Published in final edited form as:

Biochim Biophys Acta Mol Cell Biol Lipids. 2021 May ; 1866(5): 158898. doi:10.1016/j.bbalip.2021.158898.

Effect of liver total sphingomyelin synthase deficiency on plasma lipid metabolism

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Abstract

Sphingomyelin (SM) is one major phospholipids on lipoproteins. It is enriched on apolipoprotein B-containing particles, including very low-density lipoprotein (VLDL) and its catabolites, low-density lipoprotein (LDL). SM is synthesized by sphingomyelin synthase 1 and 2 (SMS1 and SMS2) which utilizes ceramide and phosphatidylcholine, as two substrates, to produce SM and diacylglyceride. SMS1 and SMS2 activities are co-expressed in all tested tissues, including the liver where VLDL is produced. Thus, neither *Sms1* gene knockout (KO) nor *Sms2* KO approach is sufficient to evaluate the effect of SMS on VLDL metabolism. We prepared liver-specific *Sms1* KO/ global *Sms2* KO mice to evaluate the effect of hepatocyte SM biosynthesis in lipoprotein metabolism. We found that hepatocyte total SMS depletion significantly reduces cellular sphingomyelin levels. Also, we found that the deficiency induces cellular glycosphingolipid levels which is specifically related with SMS1 but not SMS2 deficiency. To our surprise, hepatocyte total SMS deficiency has marginal effect on hepatocyte ceramide, diacylglyceride, and phosphatidylcholine levels. Importantly, total SMS deficiency decreases plasma triglyceride but not apoB levels and reduces larger VLDL concentration. The reduction of triglyceride levels also was observed when the animals were on a high fat diet. Our results show that hepatocyte total

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CRediT authorship contribution statement

Z.L. and Y.P.C. performed 70% of the experiments, analyzed data and modified the manuscript. M.H., K.Z., J.Z., W.W., J.C., Y.C., and G.C. performed some Western blots, lipid analysis, and mouse maintenance. Y.C. and J.D. performed native gel electrophoresis for lipoprotein separation. sectioning and staining of liver tumor, and edited the manuscript. T.S.W measured sphingolipids using LC/MS/MS, and modified the manuscript. X.C.J. conceived the ideas, designed and discussed experiments, supervised progress and wrote the manuscript.

Declaration of competing interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2021.158898.

SMS blocking can reduce VLDL-triglyceride production and plasma triglyceride levels. This phenomenon could be related with a reduction of atherogenicity.

Keywords

Sms1 liver-specific/ *Sms2* global knockout mice; Albumin-Cre recombinase; Sphingomyelin; Glycosphingolipids; Very low-density lipoprotein; High-density lipoprotein

1. Introduction

Sphingomyelin (SM) biosynthesis occurs via the actions of serine palmitoyltransferase (SPT), 3-ketosphinganine reductase, ceramide synthase, dihydroceramide desaturase, and sphingomyelin synthase (SMS) [1]. This sphingolipid pathway is essential for the life, because homozygous SPTLC1 or SPTLC2 (both subunits of SPT) deficient mice are embryonic lethal [2].

SMS has two isoforms (SMS1 and SMS2), sits downstream of SPT, and catalyzes the conversion of ceramide to SM [3,4]. SMS1 and SMS2 activities are co-expressed in all tested tissues, including the liver. Thus, neither *Sms1* KO nor *Sms2* KO approach is sufficient to evaluate the effect of SM reduction on the liver. We established *Sms1* KO mice, although there was a problem of postnatal lethality, and found that SMS1 deficiency significantly decreased SM in plasma, liver, and macrophages but had only a marginal effect on ceramide levels [5]. We also established whole body *Sms2* KO mice and found the deficient mice, with a reduction of SM in the circulation, are viable and healthy [6].

Atherosclerosis progression is initiated by atherogenic lipoprotein arterial wall lipoprotein retention and aggregation [7–10]. Much evidence indicates that SM content in the aortic wall and in plasma is closely related to atherosclerosis. It is well established that SM accumulates in atheroma formed in humans and animal models [11–16]. LDL extracted from human atherosclerotic lesions is much richer in SM than LDL from the plasma [17–20]. The ratio of SM to phosphatidylcholine (PC) is increased 5-fold in VLDL from hypercholesterolemic rabbits [21,22]. We found that plasma SM level in *Apoe* KO mice is 4-fold higher than in wild-type (WT) animals [23], and this may contribute to increased atherosclerosis [24,25]. We also found that human plasma SM level is an independent risk factors for coronary heart disease [26,27]. SM increases from 10% at birth to 48% in patients who had undergone a coronary artery bypass grafting and to 60% in patients who had plaques in their coronary arteries, and SM contributes to atherosclerosis and sudden dearth [28]. SM is also associated with increased risk of myocardial infarction [29] and human atherosclerotic plaque inflammation [30]. Given that SM is the major sphingolipid in atherogenic apoB-containing lipoproteins, including VLDL, LDL, and chylomicrons, SM biosynthesis should have important impact on VLDL production as well as atherogenesis [31–33]. Studies in hamsters suggested that *de novo* synthesized SM is secreted via the VLDL/LDL pathway in the liver [23,24]. Isolated rat hepatocytes secrete SM as a part of VLDL [25]. Although the importance of SM availability in VLDL production is well recognized, the impact of SM biosynthesis on it has never been systemically tested. In this study, we evaluated lipoprotein metabolism in hepatocyte total SMS deficient mice.

2. Materials and methods

2.1. Liver-specific Sms1 KO mouse preparation

The overall strategy for gene targeting was to delete exon 6 of *Sms1*. The strategy of is as follows: A 9.1-kb region used to construct the targeting vector was first subcloned from a positively identified C57BL/6 (RPCI23) BAC clone. A loxP- and FRT-flanked Neo cassette was inserted in intron 7, and a single loxP site was inserted immediately upstream of the 5' side of exon 6 where the initial codon (ATG) is located (Fig. 1A). We obtained homozygous targeted *Sms1* mice and prepared *Sms1*-Flox mice by crossing Flp transgenic mice to delete the Neo cassette. To prepare liver-specific *Sms1* deficient mice, we can cross our *Sms1*-Flox mice with albumin-Cre transgenic mice (Jackson laboratory). Both mice have C57BL/6 genetic background.

2.2. Western blots

The western blot procedure was similar as we did before [17].

2.3. TG production

Female WT and SMS1/SMS2 double KO mice were utilized. They were fasted for 16 h, and then injected poloxamer 407 (1 mg/g of body weight, i.p.) to block VLDL lipolysis. Plasma TG was measured at 0, 1, 2 and 7 h after P407 treatment.

2.4. Lipoprotein size determination by nuclear magnetic resonance (NMR)

We isolated plasma from female WT and SMS1/SMS2 double KO mice. The fresh mice plasma samples were sent to LabCorp (previously LipoScience). Lipoprotein size was analyzed by NMR.

2.5. Native polyacrylamide gel electrophoresis (PAGE) for plasma lipoproteins

We used the system which we reported before [34]. Two sets of samples were used. They were WT plasma and *Sms1/Sms2* double KO plasma. All samples were incubated at 37 °C for 4 h. Fifty μl of each was loaded on the PAGE gel.

2.6. Tissue SMS activity assay

The activity assay was similar as we did before [35].

2.7. Plasma lipid and lipoprotein measurements

The procedure was similar as we did before [35]. Lipoprotein profiles were obtained by fast protein liquid chromatography (FPLC), using a Sepharose 6B column. Plasma (0.2 μl) was separated by 4–15% SDS gel electrophoresis and immunoblotted with the polyclonal antibodies against apoE (Santa Cruz Biotechnology), apoB (Abcam), and apoA-I (Santa Cruz Biotechnology).

2.8. Sphingolipid analyses by LC/MS/MS

Sphingolipid levels were measured in WT and *Sms1/Sms2* double KO hepatocyte by LC/MS/MS, as previously described [36,37].

2.9. Statistical analysis

Each experiment was conducted at least three times. Data are expressed as the mean ± SD. Differences between two groups were analyzed by the unpaired two-tailed Student's *t*-test, and differences among multiple groups were assessed by ANOVA followed by the Student-Newman-Keuls test. A *P*-value <0.05 was considered significant.

3. Results

3.1. Depletion both Sms1 and Sms2 in mouse liver

Based on total SMS activity measurement on global *Sms2* KO mice [6], SMS1 and SMS2 activities are co-expressed in all tested tissues, including the liver (Supplement Fig. 1). Thus, neither *Sms1* KO nor *Sms2* KO approach is sufficient to evaluate the effect of SM reduction. We have prepared *Sms1* KO mice [5]. The homozygous KO mice have a problem of postnatal lethality (60% of the pups died within three weeks after birth). Moreover, homozygotes could not be obtained by a homozygous/ homozygous cross. Thus, it is not possible to obtain global *Sms1/Sms2* double KO mice by simply crossing *Sms1* KO mice and *Sms2* KO mice. We crossed the *Sms1*-Flox mice with global *Sms2* KO mice, yielding homozygous *Sms1*-Flox/*Sms2* KO mice; and then crossed the mice with albumin-Cre transgenic mice, yielding *Sms1* liver-specific KO/*Sms2* global KO mice. All animals are bred on a C57BL/6 genetic background. Recombination of the Floxed *Sms1* alleles (and thereby inactivation of SMS1) was achieved in essentially 90% of in the liver but not in other tissues (Fig. 1B). We also found that there are no SMS2 expression in *Sms2* KO and *Sms1/Sms2* double KO mice (Supplement Fig. 2). Importantly, the KO mice have no detectible total SMS activity (Fig. 1C). We stained 2-months old WT, *Sms2* KO, and *Sms1/Sms2* KO mouse liver sections with haematoxylin and eosin, as well as trichrome for collagen fiber. We did not find significant difference among them (Supplement Fig. 3A and 3B). We also measure plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in these 2-months old mice and found that ALT were significantly increased (Supplement Fig. 4).

3.2. Effect of SMS depletion on primary hepatocyte lipids

We next sought to measure different lipids in isolated primary hepatocytes, using LC/MS/MS. As expected, we found a dramatic reduction of SM with all tested subspecies (Fig. 2A, supplement Fig. 5). To our surprise, we did not find significant changes of total ceramide levels (Fig. 2B) and some of the subspecies are increased and some of them are decreased (Supplement Fig. 6). One obvious difference is SMS total deficiency significantly increased the levels of glycosphingolipids, including glucosylceramide, lactosylceramide, and GM3, in hepatocytes (Figs. 2C–E, supplement Figs. 7–9), and this is related with SMS1 deficiency but not SMS2 deficiency [5].

We further isolated plasma membrane from the liver of female double KO mice and controls and measured sphingolipid levels. We found that although SMS deficiency significantly reduced almost all major measured SM in the plasma membrane (Table 1), however, it had a different effect on ceramide subspecies and some ceramide levels are increase and some are decreased (Table 1).

3.3. Effect of liver SMS depletion on plasma lipid levels

We next sought to measure plasma lipid levels in 2-months old male and female mice. We found that SM and triglyceride, but not other lipid levels are significantly reduced (Table 2).

Plasma lipid distributions were also examined by fast protein liquid chromatography (FPLC) using pooled plasma. We observed that plasma triglyceride levels decreased in non-HDL fraction from female SMS double KO mice, compared with controls (Fig. 3A). There are some reduction of cholesterol and phospholipid in HDL fractions, but some induction in non-HDL (Fig. 3B and C). We obtained similar results from the male KO mice (data not shown). Next, we assessed plasma apolipoprotein levels by reducing SDS-PAGE and immuno- blotting and found that no significant changes for all tested proteins, including apoA-I, apoB, and apoE (Fig. 3D and E), suggesting the VLDL produced from the double KO mouse liver could be triglyceride-poor particles.

We next evaluated liver triglyceride production rate using Poloxamer 407 to block the clearance of VLDL from the circulation as we did before [38]. We found that WT mice have a milky plasma and liver total SMS depletion yields a less milky plasma (Fig. 4A). The deficiency also significantly reduces triglyceride production (Fig. 4B) and large triglyceride-rich lipoprotein particle (L-TRLP) levels (Fig. 4C). Moreover, the SMS depletion-mediated plasma triglyceride reduction does not cause triglyceride accumulation in the liver (Fig. 4D). Moreover, unlike liver specific-SPTLC2 deficiency [39], we did not observe jaundice in these 2-months old SMS deficient mice (data not shown).

To precisely examine the changes of lipoproteins, we utilized native polyacrylamide gel electrophoresis (PAGE) system, which we developed, to separate lipoproteins [34]. We found that the double KO mice have lower levels of VLDL and diffused LDL, which could be smaller VLDL or triglyceride-poor VLDL (Fig. 5). However, the double KO mice have no obvious changes on HDLs (Fig. 5).

In order to investigate the dietary effect on *Sms1*/*Sms2* double KO mice, we fed female mice with a high-fat/cholesterol diet (TD88137, Harlan Teklad) for two weeks. The diet increased all lipid levels, and again, total SMS depletion significantly reduced triglyceride but not other lipid levels (Figs. 6A–D).

4. Discussion

In this study, we prepared liver-specific *Sms1* KO/global *Sms2* KO mice by crossing *Sms1*-Flox/*Sms2* KO mice with albumin-Cre recombinase transgenic mice. We demonstrated that disruption of liver *Sms* genes in 2–3 months mice resulted in: 1) dramatic reduction of hepatocyte SM levels and induction of glycosphingolipids; 2) dramatic reduction of membrane SM levels but not ceramide levels; 3) significant reduction of plasma SM and triglyceride but no changes in other lipid levels; and 4) significant reduction of triglyceride-rich lipoprotein production.

One key finding of this study is that blocking SMS in mouse liver reduces the production of VLDL which transports triglyceride from the liver into the bloodstream [40]. The regulation

of VLDL secretion is poorly understood. Accumulating evidence suggests that formation of VLDL [41–43] is accomplished sequentially. The two-step model postulates that the initial product is a primordial small, dense particle formed during or immediately after apoB translation in the ER. Bulk lipid, most likely triglyceride and phospholipids, including SM and phosphatidylcholine, is incorporated into the primordial particle to form mature VLDL [44]. Multiple factors are involved in VLDL maturation. Microsomal TG transfer protein is involved in an early phase of lipid addition to apoB [45,46]. Phospholipid transfer protein may also be involved the first and second stage of VLDL lipidation [31–33]. Studies in hamsters suggest that *de novo* synthesized SM is secreted via the VLDL/LDL pathway in the liver [23,24]. Isolated rat hepatocytes secrete SM as a part of VLDL [25]. The major carriers of SM in plasma are VLDL and chylomicrons [20,47], another triglyceride-rich lipoprotein produced in enterocytes. Thus, total SMS deficiency-mediated reduction of triglyceride-rich lipoprotein production (Fig. 4B) and large VLDL (Fig. 4C), indicates that SM availability in the hepatocyte is important for mature and normal VLDL production.

The induction of glycosphingolipids in liver-specific *Sms1* KO/global *Sms2* KO mice is due to SMS1 deficiency. We have reported that global *Sms1* KO mice but not *Sms2* KO mice dramatically increased glucosylceramide and GM3 in the circulation, liver, and macrophages [5]. It is known that SMS1 can use its N-terminal sterile α-motif to interact with C-terminal of glucosylceramide synthase to form a complex which increases SM and decreases glucosylceramide levels [48]. *Sms 1* KO mice have no SMS1 protein and the complex cannot be formed. Thus, glucosylceramide can be synthesized without control and glucosylceramide, then glycosphingolipids, are accumulated dramatically. SMS2 has no sterile α-motif at its N-terminal [3] and it has no effect on glycosphingolipid biosynthesis. In fact, glycosphingolipids, such glucosylceramide and GM3, accumulation can induce lysosomal storage disorders [49].

Both SMS1 and SMS2 utilize ceramide and phosphatidylcholine, as substrates, to synthesize SM and diacylglyceride [3]. To our surprise, besides a significant reduction of SM, total SMS depletion in hepatocytes has marginal effect on ceramide (Fig. 2). This indicated that SM biosynthesis pathway plays a minor role in controlling the steady state levels of ceramide. Ceramide seems to be involved metabolic disorders [50]. However, in the present study we did not observe significant changes of ceramide at the intracellular level (Fig. 2B) and in liver plasma membranes (Table 1). Thus, ceramide levels may have negligible role in mediating VLDL production and therefore may have minor influence on atherogenicity.

As we reported previously [51], our native PAGE could separate human HDL into four fractions, however, mice have a different HDL pattern, compared with humans (Fig. 5). They have a major HDL (90%) with a similar migration rate as that of human HDL_2. Mice also have large and very large HDLs which does not exist in human plasma. We found that hepatocyte total SMS depletion has marginal effect on mouse HDLs (Fig. 5). However, the deficiency causes a reduction of VLDL and diffused pattern of LDL, which could be triglyceride-poor VLDLs. This deserves further investigation.

We and another research group reported that *Sms1* KO exhibited neonatal lethality [5,52]. Yano et al. reported [52] that SMS1 deficiency-mediated lipodystrophy is related with an

induction of oxidative stress in adipose tissues. However, global *Sms2* KO mice display no obvious abnormalities and grow to adulthood. Notably, global *Sms2* KO mice have normally functioning adipose tissue and increased insulin sensitivity compared with control mice [53]. Thus, SMS2 but not SMS1 could be a therapeutic target for metabolic diseases, including type 2 diabetes, fatty liver, and atherosclerosis [53–59]. Moreover, 2-months old double KO livers did not show abnormality (Supplement Fig. 3), although ALT was significantly increased (Supplement Fig. 4). In fact, it is well known that all statin drugs (cholesterol biosynthesis inhibitor) elevate liver enzymes to some degree, including AST and ALT [60]. This side effect did not prevent stains used as a drug fighting against hypercholesterolemia.

In summary, hepatocyte total SMS blocking can decrease SM availability during VLDL-triglyceride production, thus reducing plasma triglyceride and large VLDL levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Guanghou Shui, from LipidALL Technologies Co., Beijing, China, for his effort to measure glycosphingolipids, phosphatidylcholine, and diacylglycerol. This work was supported by grants VA Merit 000900–01, NIH HL1395821, and HL149730 to XCJ.

Abbreviations:

SMS	sphingomyelin synthase
SM	sphingomyelin
KO	gene knockout

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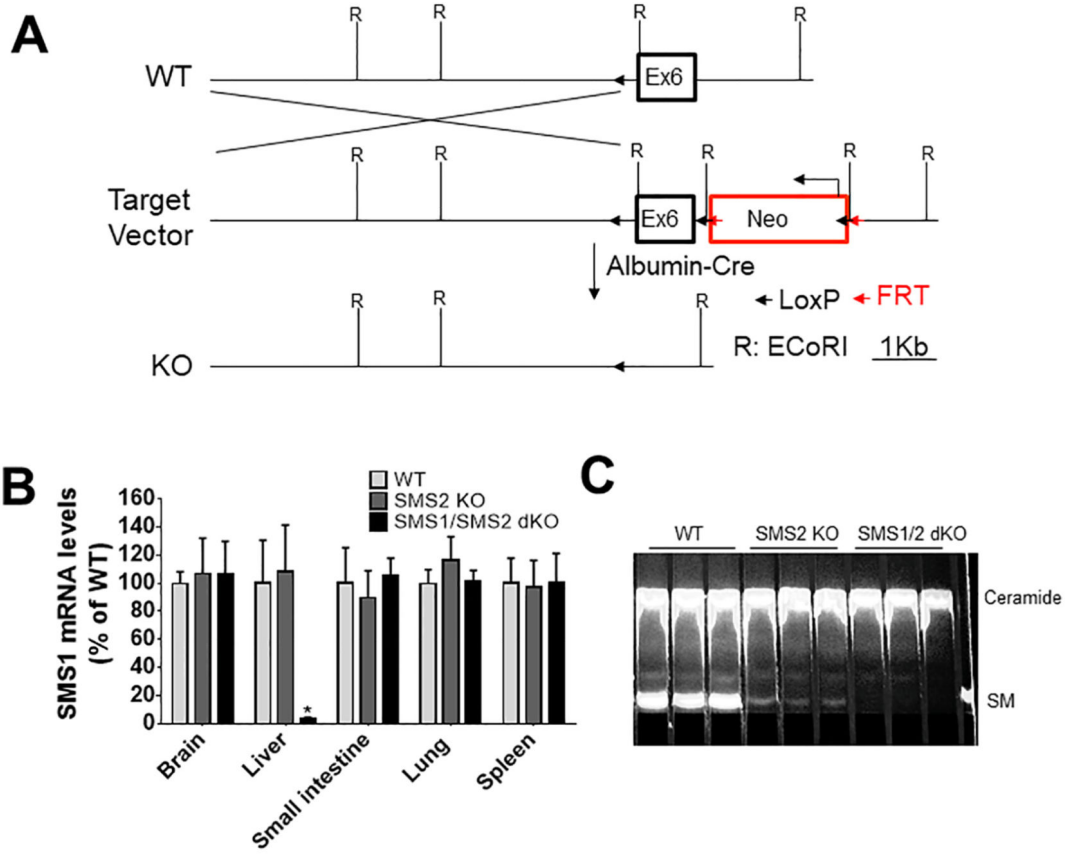

Fig. 1.

Albumin-Cre-mediated Liver-specific *Sms1* KO Mouse Preparation. (A) Strategy for liver-specific *Sms1* KO mouse preparation. (B) Real time PCR for liver SMS1 mRNA measurement. (C) Liver total SMS activity in female WT, *Sms2* KO, and *Sms1*/*Sms2* KO mice. Values are mean ± SD, $n = 3$.

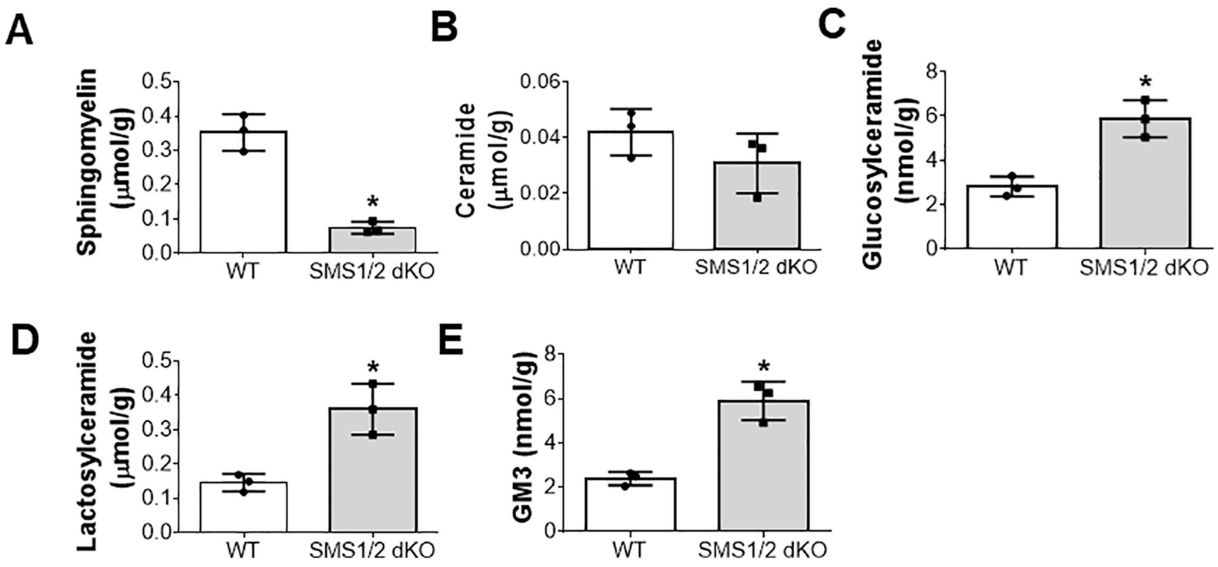

Fig. 2.

Hepatocyte lipid measurements by LC/MS/MS. Primary hepatocytes were isolated from female liver-specific *Sms1* KO/global *Sms2* KO and control mouse livers. All lipids were measured by LC/MS/MS. (A) Sphingomyelin (SM); (B) ceramide; (C) glucosylceramide; (D) lactosylceramide; (E) GM3. Values are mean ± SD, n = 3, $*P < 0.01$.

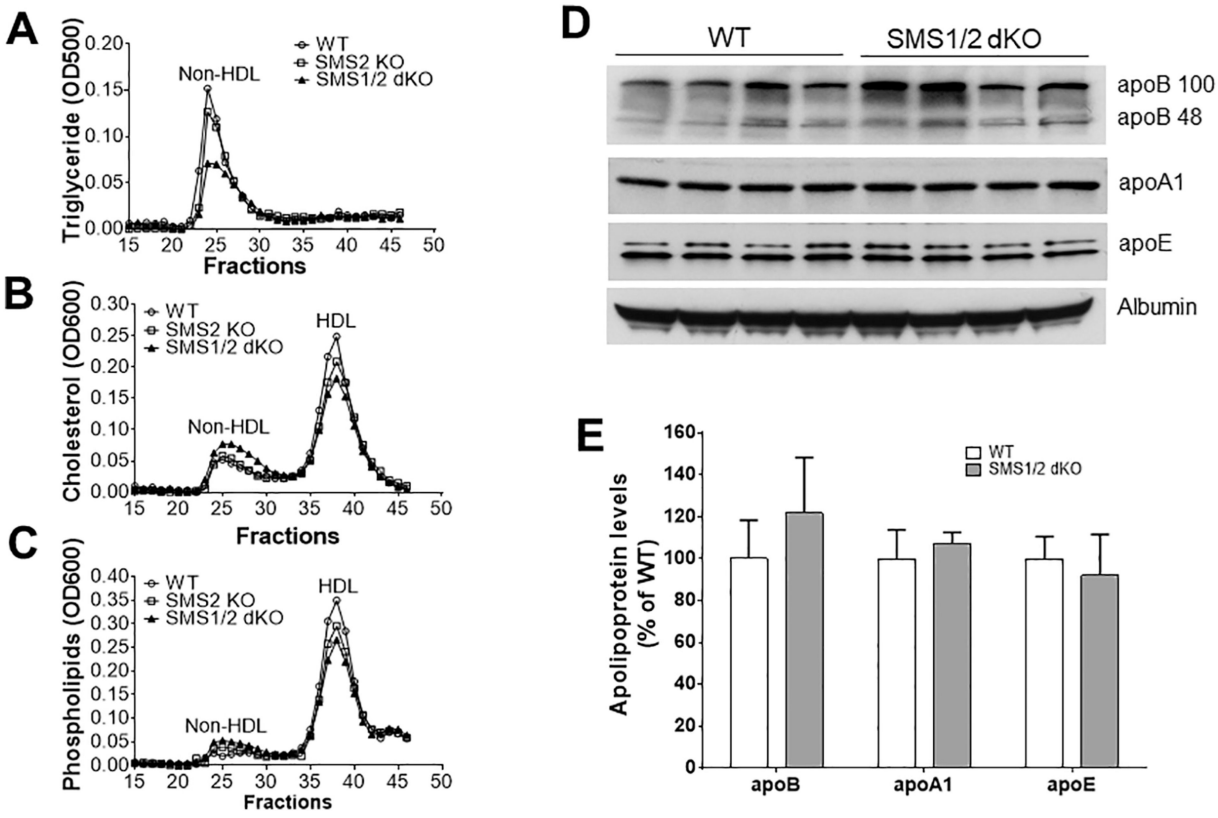

Fig. 3.

Plasma lipoprotein analysis. (A-C), FPLC for plasma lipid distribution analysis, using pooled plasma from female *Sms1*/*Sms2* double KO or *Sms2* KO or control mice. (D), ApoA-I, apoB, and apoE analysis by Western blotting. (E), quantification of all tested apolipoproteins. ApoB levels are the combination of apoB100 and apoB48. Values are mean ± SD, $n = 4$.

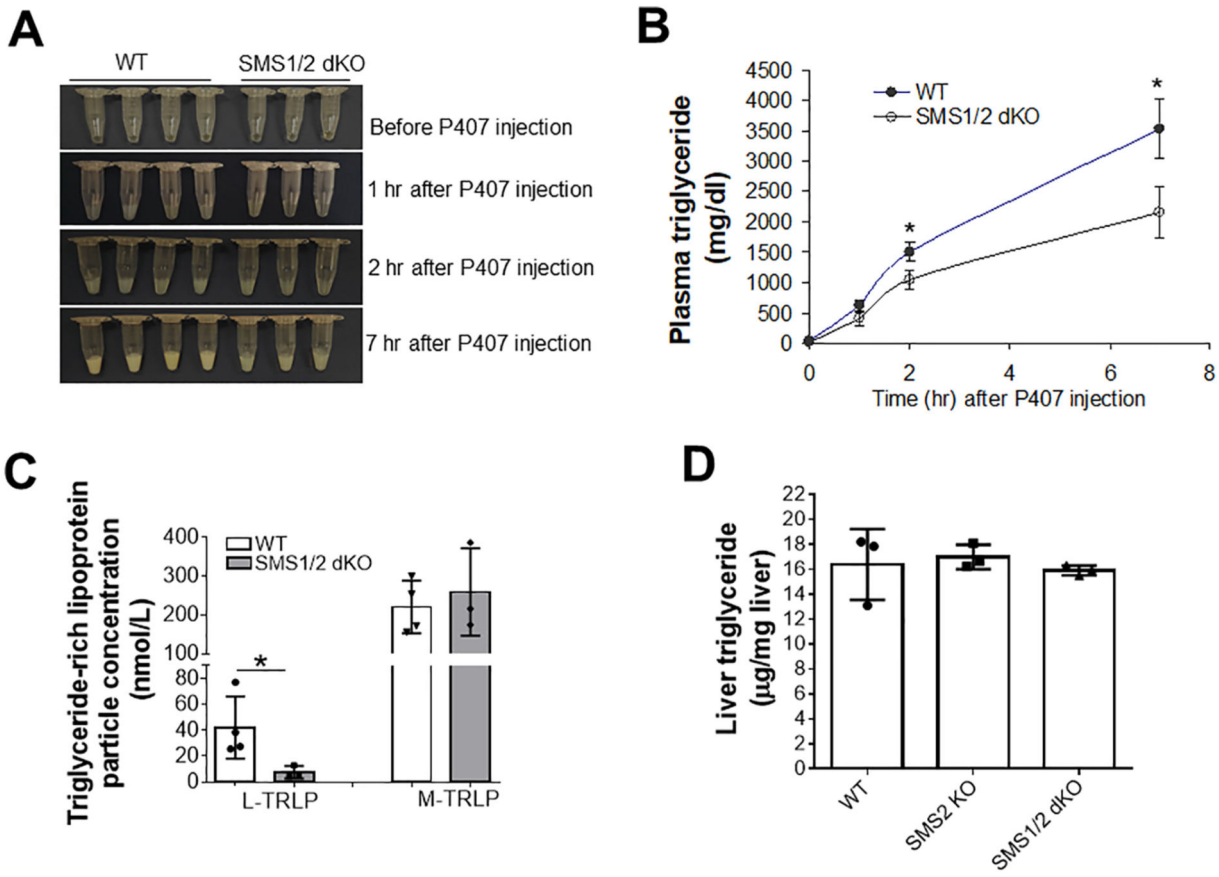

Fig. 4.

The measurements of TG production, VLDL size, and liver TG levels. Female *Sms1*/*Sms2* double KO and control mice were treated with Poloxamer 407 (1 mg/ g of body weight, i.p.). Plasma TG was measured at 0, 1, 2 and 7 h. (A) plasma at 0, 1, 2 and 7 h after 407 treatment. (B) TG production quantification. (C) VLDL size was determined by NMR. M-TRLP, 37–49 nm; L-TRLP, 50–240 nm. (D) liver TG level quantification. Value is Means ± SD, $n = 5$, $*P < 0.05$.

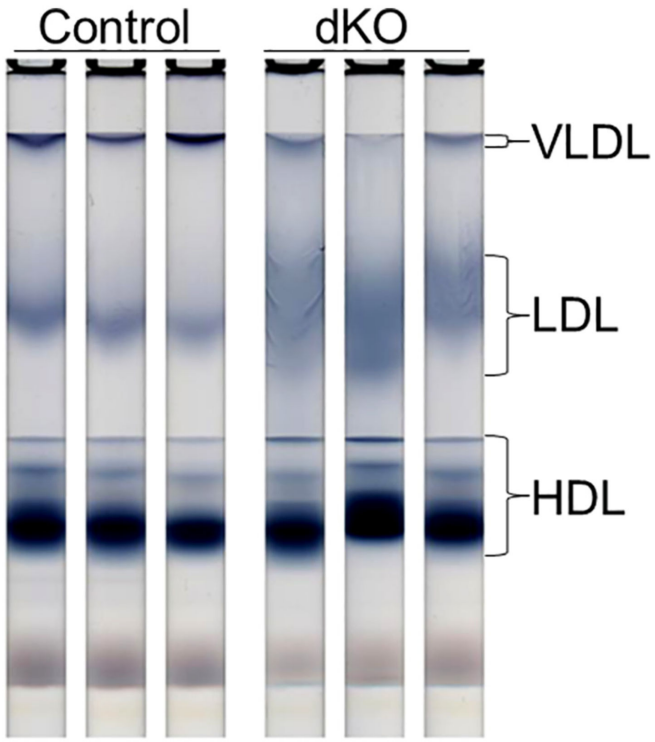

Fig. 5.

Native PAGE system to separate lipoproteins. Female mouse serum (50 μl) was pre-stained with Sudan Black and then run on the system which we reported before (Materials and methods).

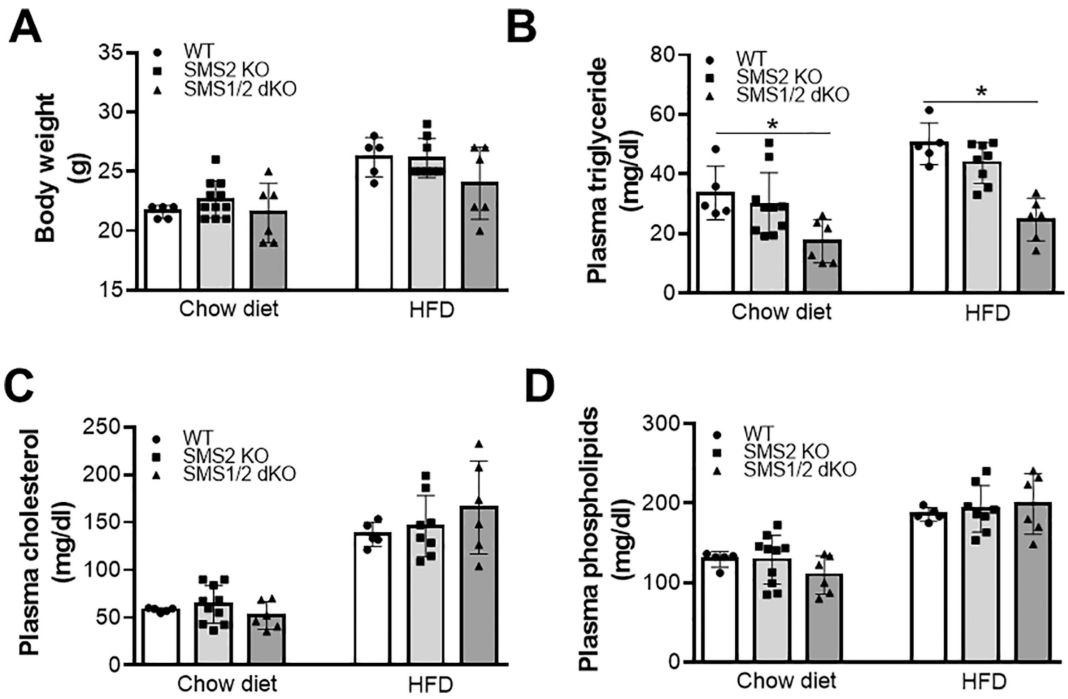

Fig. 6.

Plasma lipid analysis. Female mice were fed a high fat diet for two weeks. Body weight and plasma lipid levels were measured. (A) Body weight. (B) Triglyceride. (C) Cholesterol. (D) Phospholipids. Value is Means ± SD, $n = 5$, $*P < 0.05$.

Table 1

Sphingolipid levels on liver plasma membrane.

Mice	C16:0	C18:0	C18:1	C24:0	C24:1	C20:0	C22:0
	(pmol/mg protein)						
Sphingomyelin							
WT	163 ±23	32±4	5±1	83 ±6	175±53	–	26±9
Sms KO	76±12 *	22±2 *	4±1	22±5 *	62±16 *	–	10±3 *
Ceramide							
WT	17±3	4±1	–	36±9	38±6	4±1	33±5
Sms KO	37±6 *	7±1 *	–	19±7 *	63±11 *	3±1	11 ±4 *

Value: mean ± SD; n=5.

* P<0.01.

Table 2

Plasma lipid measurement.

Mice	SM (mg/dl)	TG	T-Chol	F-chol	PL
Male					
WT	30±5	35±6	80±7	8±2	122±8
Sms2 KO	19±3	32±4	77±5	7±1	106±12
Sms1/2 KO	12±4*	17±5*	70±9	10±3	115±10
Female					
WT	41±5	46±7	85±8	9±2	134±16
Sms2 KO	28±6	38±5	79±5	8±2	145±21
Sms1/2 KO	15±4*	23 ±6*	75±6	12±3	126±9

SM, Sphingomyelin; TG, Triglyceride; T-Chol, total cholesterol; F-Chol, free cholesterol glucosylceramide; PL, phospholipids. Value: mean ± SD; n=5.

*P<0.01.