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Dietary sphingomyelin attenuates hepatic steatosis and adipose tissue inflammation in high-fat-diet-induced obese mice☆

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Abstract

Western-type diets can induce obesity and related conditions such as dyslipidemia, insulin resistance and hepatic steatosis. We evaluated the effects of milk sphingomyelin (SM) and egg SM on diet-induced obesity, the development of hepatic steatosis and adipose inflammation in C57BL/6J mice fed a high-fat, cholesterol-enriched diet for 10 weeks. Mice were fed a low-fat diet (10% kcal from fat) (n=10), a high-fat diet (60% kcal from fat) (HFD, n=14) or a high-fat diet modified to contain either 0.1% (w/w) milk SM (n=14) or 0.1% (w/w) egg SM (n=14). After 10 weeks, egg SM ameliorated weight gain, hypercholesterolemia and hyperglycemia induced by HFD. Both egg SM and milk SM attenuated hepatic steatosis development, with significantly lower hepatic triglycerides (TGs) and cholesterol relative to HFD. This reduction in hepatic steatosis was stronger with egg SM supplementation relative to milk SM. Reductions in hepatic TGs observed with dietary SM were associated with lower hepatic mRNA expression of PPARγ-related genes: Scd1 and Pparg2 in both SM groups, and Cd36 and Fabp4 with egg SM. Egg SM and, to a lesser extent, milk SM reduced inflammation and markers of macrophage infiltration in adipose tissue. Egg SM also reduced skeletal muscle TG content compared to HFD. Overall, the current study provides evidence of dietary SM improving metabolic complications associated with diet-induced obesity in mice. Further research is warranted to understand the differences in bioactivity observed between egg and milk SM.

Keywords: Sphingomyelin; Milk; Egg; Hepatic steatosis; Obesity; Inflammation

1. Introduction

The National Center for Health Statistics reported that 36.5% of American adults were considered obese from 2011 to 2014 [1]. Obesity can contribute to the development of several noncommunicable diseases, including nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM). Concurrent with rises in obesity and an aging population, the prevalence of T2DM and NAFLD has increased dramatically in recent decades [2,3]. Obesity, particularly abdominal obesity, is often accompanied by chronic low-grade inflammation in both adipose tissue and systemic circulation, which may result in metabolic abnormalities such as insulin resistance [4,5]. Localized adipose tissue inflammation is thought to arise from a dysfunction in the normal metabolic handling of nutrients by adipocytes as a consequence of excessive adipocyte enlargement, insulin resistance and infiltration of adipose tissue with macrophages [4,5]. Insulin resistance and metabolic defects in lipid metabolism are associated with NAFLD, related to an increased flux of nonesterified fatty acids (NEFAs) from inflamed adipose tissue to the liver [6]. NAFLD consists of a broad spectrum of conditions occurring in the absence of alcohol use, including steatosis, nonalcoholic steatohepatitis (NASH) and liver cirrhosis. Hepatic steatosis has been estimated to be present in over two thirds of the obese population [7–9]. Simple steatosis is relatively benign; however, once steatosis has developed, the liver is "sensitized" to various inflammatory stimuli, which can precipitate NASH [10]. Consequently, NASH can progress to cirrhosis and may result in hepatocellular carcinoma or liver failure [11]. Currently, there is a lack of effective management options for NAFLD besides weight reduction, which can be difficult to maintain long term.

Dietary lipids, such as cholesterol and triglycerides (TGs), have been shown to exacerbate adipose tissue inflammation and NAFLD in animal models [12–16]. Dietary phospholipids, however, are a potential source of bioactive lipids which may improve cardiometabolic health [17]. Dietary sphingomyelin (SM), a sphingolipid found exclusively in animals, has been shown to dose-dependently reduce the absorption of cholesterol, TGs and fatty acids both *in vitro* [18,19] and in rat models [20,21]. Although dietary SM is often overlooked, it demonstrates potential to influence chronic diseases, such as obesity and NAFLD. Only a few studies have evaluated the effects of chronic dietary SM intake on lipid metabolism in rodents [22–25]. Little is known about an optimal intake of SM and whether an increased dietary intake of SM is beneficial to health. Consumption of dietary sphingolipids in Western diets is relatively common (0.3–0.4 g/day)

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and comes mainly from animal-based foods, including eggs and dairy [26,27].

Previously, Chung et al. [22] reported that supplementation with egg-derived SM at 0.3%, 0.6% and 1.2% (w/w) reduced hepatic TGs in a dose-dependent manner in mice on a 4-week Western-type diet. We have recently reported that milk-derived SM (0.25% w/w) reduced both serum lipids and hepatic TGs in mice on a 4-week high-fat diet (HFD) [23]. However, since both studies were relatively short term, the efficacy of dietary SM in preventing diet-induced obesity, adipose tissue inflammation and hepatic steatosis was not fully examined. Furthermore, egg SM and milk SM differ in their amide-linked fatty acid and sphingoid base compositions [28–30] and therefore may have different biological effects on lipid metabolism *in vivo* [21,23]. Thus, the current study evaluated the effects of dietary SM from both egg and milk on hepatic steatosis and obesity in C57BL/6J mice fed a cholesterol-enriched HFD for 10 weeks. We hypothesized that both sources of dietary SM would attenuate hepatic steatosis and obesity development due to their abilities to inhibit lipid absorption. Furthermore, we expected milk SM to be more effective than egg SM based on its greater potency to inhibit lipid absorption in rodent models [21].

2. Materials and methods

2.1. Animals and diets

Male C57BL/6J mice (6 weeks old) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and allowed to acclimate for 2 weeks before being fed 1 of 4 lard-based diets for 10 weeks: low-fat diet control (LFD; 10% kcal from fat; $n=10$); high-fat, high-cholesterol diet control (HFD; 60% kcal from fat, 0.15% cholesterol added by weight; $n=14$); HFD modified to contain 0.1% of milk SM added by weight (HFMSM; $n=14$) or HFD with 0.1% of egg SM added by weight (HFESM; 60% kcal from fat; $n=14$). Diet composition details are provided in Table 1. Experimental diets were made using ingredients obtained from Dyets, Inc. (Bethlehem, PA, USA). Milk SM (bovine; >99% purity) and egg SM (chicken egg; >99% purity) powders were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and were substituted for an equal weight of lard in their respective experimental groups. Fatty acid composition varied between the milk SM (34% C23:0, 21% C24:0, 20%, C22:0, 16% C16:0, 3% C24:1 and 6% unknown) and egg SM (86% C16:0, 6% C18:0, 3%, C22:0, 3% C24:1 and 2% unknown) powders. Egg SM and milk SM also vary in the distribution of sphingoid bases; egg SM contains almost entirely sphingosine (d18:1) bases, while milk SM contains a more varied distribution (d16:0 to d19:0) [30]. Based on body surface normalization and accounting for weight gain throughout the study, the experimental diets provided the equivalent of consuming approximately 405–670 mg SM/day in a 70-kg human [31].

Fresh food was provided biweekly, and mice were allowed to eat *ad libitum*. Body weights were recorded on a weekly basis, while food intake was estimated biweekly. After 10 weeks on their respective diets, mice were fasted for 6–8 h prior to blood collection by cardiac puncture following euthanasia. Blood was allowed to clot at room temperature for 30 min before serum was isolated by centrifugation (10,000×*g* for 10 min at 4°C) and then stored at −80°C. Tissues were perfused with sterile saline before being harvested, snap-frozen in liquid nitrogen and stored at −80°C. The liver, skeletal muscle (quadriceps femoris) and epididymal adipose tissues were collected from animals. Pieces of liver were harvested from the left lateral lobe for histology and lipid analysis. Liver and epididymal adipose tissues were fixed in 10% neutral-buffered formalin for at least 48 h prior to histological processing. Mice were housed in the University of Connecticut-Storrs vivarium in a temperature-controlled room and maintained in a 12-h light/12-h dark cycle. The Animal Care and Use Committee of the University of Connecticut-Storrs approved all procedures used in the current study.

Table 1
Diet composition (g/kg of diet)

Diet component	LFD (g/kg)	HFD	HFMSM/HFESM
Casein	210	265	265
L-Cystine	3	4	4
Corn starch	467	0	0
Maltodextrin	100	0	0
Sucrose	90	253.5	253.5
Lard	20	310	309
Soybean oil	20	30	30
Cellulose	37.15	64	64
Mineral mix, AIN-93G-MX (94046)	35	48	48
Vitamin mix, AIN-93-VX (94047)	15	21	21
Choline bitartrate	2.75	3	3
Cholesterol	0	1.5	1.5
Milk or egg SM	0	0	1

2.2. Serum biochemical analysis

Total serum cholesterol, TGs, NEFAs, liver enzymes and glucose concentrations were measured using commercial assays according to manufacturer instructions. Serum cholesterol, NEFAs and TGs were measured using enzymatic kits obtained from Wako Diagnostics (Richmond, VA, USA). Fasting glucose, serum alanine transaminase (ALT) and aspartate transaminase (AST) were measured using kits obtained from Pointe Scientific, Inc. (Canton, MI, USA). Serum insulin and C-C motif chemokine ligand 2 (CCL2) were measured by ultrasensitive enzyme-linked immunosorbent assay (Crystal Chem, Inc., Downers Grove, IL, USA) and magnetic bead-based assay (EMD Millipore, Billerica, MA, USA), respectively. The homeostasis model assessment (HOMA-IR) equation was used to estimate insulin resistance based on fasting serum insulin and glucose concentrations [32].

2.3. Tissue lipid extraction and analysis

Hepatic and skeletal muscle lipids were extracted using a modified Folch method as previously reported [33], with solubilization and enzymatic analysis of lipid extracts by the methods of Carr et al. [34]. Briefly, tissue lipids were extracted with chloroform:-methanol (2:1), dried under nitrogen and solubilized in Triton X-100. The solubilized lipid extracts were analyzed for total cholesterol (TC), free cholesterol (FC), choline-containing phospholipids (PL) and TGs by enzymatic methods. Cholesteryl ester (CE) mass was calculated as (TC−FC)×1.67 to account for the fatty acid moiety contribution to CE [35].

2.4. Tissue histology

Formalin-fixed liver and epididymal adipose tissues were embedded in paraffin and cut into 5-μm sections prior to staining with hematoxylin and eosin (H&E). All histological procedures were conducted at the Connecticut Veterinary Medical Diagnostic Laboratory (Storrs, CT, USA). The stained tissue sections were viewed under bright field microscopy at ×200 magnification, and images were taken with an AxioCam ICc3 camera (Zeiss, Thornwood, NY, USA). The infiltration of macrophages into adipose tissue was assessed by the counting of crown-like structures (CLSs) (three slides per animal) by a technician blinded to group assignment.

2.5. RNA isolation, cDNA synthesis and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA from liver, epididymal adipose and skeletal muscle was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Total RNA was treated with DNase I and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time qRT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) on a CFX96 real-time-PCR detection system (Bio-Rad). For liver, gene expression was normalized to the geometric mean of the reference genes, glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and beta actin, using the $2^{-\Delta\Delta Ct}$ method. The geometric mean of Gapdh and ribosomal protein, large, P0 (36B4) was used as a reference gene control for both adipose tissue and skeletal muscle. Primer sequences used for qRT-PCR analysis are listed in Table S1.

2.6. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and immunoblotting

Hepatic protein was isolated and analyzed by SDS polyacrylamide gel electrophoresis and immunoblotting as previously described [36]. Briefly, livers were homogenized in a tissue lysate RIPA buffer (Cell Signaling Technologies, Beverly, MA, USA) containing 2 mM phenylmethylsulfonyl fluoride (Sigma Aldrich, St. Louis, MO, USA). Total protein concentrations of the lysates were determined by bicinchoninic acid assay (Cell Signaling Technologies). Sixty micrograms of total protein was mixed with loading buffer containing dithiothreitol (BioRad) and loaded onto 4%–20% SDS Stain-Free gels (BioRad). Gels were activated using the Stain-Free protocol on a ChemiDoc XRS+ imager (BioRad). Protein was then transferred to a polyvinylidene difluoride membrane (Thermo Fisher Scientific, Waltham, MA, USA) through a semi-dry protocol using a Trans-Blot Turbo System (BioRad). Stain-Free blot images were captured, and membranes were blocked for 1 h at room temperature with blocking buffer (20 mM Tris base, 150 mM NaCl, 0.05% Tween-20, 5% w/v nonfat dry milk). Primary rabbit anti-mouse PPARγ antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA), diluted according to manufacturer's recommendation and incubated overnight at 4°C. After washing the membrane with TBS buffer (20 mM Tris base, 150 mM NaCl, 0.05% Tween-20), a 1:10,000 dilution of secondary HRP-anti-rabbit antibody (Thermo Fisher Scientific) was incubated with the membrane at room temperature for 1 h. Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) was used for the final imaging of the blot. PPARγ relative abundance was quantified after standardizing to total protein images obtained from the Stain-Free blot using ImageLab Software V5.1 (BioRad).