot be degraded by plasma enzymes such as lecithin cholesterol acyltransferase (LCAT) or by lipase, and that SM removal from plasma is entirely dependent on hepatic clearance mechanisms. Plasma SM tends to become enriched in atherogenic remnants of TRL (18). Based on our results (Figs. 1 and 2), plasma (or serum) and RLP SM measurements could act as markers for atherogenic remnant accumulation.

Several studies have suggested that postprandial SM and cholesterol-rich remnants may cause intracellular lipid loading. Van Lenten et al. demonstrated that RLP are taken up by monocyte-derived macrophages in the arterial wall, contributing to atherosclerotic plaque formation (19). Gianturco et al. (20) found that RLP from human serum caused cholesterol accumulation in fibroblasts. Van Lenten et al. (21) demonstrated that RLP induced foam cell formation in macrophages, while Georgopoulos et al. (22) found that postprandial TRL from diabetic subjects produced cholesterol accumulation in macrophages. Yu et al. provided specific evidence that remnants of postprandial lipoproteins, i.e., chylomicrons, are capable of inducing foam cell formation in human monocyte-derived macrophages, notably in the absence of significant oxidative modification (23).

In animal studies, it has been further confirmed that plasma SM is a risk factor for atherosclerosis. ApoE deficiency is a well-known mouse model for atherosclerosis (24), and the accumulation of RLP that are cholesterol- (24) and SM-enriched (18) in the circulation is the distinctive factor. These mice develop atherosclerotic lesions spontaneously at the age of 2 months without any dietary fat or cholesterol induction at all (24).

TG is a well known RLP clearance marker (12-14). In this study, we do not emphasize that SM-RLP is better than TG-PLP as a marker for RLP metabolism, but we emphasize that SM-RLP levels has its own specialty, in terms of atherogenicity. SM is not degraded in plasma, it becomes enriched in remnants of TRL (3). SM removal from plasma is dependent on hepatic clearance mechanisms, such as the LDL receptor, the LDL receptor–
related protein, or proteoglycan pathways. Substantial evidence now supports the role of lipoprotein SM and arterial SMase in atherogenesis (6-8). SM carried into the arterial wall on atherogenic lipoproteins is acted on by an arterial wall SMase, leading to an increase in ceramide content and promoting lipoprotein aggregation (6,7).

As humans, we spend most of our lives in the postprandial state. It is entirely possible that postprandial SM, which resides on RLP, has an impact on atherosclerosis. If these SM-rich particles accumulate in the circulation, they clearly have more opportunity to be deposited on the arterial wall and to aggregate there as a result of sphingomylinase activity (6). We cannot of course overlook other possibilities: for example, that BLp is modified in the arterial wall by processes such as oxidation, leading to the formation of macrophage foam cells and initiating atherosclerosis (25). But at this juncture it seems to us that the likelihood lies strongly in the direction of SM.
References


Table 1. Fasting and postprandial lipid and apolipoprotein parameters. All results are presented as mean ± SE.

<table>
<thead>
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<th></th>
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<th>5hr</th>
<th>7hr</th>
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<td>101±7</td>
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<td>53±2</td>
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<td>1.37±0.05</td>
<td>1.36±0.05</td>
<td>1.37±0.05</td>
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Table 2. Correlation between serum SM and other variables. A general mixed linear model was utilized to estimate the multivariate R for serum SM with serum TG, RLP SM, RLP TG, and RLP cholesterol, respectively.

<table>
<thead>
<tr>
<th>Serum TG*</th>
<th>RLP Cholesterol*</th>
<th>RLP SM</th>
<th>RLP TG</th>
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<td>Serum SM</td>
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<td>0.658†</td>
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<td>n</td>
<td>152</td>
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*Log-transformed variables were used for skewed distribution. †: p < 0.001.
Table 3. Correlation between serum TG and other variables. A general mixed linear model was utilized to estimate the multivariate R for serum TG with serum SM, RLP SM, RLP TG, and RLP cholesterol, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Serum SM</th>
<th>RLP Cholesterol*</th>
<th>RLP SM</th>
<th>RLP TG</th>
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<tr>
<td>Serum TG*</td>
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<td></td>
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</tr>
<tr>
<td>R</td>
<td>0.521†</td>
<td>0.369†</td>
<td>0.327†</td>
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<tr>
<td>n</td>
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*Log-transformed variables were used for skewed distribution. †: p < 0.01.
**Figure legends**

**Figure 1:** Postprandial TG, SM, and cholesterol levels. The serums were prepared and the TG, SM, and cholesterol concentrations were determined as described in “Material and Methods.” All results are presented as average ± standard error. Statistical analysis was based on the comparison of fasting levels with 3h, 5h, 7h, and 10h levels by ANOVA. *p<0.05.

**Figure 2:** Postprandial RLP TG, PLP SM, and RLP cholesterol levels. The RLP were prepared and the RLP TG, RLP SM, and RLP cholesterol concentrations were determined as described in “Material and Methods.” All results are presented as average ± standard error. Statistical analysis was based on the comparison of fasting levels with 3h, 5h, 7h, and 10h levels by ANOVA. *p<0.05.
Fig. 2. Schlitt et al.

A

RLP Triglyceride (mg/dl)

0 25 50 75

Fast 3h 5h 7h 10h

B

RLP Sphingomyelin (mg/dl)

0 5 10 15 20

Fast 3h 5h 7h 10h

C

RLP Cholesterol (mg/dl)

0 5 10 15 20 25

Fast 3h 5h 7h 10h