Fish Oil and Fenofibrate Prevented Phosphorylation-dependent Hepatic Sortilin 1 Degradation in Western Diet-fed Mice*

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Background: Hepatic sortilin 1 inhibits apoB secretion and lowers plasma lipids.

Results: Fish oil and fenofibrate treatments prevented fatty acid-induced hepatic Sort1 posttranslational down-regulation in Western diet-fed mice.

Conclusion: Hepatic Sort1 is an in vivo target of n-3 PUFAs and fenofibrate.

Significance: Increasing hepatic Sort1 by therapeutic approaches may improve plasma lipid homeostasis.

Diabetic dyslipidemia is characterized by hypertriglyceridemia, reduced high-density lipoprotein (HDL), and smaller and denser low-density lipoprotein (LDL). Such an atherogenic plasma lipid profile often leads to significantly higher risk of cardiovascular disease, which is the leading cause of morbidity and mortality in type 2 diabetes (1). Increased hepatic very low-density lipoprotein (VLDL) secretion is one of the major causes of diabetic dyslipidemia (2, 3). However, the underlying mechanisms of hepatic VLDL overproduction in obesity and diabetes are complex and not fully understood. Central obesity, low-grade inflammation and insulin resistance can cause abnormal lipolysis and elevated circulating free fatty acids (FFAs)2 (3). Increased hepatic FFA uptake promotes hepatic triglyceride (TG) synthesis, apoB lipidation, and VLDL assembly and secretion (4, 5). FFAs also activate Toll-like receptors (6–9) and promote cytokine production and intracellular signaling activation, which leads to hepatic insulin resistance (10, 11). Insulin promotes intracellular apoB degradation, and hepatic insulin resistance has been linked to increased apoB production (12, 13). Consistently, elevated circulating FFAs are considered as an important cardiovascular disease risk factor in diabetes (14, 15), and the most abundant saturated fatty acid palmitate may serve as a biomarker for type-II diabetes (16).

A number of recent genome-wide association studies identified SORT1 as the causal gene that is responsible for the strong and reproducible association between SNPs at the cardiovascular risk 1p13.3 locus and plasma LDL cholesterol (LDL-C) and TG levels in human populations (17–20). Sort1 is a transmembrane multiligand receptor that belongs to the family of Vps10p domain receptors (21). Sort1 mainly localizes in the trans-Golgi network and assists in sorting target proteins in the secretory or endosomal pathways. A small amount of Sort1 can also localize to the plasma membrane, where Sort1 is involved in receptor-mediated uptake processes. It has so far been shown that Sort1 interacts with and regulates the intracellular trafficking, secretion, or endocytosis of lipoprotein lipase (22), apoA-V (23), and apoB (17, 24, 25). A number of recent studies demonstrated in both mouse models and cultured cells that liver Sort1 directed apoB for pressecretory lysosomal degradation and thus inhibited

Obesity and diabetes are associated with hepatic triglyceride overproduction and hypertriglyceridemia. Recent studies have found that the cellular trafficking receptor sortilin 1 (Sort1) inhibits hepatic apolipoprotein B secretion and reduces plasma lipid levels in mice, and its hepatic expression was negatively associated with plasma lipids in humans. This study investigated the regulation of hepatic Sort1 under diabetic conditions and by lipid-lowering fish oil and fenofibrate. Results showed that hepatic Sort1 protein, but not mRNA, was markedly lower in Western diet-fed mice. Knockdown of hepatic Sort1 increased plasma triglyceride in mice. Feeding mice a fish oil-enriched diet completely restored hepatic Sort1 levels in Western diet-fed mice. Fenofibrate also restored hepatic Sort1 protein levels in Western diet-fed wild type mice, but not in peroxisome proliferator-activated receptor α (PPARα) knock-out mice. PPARα ligands did not induce Sort1 in hepatocytes in vitro. Instead, fish oil and fenofibrate reduced circulating and hepatic fatty acids in mice, and n-3 polyunsaturated fatty acids prevented palmitate inhibition of Sort1 protein in HepG2 cells. LC/MS/MS analysis revealed that Sort1 phosphorylation at serine 793 was increased in obese mice and in palmitate-treated HepG2 cells. Mutations that abolished phosphorylation at Ser-793 increased Sort1 stability and prevented palmitate inhibition of Sort1 ubiquitination and degradation in HepG2 cells. In summary, therapeutic strategies that prevent posttranslational hepatic Sort1 down-regulation in obesity and diabetes may be beneficial in improving dyslipidemia.

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2 The abbreviations used are: FFA, free fatty acid; Sort1, sortilin 1; apoB, apolipoprotein B; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TG, triglyceride; LDL-C, LDL cholesterol.
apoB secretion (17, 18, 24). In addition, plasma membrane-bound Sort1 facilitates circulating LDL uptake and catabolism in the liver independent of LDL receptor (24). Liver-specific overexpression of Sort1 decreased whereas liver-specific knockdown of Sort1 increased plasma TG and LDL-C in mice (17). In contrast, whole body sort1 knock-out mice showed either unchanged or decreased plasma LDL cholesterol. Interestingly, a recent study reported that Sort1 promotes the secretion of PCSK9 that regulates LDL receptor stability (26).

It has been shown that the causal SNP in the non-coding region modulates transcriptional factor CCAAT/enhancer-binding protein binding and thus hepatic Sort1 gene transcription, which provides a molecular basis for the negative correlation of hepatic Sort1 mRNA with plasma LDL-C in human populations (17). In addition to genetic variations of hepatic Sort1, we recently reported that hepatic Sort1 was significantly decreased in obese humans, experimental mouse models of obesity, and type-I and type-II diabetes (27). Our results showed that adenovirus-mediated liver-specific overexpression of Sort1 decreased plasma cholesterol and TG levels in obese mice (27). Our study suggested that reduced hepatic Sort1 protein may be due to elevated FFA, which activates hepatic ERK signaling to cause posttranslational Sort1 protein degradation without inhibiting Sort1 gene transcription (27).

Despite the emerging role of hepatic Sort1 in diabetic dyslipidemia, how hepatic Sort1 is regulated under obesity and diabetes or by therapeutic approaches is still largely unknown. In this study, we showed that administration of the lipid-lowering agent fish oil or fenofibrate restored hepatic Sort1 levels in Western diet-induced hyperlipidemic mice. Because obesity and diabetes are associated with reduced hepatic Sort1 levels (27), therapeutic approaches that increase hepatic Sort1 expression and function may be beneficial in improving lipid homeostasis in obesity and diabetes.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Sort1 antibodies were purchased from Abcam (Cambridge, MA). Antibodies against phospho-ERK (catalog no. 9106), total ERK (catalog no. 4695), histone 3 (catalog no. 9717), HA, phospho-MAPK substrate, and recombinant TNFα were purchased from Cell Signaling Technology (Danvers, MA). Anti-FLAG M2 antibody (free or conjugated to magnetic beads), palmitate, cycloheximide, fenofibrate, ciprofibrate, actin antibodies were from either Abcam or Sigma. Anti-FLAG M2 antibody (free or conjugated to magnetic beads), palmitate, cycloheximide, fenofibrate, ciprofibrate, Wy14643, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) were purchased from Sigma. Actin antibodies were from either Abcam or Sigma.

**Animals**—Male wild type C57BL/6J mice, ob/ob mice, and PPARα knock-out mice on C57BL/6J background (stock no. 008154) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a standard chow diet and water ad libitum and were housed in a room with a 13-h light (5 a.m. to 7 p.m.) and 11-h dark (7 p.m. to 5 a.m.) cycle. The Western diet (TD.88137) obtained from Harlan Teklad contains 21% milk fat (w/w) and 0.2% cholesterol. Purified diet AIN 93G (Harlan Teklad) contains ~8% fat and was used as a control diet. The fish oil-supplemented diet was prepared by Harlan Teklad as follows. 6% of the 8% fat in the AIN 93G control diet was replaced with fish oil, whereas 8% of the total 21% fat in the Western diet was replaced by fish oil. The fish oil-supplemented diet and the corresponding control diet had approximately equal amounts of calories from fat content. The final n-3 PUFAs (mainly DHA and EPA) in the diet were about 0.6–0.8%, consistent with previous studies (28–30). To minimize oxidation of n-3 PUFAs, fish oil-supplemented diets were replaced daily. Fenofibrate was mixed with food at 0.2% (w/w) based on previous published studies (31). Mice were fasted overnight before sacrifice. All study protocols were approved by the Institutional Animal Care and Use Committee.

**Cell Culture, Transfection, and Treatment**—The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA). Palmitate (50 mM) was first dissolved in 0.01 N NaOH to yield a clear solution, which was then added to serum-free medium containing 2% fatty acid-free bovine serum albumin (BSA) and incubated at 37 °C for 2–4 h before the addition to cells (32). Vehicle control was prepared in the same procedure without palmitate. DHA and EPA were dissolved in 100% ethanol and incubated in serum-free culture medium containing 2% BSA at 37 °C for 2 h before adding to cells. PPARα agonists for cell culture study were dissolved in DMSO. FLAG-tagged Sort1 phosphorylation mutant plasmids were generated using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Inc. Santa Clara, CA). Plasmids were transfected into HepG2 cells with Lipofectamine 2000 (Invitrogen). Cell treatments were carried out 24–48 h after transfection.

**Primary Mouse Hepatocyte Isolation and Culture**—Male C57BL/6J mice were used for primary hepatocyte isolation in the Cell Isolation Core at the University of Kansas Medical Center. A two-step collagenase perfusion method was used. After the induction of anesthesia with ketamine and xylazine, the peritoneal cavity was opened, a 24-gauge catheter was inserted into the inferior vena cava, and the perfusion was started. Instantly, the portal vein was cut to release the excess volume and pressure. The diaphragm was cut, and the superior vena cava was clamped. The liver was perfused in situ for 10 min at 37 °C with calcium and magnesium-free Hanks’ balanced salt solution (Hyclone, SH30588.02) followed by perfusion with Hanks’ balanced salt solution containing calcium, magnesium (Hyclone, SH30268.01), and 0.025 mg/ml LiberaseTM (Roche Applied Science, 0540112001) until the liver revealed signs of digestion. The liver was then placed in ice-cold calcium- and magnesium-free Hanks’ balanced salt solution and chopped with sterile scissors to release the isolated liver cells. The cell suspension was filtered through nylon gauze, centrifuged for 5 min at 50 × g at 4 °C, and then resuspended in fresh ice-cold calcium- and magnesium-free Hanks’ balanced salt solution. Hepatocyte viability was evaluated by the trypan blue exclusion method, and the number of hepatocytes was determined using a hemacytometer. Cells were plated on collagen-coated 6-well culture plates and allowed to attach for 2 h, after which the cells were then cultured in Williams’ E medium (Sigma). After about 24 h, the treatment was initiated for the additional time indicated. The isolation protocol was approved by the Institutional Animal Care and Use Committee.

**Recombinant Adenovirus**—Adenovirus vectors expressing an shRNA against human and mouse Sort1 or expressing a C-ter-
Fenofibrate Treatment Increased Hepatic Sort1 Protein in Western Diet-fed Mice in a PPARα-dependent Manner—We next investigated the mechanisms by which dietary fish oil regulates hepatic Sort1 in mice. Analysis of hepatic gene expression revealed that dietary fish oil did not affect the mRNA expression of microsomal triglyceride transfer protein in VLDL assembly and only modestly decreased hepatic SREBP-1c (sterol regulatory element-binding protein) mRNA that was induced by Western diet feeding (Fig. 3A). Under chow-fed conditions, dietary fish oil induced hepatic CD36 involved in FFA uptake. CD36 mRNA was highly induced by Western diet feeding, which was not further altered by dietary fish oil supplement (Fig. 3A). Dietary fish oil also induced hepatic fatty acid t test or analysis of variance. p < 0.05 was considered as statistically significant.

RESULTS

Fish Oil Treatment Restored Hepatic Sort1 Levels in Western Diet-fed Mice—To establish a causal relationship between decreased hepatic Sort1 and elevated plasma lipid levels in obesity, we first monitored hepatic Sort1 protein changes in response to Western diet feeding over a time course in mice. We found that a relatively short term Western diet feeding for 4 weeks already resulted in a significant decrease of hepatic Sort1 protein (Fig. 1A) but not mRNA (not shown), suggesting that down-regulation of hepatic Sort1 was coupled to the progression of Western diet-induced metabolic perturbations. To further determine whether hepatic Sort1 down-regulation may further contribute to the development of hyperlipidemia, we injected wild type mice with an adenoviral vector expressing a Sort1 shRNA (Fig. 1, B and C). Interestingly, knockdown of hepatic Sort1 by ~50–60% resulted in significantly elevated plasma TG levels (Fig. 1D) but did not affect plasma total cholesterol levels (Fig. 1D). Our previous study showed that liver-specific Sort1 overexpression significantly decreased both plasma TG and total cholesterol in ob/ob mice (27). It is possible that Sort1 specifically regulates plasma LDL-C, which accounts for a very small percentage of total plasma cholesterol in wild type mice. These results suggest that reduced hepatic Sort1 upon Western diet feeding plays a role in the development of dyslipidemia. On the other hand, whether and how hepatic Sort1 can be modulated by therapeutic approaches has never been investigated. It is known that dietary fish oil, enriched with n-3 PUFA DHA and EPA, inhibited hepatic apoB/VLDL production and were effective in lowering plasma TG in diabetic humans and animal models (34–36). We therefore asked whether Sort1 could be regulated by fish oil. Interestingly, feeding mice with fish oil-supplemented chow or Western diet for 12 weeks significantly induced hepatic Sort1 mRNA (Fig. 2A). Western diet feeding did not inhibit Sort1 mRNA but dramatically reduced Sort1 protein levels in mouse livers, which were completely restored to normal levels by fish oil (Fig. 2B). As expected, FPLC analysis showed that fish oil reduced plasma VLDL-TG by ~25% and LDL-C by ~40% in mice (Fig. 2, C and D). Taken together, these studies revealed that hepatic Sort1 is a novel target of fish oil, and increased hepatic Sort1 may be a novel mechanism linking fish oil treatment to reduced plasma TG levels in obesity and diabetes.
oxidation genes carnitine palmitoyltransferase 1 (Cpt1) and acyl-CoA oxidase 1 (Acox1) under both chow-fed and Western diet-fed conditions (Fig. 3B). In addition, dietary fish oil prevented Western diet-induced proinflammatory cytokine expressions in mouse livers (Fig. 3C), consistent with the anti-inflammatory role of n-3 PUFAs (37). Because CD36, Cpt1, and Acox1 are known PPARα target genes (38) and n-3 PUFAs are known ligands for PPARα (39), we therefore asked whether induction of hepatic Sort1 by dietary fish oil may be mediated by PPARα activation. To test this, we first fed wild type mice either a chow diet or a Western diet for 3 weeks, followed by fenofibrate administration for an additional 3 weeks. As expected, fenofibrate treatment reduced plasma TG levels by ~50% in chow-fed mice (Fig. 4A). Western diet feeding resulted in a ~2-fold increase in plasma TG, which was decreased by ~80% by fenofibrate treatment (Fig. 4A). Fenofibrate did not lower plasma TG in PPARα knock-out mice, suggesting that the TG-lowering effect of fenofibrate was largely mediated by PPARα (Fig. 4A). Interestingly, fenofibrate treatment indeed induced hepatic Sort1 mRNA of Sort1 and Acox1 (positive control) under both chow-fed and Western diet-fed mice (Fig. 4B). Western diet feeding did not reduce hepatic Sort1 mRNA in wild type mice (Fig. 4B). Unexpectedly, Western diet-fed PPARα knock-out mice showed a ~70% reduction in Sort1 mRNA compared with Western diet-fed wild type mice (Fig. 4B). Fenofibrate did not induce Acox1 mRNA but still induced Sort1 mRNA in PPARα knock-out mice (Fig. 4B). Western blot analysis showed that fenofibrate did not significantly increase hepatic Sort1 protein in chow-fed mice despite its induction of Sort1 mRNA (Fig. 4C). However, fenofibrate treatment completely restored hepatic Sort1 protein that was repressed by Western diet feeding (Fig. 4C). Western diet-fed PPARα knock-
out mice showed much lower hepatic Sort1 protein levels compared with Western diet-fed wild type controls, and fenofibrate treatment failed to increase hepatic Sort1 protein in PPAR\alpha knock-out mice (Fig. 4D). Fenofibrate significantly reduced circulating FFA levels (Fig. 4E), which may explain the increased hepatic Sort1 protein. In contrast to these in vivo observations, however, treatment of primary hepatocytes or HepG2 cells with PPAR\alpha agonist fenofibrate, ciprofibrate, and Wy14643 only induced the positive control Acox1 but did not induce Sort1 mRNA or protein (Fig. 5, A and B). Treatment of DHA or EPA, which are also PPAR\alpha ligands, did not induce Sort1 protein in primary mouse hepatocytes in vitro (Fig. 5C). A short term treatment of fish oil for 1 week did not affect hepatic Sort1 mRNA or protein levels in mice either (Fig. 5, D and E). In summary, these results suggest that fenofibrate effect on hepatic Sort1 protein levels was PPAR\alpha-dependent. Sort1 was not a direct transcriptional target of PPAR\alpha, and fenofibrate induction of Sort1 mRNA in mouse livers was PPAR\alpha-independent. Importantly, these results suggest that the beneficial effects of dietary fish oil and fenofibrate mainly reside in reversing the Western diet-induced posttranslational Sort1 down-regulation, which was probably secondary to metabolic and signaling changes caused by dietary fish oil and fenofibrate activation of PPAR\alpha in the liver.

\textit{n-3 PUFA}s and \textit{Fenofibrate Regulate Hepatic Sort1 through the Modulation of Fatty Acid Metabolism and Signaling in Western Diet-fed Mice—}Elevated circulating saturated fatty acids, via activation of intracellular signaling pathways, have been critically implicated in the pathogenesis of inflammation, insulin resistance, and hyperlipidemia in obesity and diabetes (6–9). Because our recent study suggested that elevated saturated fatty acid activated ERK signaling to cause posttranslational Sort1 down-regulation (27), we tested the hypothesis that n-3 PUFA}s and fenofibrate increased hepatic Sort1 protein in Western diet-fed mice by modulation of fatty acid metabolism and signaling in the liver. In support of this hypothesis, we first showed that hepatic Sort1 protein, but not mRNA, was markedly decreased in PPAR\alpha knock-out mice that had impaired fatty acid metabolism and thus elevated fatty acid levels (Fig. 6, A–D). In addition, Western diet feeding increased plasma and hepatic FFA levels, whereas dietary fish oil significantly decreased plasma and hepatic FFA levels in mice (Fig. 7, A and B). Furthermore, we found that treatment of HepG2 cells with palmitate reduced Sort1 protein, and co-treatment of HepG2 cells with DHA or EPA completely prevented palmitate down-regulation of Sort1 (Fig. 7C). When cells were pretreated with cycloheximide to block protein synthesis, palmitate treatment accelerated Sort1 protein degradation, which was blocked by DHA treatment (Fig. 7D). Our previous study showed that palmitate inhibition of Sort1 protein was dependent on ERK activation in HepG2 cells (27). Consistently, treating HepG2 cells with palmitate rapidly induced ERK phosphorylation, which was attenuated by DHA treatment (Fig. 7, E and F). These results are consistent with recent findings that increased cellular content of n-3 PUFA}s can prevent inflammatory signaling activation in many cell types, including immune cells, prob-

\textbf{FIGURE 2.} \textit{Dietary fish oil increased hepatic Sort1 protein in Western diet-fed mice.} Male C57BL/6J mice were fed a chow diet (C) or Western diet (WD) with or without fish oil (FO) supplement for 12 weeks. A, liver Sort1 mRNA (A) was measured by real-time PCR. Real-time PCR results are plotted as mean \pm S.E. (error bars); \(n = 5–6, p \leq 0.05\) is considered as statistical significance; *, versus chow-fed group; #, versus Western diet-fed group. B, liver Sort1 protein was determined by Western blot. Bottom, band intensities were quantified by ImageJ software. \(p \leq 0.05\) is considered as statistical significance; *, versus chow-fed group. C and D, FPLC analysis of plasma lipoprotein profile. Pooled plasma samples from 5–6 mice/group were used.
ably by modulating membrane lipid composition and lipid raft structures (40, 41).

Phosphorylation of Sort1 Regulates Sort1 Protein Stability—Our results obtained so far suggest that hepatic Sort1 was highly sensitive to FFA-induced posttranslational down-regulation, and fish oil and fenofibrate increased hepatic Sort1 in obese mice through their ability to reduce circulating fatty acid levels and to antagonize FFA signaling activation. However, how elevated FFA caused Sort1 protein degradation is still not clear. To obtain further mechanistic insights, we asked whether decreased Sort1 protein in obese mice could be a result of altered Sort1 protein phosphorylation. To test this, we expressed a FLAG-tagged human Sort1 in wild type and ob/ob mice using adenovirus-mediated gene delivery. After 7 days, immunoprecipitated FLAG-Sort1 protein was used for LC/MS/MS-based analysis of Sort1 phosphorylation. This analysis revealed that a serine residue (Ser-793) within the Sort1 C-terminal cytoplasmic tail was uniquely phosphorylated in obese mice but not in WT mice (Table 1 and Fig. 8A). In addition, Ser-825 of Sort1 was phosphorylated in both obese and wild type mice. To determine whether phosphorylation at these serine residues alters Sort1 protein stability, plasmids expressing wild type and non-phosphorylatable mutant Sort1 were transfected into HepG2 cells. As shown in Fig. 8B, when protein synthesis was blocked by cycloheximide treatment, Sort1 with an S793A mutation showed significantly increased stability, whereas the S825A mutation did not seem to increase Sort1 stability. The lysine residue Lys-818 on Sort1 (Fig. 8A) was previously shown to be a ubiquitination site that mediates Sort1 protein turnover (42). As a positive control, we also showed that Sort1 with a K818R mutation showed increased protein stability, suggesting the involvement of ubiquitination-mediated cellular Sort1 degradation (Fig. 8B). Furthermore, we showed that both Sort1 with the S793A mutation and Sort1 with the K818R mutation were resistant to palmitate inhibition in HepG2 cells (Fig. 8C), suggesting that palmitate-induced Sort1 protein degradation may require phosphorylation at Ser-793 and ubiquitination at Lys-818. Furthermore, a cell-based ubiquitination assay showed that Sort1 was ubiquitinated under basal conditions in HepG2 cells, consistent with ubiquitination-mediated Sort1 cellular turnover (Fig. 8D). Palmitate treatment caused increased ubiquitination of wild type Sort1. S793A mutation did not seem to alter Sort1 ubiquitination under basal conditions but did prevent palmitate-induced ubiquitination, suggesting a possible interaction between phosphorylation of Ser-793 and palmitate-induced Sort1 ubiquitination. Consistent with the previous report (42), the K818R mutation resulted in a dramatic reduction of Sort1 ubiquitination under both basal and palmitate-treated conditions, which confirmed that Lys-818 is the major ubiquitination site on Sort1. S793A mutation did not seem to alter Sort1 ubiquitination under basal conditions but did prevent palmitate-induced ubiquitination, suggesting a possible interaction between phosphorylation of Ser-793 and palmitate-induced Sort1 ubiquitination. Consistent with the previous report (42), the K818R mutation resulted in a dramatic reduction of Sort1 ubiquitination under both basal and palmitate-treated conditions, which confirmed that Lys-818 is the major ubiquitination site on Sort1. To further test if palmitate increases Sort1 phosphorylation at Ser-793, we immunoprecipitated FLAG-Sort1 protein from HepG2 cells treated with vehicle or palmitate for 4 h. LC/MS/MS analysis showed that Ser-793 was phosphorylated in non-treated HepG2 cells, and the phosphorylation level was increased by ~2.7-fold upon palmitate treatment (Fig. 8E and Table 2). The LC/MS/MS analysis was further confirmed by an anti-phosphoserine antibody in Western blot (Fig. 8F).
This study demonstrated in a number of in vivo and in vitro models that hepatic Sort1 was highly sensitive to fatty acid-induced posttranslational down-regulation and was markedly decreased in obesity and diabetes. How liver Sort1 lowers plasma cholesterol and TG is not fully understood, but current evidence suggests that the lipid-lowering effect of liver Sort1 may be mediated via its ability to modulate hepatic apoB production and cellular uptake (17, 24, 43). Our previous study also confirmed that overexpression of Sort1 in HepG2 cells or mouse hepatocytes inhibited apoB secretion into the culture medium (27). Therefore, down-regulation of Sort1 in obesity and diabetes linked elevated circulating FFA, a hallmark change in insulin-resistant states, to hepatic apoB overproduction, another characteristic feature of diabetes. In response to increased hepatic fatty acid influx, decreased Sort1 will attenuate lysosome-dependent presecretory apoB degradation and thus increase the availability of apoB for VLDL assembly and secretion. FFA down-regulation of Sort1 may be an integrative part of the hepatic regulatory network that coordinately controls apoB production, lipogenesis, and VLDL secretion in response to FFA oversupply. Such mechanisms may play an adaptive role in alleviating fat accumulation and “lipotoxic” damage to the liver but at the cost of developing diabetic hypertriglyceridemia.

Another new finding from this study is that hepatic Sort1 is a novel target of fish oil and fenofibrate. Both treatments fully restored hepatic Sort1 levels in Western diet-fed mice. It is thought that the lipid-lowering effects of fish oil are probably mediated by the active ingredient n-3 PUFAs, but the mechanism of n-3 PUFA-mediated plasma TG lowering is not fully understood. A large number of studies found that n-3 PUFA

\[\text{FIGURE 4. Fenofibrate increased hepatic Sort1 protein in Western diet-fed mice in a PPAR\alpha-dependent manner.} \]

Male C57BL/6J wild type mice and PPAR\alpha knock-out mice were fed a chow diet or a Western diet for 3 weeks and then a chow or Western diet containing 0.2% (w/w) fenofibrate (F) for an additional 3 weeks. A, plasma TGs were measured by assay kits. Results are plotted as mean ± S.E. (error bars); n = 3–5. p ≤ 0.05 is considered as statistical significance; *, versus chow-fed group; #, versus untreated group on the same diet. NS, not significant. B–D, hepatic Sort1 mRNA (B) and protein (C and D) were measured by real-time PCR or Western blot, respectively. Results of mRNA expression are plotted as mean ± S.E.; n = 3–5. p ≤ 0.05 is considered as statistical significance; *, versus untreated group on the same diet and of same genotype. E, plasma free fatty acid levels were measured by assay kits. Results are plotted as mean ± S.E.; n = 3–5. p ≤ 0.05 is considered as statistical significance; *, versus chow fed group; #, versus untreated group on the same diet.
treatment was consistently associated with reduction of hepatic apoB and VLDL production (44). Previous studies have shown that n-3 PUFAs may induce apoB degradation through a process involving lipid peroxidation and autophagy/lysosome-mediated protein degradation (34). Our study suggests that increased hepatic Sort1 upon fish oil administration may be a novel molecular link between n-3 PUFA and decreased hepatic apoB production. However, it should be noted that the positive effects of fish oil on hepatic Sort1 expression were only seen in vivo and not in vitro (Fig. 5). In addition, fish oil restored hepatic Sort1 levels under Western diet-fed conditions but did not directly induce hepatic Sort1 protein under basal chow-fed conditions. This is in contrast to the fact that n-3 PUFA treatment also promoted apoB degradation in in vitro cultured cells (34), suggesting that the mechanisms by which n-3 PUFAs regulate hepatic apoB metabolism are pleiotropic. Our results suggest that the effect of fish oil on hepatic Sort1 levels was at least in part mediated through reducing circulating and hepatic FFA as well as antagonizing FFA signaling. These effects are also consistent with the insulin-sensitizing and anti-inflammatory roles of n-3 PUFAs (45, 46). n-3 PUFAs have been shown to regulate hepatic Sort1 by n-3 PUFA and PPARα

FIGURE 5. Sort1 was not induced by PPARα ligands in hepatocytes in vitro. A, primary mouse hepatocytes from C57BL/6J mice were treated with 50 μM fenofibrate (F), 50 μM ciprofibrate (Cipro), or 10 μM Wy14643 (Wy) as indicated for 24 h. Sort1 and Acox1 mRNA were measured by real-time PCR. Real-time PCR results are plotted as mean ± S.E. (error bars); n = 3. p ≤ 0.05 is considered as statistical significance; *, versus control. B, Sort1 protein in mouse primary hepatocytes was treated with PPARα ligands as in Fig. 5A for 24 h. C, primary mouse hepatocytes were treated with DHA or EPA for 24 h. Sort1 protein was measured by Western blot. D and E, C57BL/6J male mice were fed a chow or a Western diet with or without fish oil (FO) supplement for 1 week. Hepatic Sort1 protein and mRNA were measured by Western blot or real-time PCR, respectively. Results of mRNA expression are plotted as mean ± S.E.; n = 3–4.

FIGURE 6. PPARα knock-out mice show reduced hepatic Sort1. Chow-fed male wild type and PPARα knock-out mice were fasted overnight and sacrificed. A and B, plasma and hepatic FFA levels were determined by assay kits. C and D, hepatic Sort1 protein and mRNA were measured by Western blot and real-time PCR, respectively. Results are plotted as mean ± S.E. (error bars); n = 3–4. p ≤ 0.05 is considered as statistical significance; *, versus wild type.
FIGURE 7. n-3 PUFA s prevented palmitate-induced posttranslational Sort1 degradation by antagonizing ERK signaling activation. A and B, male C57BL/6J mice were fed a chow diet (C) or Western diet (WD) with or without fish oil (FO) supplement for 12 weeks. Fasting hepatic and plasma free fatty acid levels were measured. Results are plotted as mean ± S.E. (error bars); n = 5–6. p ≤ 0.05 is considered as statistical significance; *, versus chow-fed group; #, versus WD fed group.

C, HepG2 cells were treated with 0.2 mM palmitate (PA) and 0.025 or 0.05 mM DHA or EPA, as indicated, for 16 h. Sort1 protein was measured by Western blot. H3, histone 3 as loading control. D, HepG2 cells were pretreated with 40 μg/ml cycloheximide (CHX) for 1 h, followed by 0.5 mM palmitate and 0.05 mM DHA, as indicated, for 8 h. Sort1 protein was then measured by Western blot. E and F, HepG2 cells were treated with 0.5 mM palmitate and 0.05 mM DHA as indicated for 30 min or 6 h. Total and phosphorylated ERK (T-ERK and P-ERK, respectively) was measured by Western blot. C–F, mean band intensity of three independent blots was plotted. *, p ≤ 0.05.

TABLE 1
LC/MS/MS identification of Sort1 phosphorylation sites in mouse liver
Phosphorylated serines are marked with an underline and asterisk. MI score, Mascot ion score; ND, not detected.

| Site       | Peptide sequence            | Wild type       | Spectral count | MI score | Δppm | ob/ob       | Spectral count | MI score | Δppm |
|------------|----------------------------|-----------------|----------------|----------|------|-------------|----------------|----------|------|
| Ser-793    | (R)FLVHRYS*VLQQHAEANGVDGVDALDTASHTNK | ND              | ND             | ND       | ND   | 2.5E8       | 58.3          | 0.46     |      |
| Ser-825    | (S)GYHDDS*DEDLLI(−)        | 3.9E8           | 49.9           | −0.26    | 2.6E8| 50.5        | −1.34          |          |      |
inhibit ERK activation in a number of cell types (47, 48). It is hypothesized that n-3 PUFAs may directly modulate the cell membrane lipid microenvironment, interfere with Ras palmitoylation, and thus antagonize inflammatory signaling activation. Our studies in fenofibrate-treated mice and in PPARα knock-out mice provide additional support for a role of elevated fatty acids in hepatic Sort1 down-regulation. It is generally thought that activation of PPARα by fibrates transcriptionally induces genes in the fatty acid oxidation pathway, which decreases the availability of fatty acids for apoB lipidation and

![FIGURE 8. Phosphorylation of Sort1 at Ser-793 regulates Sort1 protein stability. A, diagram illustrating the identified phosphorylation sites on Sort1. TM, transmembrane domain. Bottom, representative spectrum of Sort1 from ob/ob mouse liver showing phosphorylation at Ser-793. B, HepG2 cells in a 12-well plate were transfected with wild type and mutant Sort1 plasmids (1 μg/well). After 24 h, cells were treated with cycloheximide (CHX) at 40 μg/ml for the indicated time. Western blot was performed with anti-FLAG antibody to detect FLAG-Sort1 protein. Mean band intensity of three independent blots was plotted to illustrate protein degradation rate. p < 0.05 is considered as statistical significance; *, versus WT at the same time point. C, HepG2 cells were transfected with wild type and mutant Sort1 expression plasmids. After 24 h, cells were treated with 0.5 mM palmitate for 8 h, and FLAG-Sort1 was detected in Western blot with anti-FLAG antibody. Bottom, mean band intensity of three independent blots was plotted. p < 0.05 is considered as statistical significance; *, versus WT control. D, cell-based ubiquitination assays were performed as described under “Experimental Procedures.” L, total lysate from HepG2 cells transfected with plasmid expressing HA-tagged ubiquitin and used as a positive control for HA-ubiquitin. NC, negative control. HepG2 cells transfected with HA-ubiquitin plasmid but not HA-ubiquitin plasmid and subjected to immunoprecipitation with anti-FLAG antibody-conjugated magnetic beads. PA, palmitate (0.5 mM, 4 h). E, LC/MS/MS analysis of the relative Sort1 Ser-793 phosphorylation level in HepG2 cells treated with 0.5 mM palmitate (PA) for 4 h. F, HepG2 cells were transfected with plasmids expressing FLAG-Sort1 WT or S793A mutant and then treated with 0.5 mM palmitate for 4 h. FLAG-Sort1 WT or S793A mutant protein was then immunoprecipitated by FLAG antibody. Anti-phosphoserine antibody was used in a Western blot to measure serine phosphorylation of the immunoprecipitated Sort1 protein. NC, negative control. HepG2 cells without transfection of FLAG-Sort1 plasmid. Mean relative band intensity of three independent blots was measured by densitometry and shown in the bottom panel. p < 0.05 is considered as statistical significance; *, versus untreated WT Sort1. IP, immunoprecipitation.
VLDL assembly (49). This study revealed that PPARα activation may concomitantly inhibit apoB production by increasing hepatic Sort1 levels. Coordinate regulation of apoB degradation and hepatic fatty acid oxidation ensures inhibition of hepatic VLDL secretion without causing hepatic fat accumulation.

Because both fish oil and fenofibrate prevented posttranslational liver Sort1 down-regulation in Western diet-fed mice, the downstream mechanisms that caused posttranslational Sort1 degradation in obesity are still not clear. This study first addressed this question by identifying differential phosphorylation of liver Sort1 at Ser-793 between WT and obese mice and further demonstrated a negative relationship between phosphorylation of Sort1 at Ser-793 and Sort1 protein stability. These results provided new insights into the Sort1 down-regulation by palmitate in vitro and in obese and diabetic conditions in vivo. However, several questions remain to be addressed by additional studies in the future. First, we showed in our previous study that ERK inhibitor prevented palmitate inhibition of Sort1 protein in cultured cells and that injection of an ERK inhibitor increased hepatic Sort1 in both wild type and ob/ob mice (27), demonstrating that ERK activation inhibits Sort1. However, Ser-793 is not a conserved ERK phosphorylation site, and our preliminary results did not indicate that palmitate-activated ERK directly phosphorylates Sort1 (not shown). It is possible that ERK may activate another downstream kinase(s) that phosphorylates Sort1 at Ser-793. We also cannot rule out the possibility that Ser-793 is phosphorylated by a kinase completely independent of ERK signaling in obese mouse livers. Second, how phosphorylation at Ser-793 may reduce Sort1 stability is not known. One possibility suggested by this study is that phosphorylation at Ser-793 may create a motif for E3 ligase and subsequently increased ubiquitination. Such a motif may concomitantly inhibit apoB production by increasing hepatic VLDL secretion without causing hepatic fat accumulation.

In summary, this study provides new mechanistic insights into the regulation of hepatic Sort1 in obesity and diabetes and further suggests that therapeutic strategies that could prevent posttranslational Sort1 down-regulation or stimulate hepatic Sort1 expression may be beneficial in improving plasma lipid homeostasis in obesity and diabetes.

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## Table 2

### LC/MS/MS identification of Ser-793 phosphorylation in HepG2 cells

Phosphorylated serines are marked with an underline and asterisk. MI score, Mascot ion score.

| Site     | Peptide sequence                  | Localization probability | MI score | Δppm | Ascore | Start | Stop |
|----------|-----------------------------------|--------------------------|----------|------|--------|-------|------|
| Ser-793  | YS*VLQQHAEANGVDGVDALDTASHTNK      | 100%                     | 81.04    | 2.97 | 27.96  | 792   | 818  |
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