Dietary inulin decreases circulating ceramides by suppressing neutral sphingomyelinase expression and activity in mice

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Abbreviations
AcCa: acylcarnitine; Akr: aldo-keto reductases; Ccl5: C-C motif chemokine ligand 15; Ccl19: C-C motif chemokine ligand 19; ChE: cholesterol ester; CL: cardiolipin; CoQ: coenzyme Q; DEGs: differentially expressed genes; DHCer: dihydroceramide; EV: extracellular vesicle; FPLC: fast protein liquid chromatography; Itk: interleukin-2-inducible T-cell kinase; Gpx6: glutathione peroxidase 6; HexCer: glucosylceramide; MG: monoglycerides; MTBE: methyl tertiary-butyl ether; Naip1: NLR family apoptosis inhibitory Protein; PAI1: plasminogen activator inhibitor 1; PPEE: posterior probability of equal expression; QC: quality control; Smpd3: sphingomyelin phosphodiesterase 3.
Abstract

Elevated circulating levels of ceramides are associated with increased risk of cardiometabolic diseases, and ceramides may play a causative role in metabolic dysfunction that precedes cardiac events, such as mortality as a result of coronary artery disease. Although the mechanisms involved are likely complex, these associations suggest that lowering circulating ceramide levels could be protective against cardiovascular diseases. Conversely, dietary fibers, such as inulin, have been reported to promote cardiovascular and metabolic health. However, the mechanisms involved in these protective processes also are not well understood. We studied the effects of inulin on lipid metabolism with a model of atherosclerosis in LDL receptor–deficient mice using lipidomics and transcriptomics. Plasma and tissues were collected at 10 days and/or 12 weeks after feeding mice an atherogenic diet supplemented with inulin or cellulose (control). Compared with controls, inulin-fed mice displayed a decreased C16:0/C24:0 plasma ceramide ratio and lower levels of circulating ceramides associated with VLDL and LDL. Liver transcriptomic analysis revealed that Smpd3, a gene that encodes neutral sphingomyelinase (NSMase), was downregulated by 2-fold in inulin-fed mice. Hepatic NSMase activity was 3-fold lower in inulin-fed mice than in controls. Furthermore, liver redox status and compositions of phosphatidylserine and free fatty acid species, the major factors that determine NSMase activity, also were modified by inulin. Taken together, these results showed that, in mice, inulin can decrease plasma ceramide levels through reductions in NSMase expression and activity, suggesting a mechanism by which fiber could reduce cardiometabolic disease risk.

Keywords: ceramide, fiber, inulin, lipidomics, transcriptomics, sphingomyelinase
**Introduction**

Soluble fibers such as inulin-type fructans are natural components of several edible fruits and vegetables, and the average individual daily consumption has been estimated to be between 1 and 11 g in Europe and the United States (1, 2). A very recent meta-analysis found that people consuming high levels of dietary fiber have a lower risk of cardiovascular mortality and incidence of common non-communicable diseases, including coronary heart disease and type 2 diabetes, than those eating less fiber (3). Dietary supplementation with inulin-type fructans has been associated with the reduction of cardiovascular disease risk in clinical trials (4, 5). Accumulating evidence has revealed that dietary inulin can modulate gut microbiota composition and generate short chain fatty acids, which are associated with beneficial effects on host lipid metabolism, such as decreased circulating cholesterol and triglyceride levels (6-8). However, the effects of inulin on systemic lipid metabolism remain to be investigated. Identification of these mechanisms might lead to improved approaches for dietary interventions to reduce cardiovascular disease risk.

Ceramides (Cer) are amide linked fatty acid esters of sphingosine. They are precursors of complex sphingolipids. Both *in vitro* and *in vivo* studies have shown that the aberrant accumulation of Cer lead to the activation of several signaling systems that induce insulin resistance, hepatic steatosis, and cardiometabolic disorders (9-12), suggesting that Cer plays a causative role in the metabolic dysfunction that precedes cardiovascular events. Recently, several studies reported divergent associations of distinct plasma Cer with cardiovascular disease mortality in patients with coronary artery disease (13-15). Plasma Cer species are prospective biomarkers of adverse cardiovascular events with tests now being offered by the Mayo clinic (16). Cer can be produced by three different pathways: (1) *de novo* synthesis from palmitate and serine in four sequential reactions in the endoplasmic reticulum, (2) hydrolysis of sphingomyelin catalyzed by sphingomyelinase (SMase), and (3) a salvage pathway in which sphingosine can be resterified to form Cer. Pharmacological inhibition or genetic ablation of enzymes driving Cer synthesis ameliorates atherosclerosis, insulin resistance, and cardiomyopathy (17-21). Although there are
no pharmacological strategies that safely and effectively manipulate Cer in humans, dietary interventions via the Mediterranean diet (22) and Nordic diet (23), which contain high levels of dietary fiber, are associated with reductions in plasma Cer levels. Additionally, a fruit and vegetable diet based intervention designed according to the USDA MyPlate guidelines was shown to be effective at reducing plasma Cer (24). However, the mechanism(s) responsible for these dietary effects remains unclear.

Systems biology is central to the biological and medical sciences (25). The advent of genotyping arrays enabled studies of global transcriptome. Metabolomic/lipidomic analysis, as the last step in a series of changes following external stimuli insult or in pathological states, can directly reveal phenotypic changes of metabolites in living systems. LC-MS based metabolomic/lipidomic approaches can rapidly and sensitively detect metabolic changes. In the present study, the effects of inulin on lipid metabolism were elucidated in low density lipoprotein receptor knock-out (Ldlr⁻/⁻) mice. Using lipidomics and transcriptomics, the present study demonstrates that inulin feeding can decrease circulating Cer and this is associated with downregulation of NSMase expression and activity. These results provide evidence for a mechanism by which dietary fiber reduce cardiovascular risk and disease.
Materials and Methods

Chemicals

Lipid internal standards including LPC 17:0, PC 18:0-d13, SM 18:1-d9, PS 17:0, PE 17:0, and cholesterol-d7 were purchased from Avanti Polar Lipids (Alabaster, AL). Cer 24:1-d7 was obtained from Cayman Chemical (Ann Arbor, MI). Other chemicals and solvents were of reagent grade, except for the HPLC-grade solvents used for LC-MS analysis.

Animal Experiments

Seven-week-old male Ldlr⁻/⁻ mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and allowed to acclimate for 1 week. All animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of University of Kentucky. Efforts were made to minimize the suffering of experimental animals. Animals were housed in plastic cages with corncob bedding, five mice per cage, in an environmentally controlled mouse room. All mice received diets and tap water ad libitum throughout the experiment. Mice were randomly divided into study groups (n = 10 per group) with half of them receiving the atherogenic Clinton/Cybulsky diet containing 8% cellulose (Research Diets, New Brunswick, New Jersey; Product No. D01061401C) or with the other half receiving the atherogenic Clinton/Cybulsky diet containing 8% inulin. The Ldlr⁻/⁻ mouse model has been previously shown to develop hyperlipidemia after feeding on the base atherogenic diet (26). The contents of fiber in diet was chosen to represent a recommended human high fiber consumption that would confer health benefits (3). Further details of the animal diet are described elsewhere (Hoffman J., et.al, Prebiotic inulin consumption reduces dioxin-like PCB 126-mediated hepatotoxicity and gut dysbiosis in hyperlipidemic Ldlr deficient mice, submitted to Environmental Pollution). After 10 days of feeding, blood was collected from mouse tail veins into tubes added with ethylenediaminetetraacetic acid, and plasma was separated after centrifugation at 3000×g for 15 min at 4°C. At the completion of week 12, animals were anesthetized using isoflurane prior to euthanasia. Plasma samples were harvested and frozen.
in liquid nitrogen. Tissues were harvested for mRNA and protein and all samples were stored at -80 °C prior to analysis.

**Lipidomic Analysis of Plasma and Liver**

Plasma and liver samples were extracted using a method reported before (27). Briefly, 20 mg liver tissues were homogenized in 200 µL of 0.1% ammonium formate. The homogenate was spiked with internal standard solution and mixed with 1.47 mL of methanol. Then, 5 mL of methyl tertiary-butyl ether (MTBE) was added and the sample was shaken for 1 h at room temperature. Phase separation was induced by adding 1.25 mL of H₂O, and the mixture was centrifuged for 10 min at 3000×g at 4°C. The upper lipid phase was then collected and dried under nitrogen. The lipid residue was dissolved in 400 µL of mixed solvent of chloroform and methanol (2:1, v/v). For plasma samples, 20 µL of plasma was extracted with 500 µL of MTBE twice and the combined extract was processed as above. Lipidomic analysis was performed using an Ultimate 3000 ultra high performance liquid chromatography (UHPLC) system coupled to a Thermo Q-Exactive Orbitrap mass spectrometer equipped with a heated electrospray ion source (Thermo Scientific, CA, USA). Lipid extracts were separated on a Waters ACQUITY BEH C8 column (2.1 × 50 mm, 1.7 µm) with the temperature maintained at 40°C. The flow rate was 250 µL/min, and the mobile phases were consisted of 60:40 water/acetonitrile (A), and 90:10 isopropanol/acetonitrile (B), both containing 10 mM ammonium formate and 0.1% formic acid. The samples were eluted with a linear gradient from 32 % B to 97 % B over 25 min, maintained at 97 % B for 4 min and re-equilibration with 32 % B for 6 min. The sample injection volume was 5 µL. The mass spectrometer was operated in positive and negative ionization modes. The full scan and fragment spectra were collected at a resolution of 70,000 and 17,500, respectively. Quality control (QC) samples prepared from pooled mouse plasma and liver extracts were used to monitor the overall quality of the lipid extraction and mass spectrometry analyses. QC samples were included in batches of analytical samples during the course of the study. The average coefficient of variation of major lipids detected in the QC samples was 20%. Data analysis and lipid identification were performed using the software Lipidsearch 4.1 (Thermo Fisher, CA, USA).
Within a lipid class, MainArea values output by LipidSearch were assigned to each fatty acid moiety of a given lipid species and summed using Microsoft Excel PivotTable. The peak areas of interested lipids and internal standards were further analyzed using Thermo Xcalibur 4.0 QuanBrowser. The peak areas for each lipid class were normalized by internal standards. For liver samples the data was further normalized to tissue weight.

**Plasma fractionation using fast protein liquid chromatography (FPLC)**

Fractionation of plasma into lipoprotein-associated pools was performed as described previously (28). Briefly, plasma samples (n = 3/group) collected after 12-weeks of feeding were pooled for each group, and 50 µL of pooled plasma samples were fractionated by fast protein liquid chromatography using a calibrated Superose 6 increase (10/300GL; GE Healthcare) column attached to an Agilent 1100 HPLC system. The column was equilibrated and eluted with PBS at a flow rate of 0.5 mL/min. Eluate was collected into a 96 deep-well plate using an Agilent 1200 (G1364B) collector as 21 fractions which include VLDL, LDL, and HDL. Lipids in plasma fractions were analyzed using UHPLC-Q Exactive MS as described above.

**Transcriptome and bioinformatics analysis of mouse liver**

Mouse liver samples were homogenized and total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific Inc, Waltham, MA, USA). The quality of RNA was assessed using the Agilent 2100 Bioanalyzer system and three samples from each group with a RNA Integrity Number greater than 8 were used for RNA library construction. The library was sequenced by the BGI Americas (San Jose, CA). Single-end reads of 50 bp read-length were sequenced on the BGISEQ-500RS sequencer. The "dirty" raw reads were firstly removed which were defined as reads that are at low quality, containing the sequence of adaptor, and high content of unknown bases. After filtering, the remaining reads are stored as FASTQ format. Quality-filtered sequence reads were mapped to mouse genome reference MM10. Readcount data were then subjected to differential expression analysis using EBSeq method. Genes with a fold change inulin/control ≥ 2 and posterior probability of equal expression (PPEE) ≤ 0.05 were considered as
differentially expressed genes (DEGs). The DEGs were subjected to GO functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using algorithm developed by BGI. The calculated P-value goes through Bonferroni Correction, taking corrected P-value \( \leq 0.05 \) as a threshold.

**Real-time PCR**

Total RNA was extracted as described above. cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). PCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the Taqman Fast Advanced Master Mix (Thermo Fisher Scientific Inc). Primer sequences from Taqman Gene Expression Assays (Thermo Fisher Scientific Inc) were as follows: sphingomyelin phosphodiesterase 1 (Smpd1); (Mm00488318_m1), sphingomyelin phosphodiesterase 2 (Smpd2); (Mm01188195_g1), sphingomyelin phosphodiesterase 3 (Smpd3); (Mm00491359_m1), sphingomyelin synthase 1 (Sgms1); (Mm00522643_m1), sphingomyelin synthase 2 (Sgms2); (Mm00512327_m1) and beta actin (Actb); (Mm02619580_g1). The levels of mRNA were normalized relative to the amount of Actb mRNA, and expression levels in mice fed control diet were set at 1. Gene expression levels were calculated according to the \( 2^{-\Delta\Delta Ct} \) method (29).

**Measurement of NSMase activity**

Liver tissue was homogenized in 40 volumes of buffer containing 25 mM Tris-HCl, 150 mM NaCl, and 1% Triton X-100, pH 7.4. NSMase activity was determined using the assay kit from Echelon Biosciences (Salt Lake City, UT) following manufacturer’s instructions. Dithiothreitol at 5mM was added to the incubation mixture to inhibit any acid sphingomyelinase activity (30). In this assay, NSMase converts sphingomyelin substrate to phosphocholine, which is hydrolyzed by alkaline phosphatase and reacted with choline oxidase to generate hydrogen peroxide that is measured colorimetrically at 595 nm. A standard curve of absorbance values of known amounts of choline standards was generated. Sphingomyelinase activity in the samples (nmol/min/mL) was calculated from their corresponding
absorbance values via the standard curve. The enzyme activity was normalized using protein levels quantified by the bicinchoninic acid assay.

**Statistical Analyses**

Partial least-squares discriminant analysis (PLS-DA) of lipidomic data was used to identify initial trends and clusters in data sets, which was performed using the Metaboanalyst 4.0 web portal (www.metaboanalyst.ca). Statistical analyses of RT-qPCR gene expression, enzyme activity, and lipidomic data were performed using the GraphPad Prism version 7.04 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Comparisons between groups were made by unpaired Student’s t-test considering significance at the level of \( P < 0.05 \). Data are expressed as mean ± SEM.
Results

*Lipidomic analysis of plasma after inulin feeding for 10 days and 12 weeks*

Lipid extracts of plasma collected 10 days and 12 weeks after initiation of feeding the inulin or cellulose (control) enriched diet were analyzed using UHPLC-Q Exactive MS. A total of 768 and 747 lipid species were identified in the plasma obtained after 10 days and 12 weeks of feeding, respectively, with coverage of 13 different lipid subclasses across all samples. A Partial Leas Squares Discriminant Analysis (PLS-DA) was performed to identify groups of lipids that contribute to the differential effects of inulin diet on the mouse plasma lipidome. There were clear differences between inulin and cellulose fed mice in plasma samples obtained after 10 days but not in those after 12 weeks of feeding (Supplemental Figure S1), indicating considerable variation in the plasma lipid composition at 10-day after treatment, and this variation was blunted at 12-weeks. Further analysis of lipidomic data by unpaired Student t-test revealed that sphingolipids and glycerolipids were the most significantly changed lipid classes between inulin-fed and control mice (Figure 1). In plasma samples obtained at 10-day after feeding, the total level of DG, monoglycerides (MG), SM and Cer were decreased compared with the control group. In plasma samples collected at 12-week, the levels of Cer, cholesterol esters (ChE) and LPC were significantly lower in inulin-fed mice than control mice. Our further studies therefore focused on investigating possible mechanisms linking dietary inulin supplementation to decreases in plasma Cer. In samples obtained after 10-days of feeding, five major Cer species were present at 39-51% lower levels in inulin-treated mice than those in control mice (Figure 2A). After 12-weeks of feeding, the major Cer species decreased in inulin-fed mice included Cer 16:0, 20:0, and 24:1, with levels present at 47% lower than those in control mice (Figure 2B). Recent studies have suggested that the ratios between Cer in plasma are more closely related with diseases than the total amounts, particularly the long-chain Cer 16:0, in relation to very-long-chain Cer 24:0 (31). In the present study, the Cer 16:0/C24:0 ratio was calculated and there was no significantly difference between the two groups in samples obtained at day-10 after feeding. However, a decreased ratio in the inulin group was observed in samples obtained after 12-weeks of feeding (Figure
2C). SM is a major precursor for Cer and can be hydrolysis by sphingomyelinase to produce Cer. Among the major SM species in plasma, the level of SM 16:0 decreased by 31% after 10 days of inulin treatment (Figure 3A). After 12-weeks, the levels of SM 20:0 and 22:0 were increased by 74% and 124% respectively, in inulin-fed mice compared with controls (Figure 3B). In both 10-day and 12-week plasma samples, ratios of Cer/SM were more than 42% lower in inulin-fed mice than control mice (Figure 3C-D). Combined, these data suggest that inulin influences plasma level of sphingolipids, especially Cer within 10 days after dietary intervention, and the effect persisted for at least 12 weeks.

**Effect of dietary inulin on liver lipidome**

Although Cer can be synthesized in virtually every organ, the liver is a key site for Cer production. Additionally, different organs have different Cer profiles, and the Cer composition in plasma is most similar to that in liver (32). Therefore, mice liver samples were analyzed using the lipidomic method. Inulin feeding was not associated with significant changes in Cer and SM levels in liver but significantly altered the contents of DG and cardiolipin (CL) (Figure 4). CL is an essential phospholipid that makes up to 15%–20% of the inner mitochondrial membrane and is linked to mitochondrial function (33, 34). Unlike other hepatic phospholipid classes which showed no differences after inulin intervention, CL levels were increased by more than 43% in the inulin fed group, especially CL 72:6, 72:7, 74:9, 74:10, 76:6, 78:11, and 79:9 (Supplemental Figure S2). It was found that although there was no significantly changes in the total levels of phosphatidylserine (PS) and free fatty acid (FFA) (Figure 4), liver composition of PS and FFA species with various fatty acyl chains were changed after inulin feeding, as demonstrated by no less than 48% increases in levels of PS species with long fatty acyl chains (total carbon n ≥ 40) and polyunsaturated bonds (double bond n ≥ 5). On the contrary, PS species with medium fatty acyl chains (total carbon n ≤ 34) and fewer double bonds (double bond n = 1 or 2) were decreased by more than 34% (Figure 5A). In all of the 23 FFA species detected in liver, inulin feeding reduced the levels of 11 unsaturated fatty acids by 25-51% (Figure 5B), Taken together, inulin feeding increased liver contents of CL and changed the hepatic compositions of PS and FFA.
Effect of dietary inulin on lipoprotein-associated sphingolipid levels

Cer is a component of lipoproteins and 98% of plasma Cer is associated with lipoprotein subfractions (35). In control diet fed mice, Cer was mostly distributed in VLDL and LDL, with less than 14% of Cer associated with HDL (Figure 6A). Cer with different fatty acyl chains distributed differentially in lipoproteins. Long chain Cer16:0 was equally distributed in VLDL (46%) and LDL (40%), whereas more than 60% of very long chain Cer 20:0, 22:0, 24:0 and 24:1 were distributed in VLDL, and 17-31% was associated with LDL. In inulin-fed mice, VLDL and LDL-associated Cer decreased by 4- to 7-fold compared with the control group, and little effect on HDL-Cer was observed in inulin-fed mice compared with controls (Figure 6A-F), suggesting that dietary inulin decreases VLDL- and LDL- but not HDL-associated Cer. Dihydroceramide (DHCer), SM, and glucosyleramide (HexCer) are either precursor or downstream metabolite of Cer. In control diet fed mice, these sphingolipids demonstrated similar lipoprotein distributions as Cer with about 50-60% in VLDL, 30% in LDL and less than 15% in HDL fraction (Supplemental Figure S3). After inulin treatment, the VLDL associated SM, DHCer and HexCer were decreased by 1.8- to 5.2-fold compared with controls. There were slight decreases in LDL associated DHCer and HexCer, however no obvious changes in HDL associated DHCer and HexCer were observed. On the contrary, HDL associated SM was increased by 1.6-fold, and there was no change in LDL associated SM in inulin fed mice compared with controls (Supplemental Figure S3).

Transcriptomic Analysis of Liver after Inulin Feeding

To understand the molecular mechanisms underlying the effect of inulin on lipid levels, comparative transcriptomic analysis was performed (n = 3/group). A total of 59 DEGs were identified in the inulin-fed mice as compared to the control group, with 47 downregulated and 12 upregulated (Supplemental Table S1). The DEGs were then subjected to an analysis of their biological functions altered by inulin feeding. As shown in Figure 7, the top two ranked functions that were modified by inulin were signal transduction and the immune system, and five genes in these two categories related to inflammation, including interleukin-2-inducible T-cell kinase (Itk), NLR family apoptosis inhibitory Protein (Naip1), plasminogen...
activator inhibitor 1 (PAI1), C-C motif chemokine ligand 15 (Ccl5) and C-C motif chemokine ligand 19 (Ccl19), were all downregulated by inulin feeding (Supplemental Table S1). These results suggested that inulin may modify the inflammatory process through down-regulation of these genes. Furthermore, three genes associated with lipid metabolism were changed due to inulin feeding, including Aldo-keto reductases (Akr), sphingomyelin phosphodiesterase 3 (Smpd3), and glutathione peroxidase 6 (Gpx6) (Supplemental Table S1). Smpd3 encodes neutral sphingomyelinase (NSMase) that catalyzes the hydrolysis of SM to form Cer. Hepatic expression of Smpd3 was downregulated in inulin fed mice compared with controls. In order to validate the findings from the transcriptomic analysis, qPCR analysis was performed. Five genes involved in SM-Cer pathways including Smpd1, Smpd2, Smpd3, Sgms1, and Sgms2 were examined. We found that the results of qPCR analysis were well matched with the transcriptomic data, with Smpd3 being the only gene that was downregulated in inulin-fed mouse liver (Figure 8A).

**Measurement of NSMase enzymatic activity**

To investigate the functional relevance of downregulated hepatic gene expression of Smpd3 in inulin-fed mice, we sought to determine if inulin feeding leads to changes of NSMase activity in mouse livers. Measurements of catalytic activity revealed that the NSMase activity in inulin-fed mice was reduced by about 4-fold compared with control mice with P value of 0.005 (Figure 8B).
Discussion

Plasma and hepatic lipid (i.e cholesterol and triglyceride) profiles are well understood to change extensively with dietary inulin intervention in humans (8). We used sophisticated lipidomics to analyze effects of dietary inulin supplementation on not only common and abundant lipids, but also several less abundant lipid classes and species in a well characterized mouse model. Our study identified Cer as a major circulating lipid class that was downregulated by inulin feeding. Most importantly, the ratio of Cer 16:0/24:0 was decreased in mice after 12-weeks of inulin feeding. Lipoprotein analysis demonstrated that inulin feeding decreased VLDL and LDL associated Cer but had little effect on HDL-Cer. We also found that CL is a major lipid class in the liver that differed significantly between the control and inulin groups. Furthermore, hepatic composition of anionic lipids that control NSMase activity were modified due to inulin feeding. This study describes how plasma and hepatic lipid profiles are altered during inulin feeding and highlights the possible implications of these changes on hepatic NSMase activity.

Recent studies revealed that plasma Cer levels correlate strongly with adverse cardiovascular events, and Cer containing the C16:0, C18:0, and C24:1 fatty acyl chains display an independent predictive value of plaque instability and/or future fatality (36). Particularly, high Cer 16:0/24:0 ratios are strongly associated with major adverse cardiac events as well as other metabolic defects including coronary artery disease and insulin resistance (31). Our results indicated that the short-term (10 days) feeding of inulin could lower plasma levels of Cer but had no effects on the ratio of Cer 16:0/24:0. However, after long-term (12 weeks) inulin treatment, not only was the plasma levels of Cer lowered, but also the ratio of Cer 16:0/24:0 was decreased compare with cellulose-fed control mice. These results suggested a beneficial effect of long-term inulin dietary intervention. Cer can be produced from SM catalyzed by SMases, which are classified based on their pH optima of activity into acid, neutral, and alkaline subtypes, and NSMase appears to be the predominant enzyme involved in cell growth, apoptosis, and inflammation (37, 38). We found that that dietary inulin feeding lowered plasma Cer levels in an atherogenic mouse model, and this effect was associated with downregulated Smpd3 expression and NSMase activity. Previous studies
showed that NSMase is activated by unsaturated FA and anionic phospholipids, and specifically by PS (39, 40). In addition, it is a redox sensitive enzyme with TNF-α, oxidative stress, and inflammation being potent activators and antioxidants like glutathione acting as strong inhibitors (41). In inulin fed mice, the hepatic levels of polyunsaturated free fatty acids were significantly reduced compared with control mice, especially linoleic acid (C18:2) (Figure 5B), which is an established stimulator of NSMase (39). Additionally, although there was no difference in the total level of hepatic PS between inulin and control mice, the PS molecular species were significantly modified by the inulin feeding. A decreased level of PS with medium fatty acyl chain lengths (PS 32:0, 34:1, 34:2) and increased level of PS with long polyunsaturated fatty acyl chains (PS 40:6, 42:7, 43:5) were observed in livers from inulin-fed mice compared with controls (Figure 5A). Recent structural analysis revealed two binding sites for PS in NSMase (39, 42), which are critical for the activation of NSMase. Therefore, inulin-induced alterations in FA and PS levels and molecular species composition could potentially contribute to decreased NSMase activity.

It was reported that aggregation-prone human LDL particles were enriched with Cer (43). Additionally, Cer transported in LDL was elevated in the plasma of obese patients with type 2 diabetes and correlated with insulin resistance (35). This suggests that LDL associated Cer is involved in the progression of cardiometabolic diseases, and reducing Cer packaging into lipoprotein may provide cardiovascular health benefit. In the present study, inulin-fed mice showed decreased levels of Cer in both VLDL and LDL, and this may partially be due to reduction of plasma VLDL particles by dietary inulin as reported previously (44). This is also supported by our observation that VLDL cholesterol (VLDL-C) was decreased in mice fed with inulin for 12 weeks, whereas LDL-C and HDL-C were not significantly affected by diet (Supplemental Figure S4). Additionally, liver lipids including TG and ChE, both of which important in VLDL assembly were depleted by inulin feeding (Figure 4), and this could subsequently lead to decreased production of VLDL in the liver. It is interesting that in response to dietary inulin SM demonstrated different lipoprotein profile compared to Cer, with more being
incorporated into HDL whereas no significant changes were observed in the LDL (Supplemental Figure S3). Increased level of HDL-SM is an inverse risk factor for coronary heart disease (45), therefore enhanced incorporation of SM in HDL by inulin could be associated with beneficial health effects. Further studies are warranted to understand the mechanism of how inulin can modify sphingolipids packaging into lipoproteins.

CL is a unique phospholipid almost exclusively located at the level of the inner mitochondrial membrane (46). This phospholipid is known to be intimately involved in several mitochondrial bioenergetic processes. CL is particularly susceptible to reactive oxygen species attack due to its high content of unsaturated fatty acids (47). In the livers of inulin-fed mice, the levels of CL were significantly higher compared to control mice (Figure 4 and Supplemental Figure S2). Transcriptomics analysis demonstrated that several hepatic genes related to inflammation were downregulated (Figure 7), among these were PAI1 and Ccl5 which are known to be positively related to redox status (48, 49). We hypothesize that the increased CL observed in inulin-fed mice was indicative of enhanced mitochondrial function, which may be augmented by inulin feeding to maintain redox homeostasis in response to oxidative stress in this atherogenic mouse model. Since NSMase is a redox-sensitive enzyme (37), the effect of inulin on redox balance may also contribute to the lower NSMase activity observed in inulin-fed mice.

It is interesting that although inulin feeding decreased plasma levels of Cer (Figure 2), it did not alter Cer or SM contents in liver (Figure 4) and intestine (data not shown), two major organs for Cer biosynthesis. Similar observations were reported in studies using an NSMase inhibitor GW4869 (50-52). Systemic administration of GW4869 to mice did not alter the Cer or SM content in liver but did decrease circulating ceramide levels (50-52). This might because Cer flux between the liver and plasma was decreased by NSMase inhibition without changing steady state levels in the liver. Cer could be generated through three pathways, including de novo synthesis from long-chain fatty acids, from the SMase catalyzed hydrolysis of SM, and via the salvage pathway. Our lipidomics analysis of liver samples
revealed that the contents of DHCer species were increased in inulin fed mice (Supplemental Figure S4), and this partially support our hypothesis that de novo synthesis is a compensatory pathway for Cer synthesis after SMase being inhibited, therefore the steady state levels of Cer in liver could be maintained.

Inulin-type fructans have been demonstrated to improve cardiovascular health through a number of mechanisms, including modifying de novo lipid synthesis, increasing muscle lipoprotein lipase enzyme activity, enhancing the production of short-chain fatty acids, increasing of fecal excretion of bile salts and cholesterol (7, 53), and reversing endothelial dysfunction via activation of the nitric oxide synthase pathway (8). Our study identified a novel mechanism by which dietary inulin alters sphingolipid metabolism through downregulation of NSMase activity and thereby decreasing circulating Cer levels. Additionally, we observed that VLDL cholesterol (VLDL-C) was decreased in mice fed with inulin for 12 weeks, and LDL-C and HDL-C were not significantly affected (Supplemental Figure S5), which was consistent with data reported by Trautwein et. al (44). Furthermore, sphingolipid profiles in VLDL and LDL were significantly modified in inulin fed mice compared with control group (Figure 6). Those results indicate that inulin could potentially change lipoprotein compositions and functions, and how this will contribute to anti-atherogenic effects of inulin is largely unknown. A long-term study is warranted to further explore impacts of inulin on lipoproteins, Cer, and the pathology of atherosclerosis.

In summary, we demonstrated that in an atherogenic mouse model, inulin feeding decreased plasma levels of Cer within 10 days, and this effect persisted to at least 12 weeks. Lipoprotein analysis indicated that inulin decreased VLDL and LDL associated Cer. Transcriptomics and qPCR revealed that inulin feeding downregulated hepatic Smpd3 expression. NSMase activity was lower in inulin-fed mice compared to the controls. In addition to decreased expression this could partially be due to decreases in NSMase activating anionic lipids (PS and FFA) and perhaps also to suppression of oxidative stress in the liver. These findings suggest that dietary fiber might be an effective way to reduce plasma Cer in humans, which could have beneficial effects on the cardiovascular risks and metabolic diseases that have been associated with elevated circulating Cer levels.
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Figures

A

Figure 1. Fold changes of plasma lipids in inulin-fed Ldlr−/− mice compared with control mice after 10-days (A) and 12-weeks (B) of atherogenic diet feeding (n=9-10/group). Lipidomic analysis was performed using UHPLC-Q Exactive mass spectrometry. Comparisons between groups were made by unpaired Student’s t-test considering significance at the level of P < 0.05. ChE: cholesterol ester; AcCa: acylcarnitine
Figure 2. Ceramide species in the plasma of Ldlr<sup>−/−</sup> mice fed on atherogenic diet supplemented with inulin or cellulose (control). A: after 10 days of feeding; B: after 12 weeks of feeding. C: the ratios of Cer16:0/24:0 after 10 days and 12 weeks of feeding. Ceramide and sphingomyelin levels were detected by UHPLC-Q Exactive MS. Means ± SEM are shown (n = 9-10/group). ****, P < 0.0001 ***, P < 0.001, **, P < 0.01 (unpaired Student’s t test).
Figure 3. The effects of inulin on plasma sphingomyelin (A, B) and ceramide/sphingomyelin molar ratio (C, D) in Ldlr−/− mice. A and C: after 10 days of atherogenic diet feeding. B and D: after 12 weeks of atherogenic diet feeding. Means ± SEM are shown (n = 9-10/group). ****, P < 0.0001 ***, P < 0.001, **, P < 0.01; *, P < 0.05 (unpaired Student’s t test).
Figure 4. Changes in \(Ldl^{-/}\) mice liver lipidome after 12 weeks feeding of inulin or cellulose (control) supplemented atherogenic diet (n=9-10/group). Lipidomic analysis was performed using UHPLC-Q Exactive mass spectrometry. Comparisons of total lipid levels between groups were made by unpaired Student’s \(t\)-test considering significance at the level of \(P < 0.05\). CoQ: coenzyme Q; CL: cardiolipin.
Figure 5. Inulin feeding modifies hepatic composition of phosphatidylserine (PS, A) and free fatty acid (FFA, B) in Ldlr−/− mice. FFA and PS levels were measured by UHPLC-Q Exactive MS as described in the methods. Means ± SEM are shown (n = 9-10). **, P < 0.01; *, P < 0.05; a: P=0.06; b: P=0.08 (unpaired Student’s t test).
Figure 6. Changes in plasma lipoprotein associated Cer after 12 weeks feeding of inulin or cellulose (control) supplemented atherogenic diet to Ldlr−/− mice. Plasma samples from three mice in each group were pooled and lipoproteins including VLDL, LDL and HDL were fractionated using FPLC. Cer in samples were detected by UHPLC-Q Exactive MS. (A) Comparison of individual Cer species in the total VLDL-, LDL-, and HDL-associated fractions of inulin and control diet fed mice. (B-F) Levels of individual Cer species present in each fraction obtained from FPLC.
**Figure 7.** Differential expressed hepatic genes in inulin-fed mice vs control mice. Genes with a fold change inulin/control $\geq 2$ and posterior probability of equal expression (PPEE) $\leq 0.05$ were considered as differentially expressed genes (DEGs). The DEGs were subjected to GO functional enrichment analysis. The calculated P-value goes through Bonferroni Correction. The pathways that have more than 2 genes enriched are shown. See also Supplemental Table S1 for raw data.
Figure 8. Effects of Inulin on *Smpd3* gene expression and NSMase activity in *Ldlr<sup>−/−</sup>* mice liver. (A) Quantitative PCR was performed to confirmed the effects of inulin on sphingolipids metabolism genes. *Smpd3* is the only gene that was downregulated in inulin-fed mice. (B) Liver NSMase activity was investigated as describe in the methods. Bars represent mean ± SEM of four samples in each group. **, P < 0.01 (unpaired Student’s *t* test).