INHIBITION OF HEPATIC SULF2 IN VIVO: A NOVEL STRATEGY TO CORRECT DIABETIC DYSLIPIDEMIA

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ABSTRACT

Background - Type 2 diabetes mellitus (T2DM) impairs hepatic clearance of atherogenic postprandial triglyceride-rich lipoproteins (TRL). We recently reported that livers from T2DM db/db mice markedly overexpress glucosamine-6-O-endosulfatase-2 (SULF2), an enzyme that removes 6-O sulfate groups from heparan sulfate proteoglycans (HSPGs) and suppresses uptake of TRLs by cultured hepatocytes. In the present study, we evaluated whether Sulf2 inhibition in T2DM mice in vivo could correct their postprandial dyslipidemia.

Methods and results - Selective second-generation antisense oligonucleotides (ASOs) targeting Sulf2 were identified. Db/db mice were treated for 5 weeks with Sulf2 ASO (20 or 50 mg/kg per week), non-target ASO, or phosphate buffered saline (PBS). Administration of Sulf2 ASO to db/db mice suppressed hepatic Sulf2 mRNA expression by 70-80%, i.e., down to levels in non-diabetic db/m mice, and increased the ratio of tri- to di-sulfated disaccharides in hepatic HSPGs (p<0.05). Hepatocytes isolated from db/db mice on non-target ASO exhibited a significant impairment in VLDL binding that was entirely corrected in db/db mice on Sulf2 ASO. Sulf2 ASO lowered the random, non-fasting plasma triglyceride (TG) levels by 50%, achieving non-diabetic values. Most importantly, Sulf2 ASO treatment flattened the plasma TG excursions in db/db mice after corn-oil gavage (iAUC 1500±470 (mg/dL)·h for non-target ASO versus 160±40 (mg/dL)·h for Sulf2 ASO (p<0.01).

Conclusion - Despite extensive metabolic derangements in T2DM mice, inhibition of a single dysregulated molecule, SULF2, normalizes the VLDL-binding capacity of their hepatocytes and abolishes postprandial hypertriglyceridemia. These findings provide a key proof-of-concept in vivo to support Sulf2 inhibition as an attractive strategy to improve metabolic dyslipidemia.
INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) and related syndromes is rising at an alarming pace worldwide, and the overwhelming majority of affected individuals die from accelerated atherosclerotic cardiovascular disease.\(^1,2\) Atherosclerosis is exacerbated in these patients in large part from their characteristic dyslipidemia, which includes increased fasting levels of very low density lipoprotein (VLDL) and its major component triglyceride (TG), as well as impaired clearance of postprandial triglyceride-rich lipoprotein (TRL) remnants.\(^3,4\) Atherosclerosis arises from the subendothelial retention of these lipoproteins, and increased plasma levels of VLDL and particularly postprandial TRL-remnants have been linked to atherosclerotic cardiovascular events in human cohorts.\(^5,6\)

Unfortunately, current therapeutic strategies have shown limited success in lowering fasting or postprandial TRL concentrations as a way to reduce cardiovascular morbidity or mortality. A major step forward in atherosclerotic cardiovascular risk reduction has been achieved in T2DM by the introduction of statins, a class of medicines that lower plasma levels of LDL cholesterol.\(^10,11\) Nonetheless, T2DM subjects treated with optimal statin therapy, exhibit considerable residual risk for cardiovascular disease, which may occur in part because statins lower TRL levels by only 10-25%.\(^12\) Although fibrates are widely used in the treatment of hypertriglyceridemia, there is no definitive evidence that fenofibrate, when added to statin therapy, reduces the risk of coronary events in subjects with T2DM.\(^13,14\) In addition, we lack therapeutic strategies that specifically restore postprandial remnant lipoprotein clearance to normal in T2DM.

Healthy metabolism of TRLs involves a series of steps that culminate in uptake of TRL-remnants by hepatocytes.\(^1,5,16\) During the past decades, we and others\(^10,19\) have implicated hepatic heparan sulfate proteoglycans (HSPGs) in TRL removal, specifically, the syndecan-1 HSPG.\(^20,21\) The syndecan-1 HSPG comprises a single-span transmembrane core protein that has three extracellular covalent attachment sites for heparan sulfate (HS),\(^22\) which is an unbranched polysaccharide that captures lipoproteins. Roughly 50 genes are involved in HSPG assembly and disassembly, affecting core protein expression, HS side-chain length, epimerization of glucuronyl residues, and sites and extent of sulfation.\(^24\) To molecularly characterize HSPG defects in T2DM liver, we recently used a highly annotated glycomic microarray to compare hepatic expression profiles in obese, T2DM \(db/db\) mice versus lean, non-diabetic \(db/m\) controls.\(^25\) Despite the complexity of HSPG biology, just one gene was identified whose dysregulation could impair syndecan-1 HSPG structure or function: the HS glucosamine-6-O-endosulfatase-2 (Sulf2).\(^26\) This gene encodes an enzyme, SULF2, that removes 6-O sulfate groups from HSPGs.\(^26,27\) Livers of obese T2DM mice were found to markedly overexpress SULF2, and SULF2 was shown to inhibit the catabolism of TRLs by cultured liver cells.\(^25\) Moreover, hepatic Sulf2 mRNA expression was positively related to plasma TG levels.\(^25\) These experimental findings imply that SULF2-mediated disruption of hepatic HSPGs may contribute to impaired TRL clearance in T2DM.
In the present study, we evaluated whether inhibition of this single overexpressed target, Sulf2, could correct the characteristic postprandial dyslipidemia of T2DM mice in vivo. To address this question, second-generation antisense oligonucleotides (ASOs) were identified that selectively inhibit hepatic Sulf2 mRNA expression. We studied the effects of Sulf2 inhibition in vivo on hepatic HSPG sulfation, binding ofTRLs to isolated primary hepatocytes, and most importantly, plasma TG excursions following corn-oil gavage under diabetic conditions.

METHODS

Antisense Oligonucleotides

Antisense therapy relies on base-pair hybridization through which ASOs selectively bind to their complementary mRNA target. This binding typically results in selective, catalytic degradation of the target mRNA by RNase H and thereby reduces levels of the encoded protein. All ASOs used in these studies were 20 nucleotides in length and chemically modified with phosphorothioate in the backbone and 2′-O-methoxyethyl on the wings with a central deoxy gap (5-10-5 gapmer). Oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer (PerkinElmer Life and Analytical Sciences-Applied Biosystems) and purified as previously described.

To identify a potent Sulf2 ASO for experiments in mice, a series of ASOs was designed and tested in primary mouse hepatocytes for their relative abilities to suppress Sulf2 mRNA levels. From these experiments, the optimal Sulf2 ASO was selected, and its efficacy was then verified by its ability to suppress hepatic Sulf2 mRNA levels in wild-type C57BL6 mice. An oligonucleotide that is not complementary to any known murine RNA sequence was used as non-target ASO. In C57BL6 mice (Jackson Laboratory, Bar Harbor, ME, USA), Sulf2 ASO treatment for four weeks (described below) resulted in an 80% ± 3% reduction of hepatic Sulf2 mRNA levels compared to levels after administration of the non-target ASO (two-sided, unpaired Student’s t test, p<0.0001, n=4/group).

Animals and oligonucleotide dosing

Seven-week-old male T2DM db/db (Lepr<sup>db/db</sup>) mice and lean non-diabetic control db/m mice from the same colony on the C57BLKS background, were used (Jackson Laboratory, Bar Harbor, ME, USA). Animals were injected intraperitoneally twice weekly with Sulf2 ASO (10 or 25 mg/kg per dose, i.e., 20 or 50 mg/kg per week), non-target ASO (50 mg/kg per week), or PBS for 5 weeks. The animals were housed in micro-isolator cages on a constant 12-hour light-dark cycle with controlled temperature and humidity and were given access to food and water ad libitum (Purina LabDiet #5008). Two days after the final dose, mice were weighted, and plasma samples were taken for in-house assays of plasma glucose, insulin, and markers of liver function, as well as plasma lipids (Olympus Analyser). Plasma insulin levels were analyzed using a commercially available Elisa (Crystal Chem Inc, 90080). HOMA-IR was defined as [fasting plasma insulin (μU/mL) * fasting plasma glucose (mmol/L)] /22.5. All animal procedures were approved by the Institutional Animal Care and Use Committee.
Measurements of hepatic mRNA levels
Mouse livers were homogenized in guanidine isothiocyanate solution (Invitrogen) supplemented with 8% 2-mercaptoethanol (Sigma). Total RNA was prepared using RNeasy mini kits (Qiagen) and reversed transcribed with cDNA synthesis kit (Bio-Rad). Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assays for Suls were performed using an ABI Prism 7700 sequence detector (Applied Biosystems). The sequences of primers and probe for mouse Sulf2 were:

5'-TGGACGGTGAGATATACCACGTA-3' (forward),
5'-CAGTGCGGCTTGCTAAGGTT-3' (reverse),
and F-5'-CTTGGATACGTGCTCAGCCC5'-Q (probe) (Integrated DNA Technologies).

The primers for mouse Sulf1 were:
5'-TCATTCGTGGTCCAAGCATAGA-3' (forward),
5'-TGGTAGGAGCTAGGTCGATGTTC-3' (reverse),
and F-5'-CCAGGGTCGATAGTCCCACAGATTGTTC-3' (probe).

18S RNA was used to normalize gene expression, primers:
5'-Gcaattattccccatgaacg-3' (forward) and 5'GGGACTTAATCAACGCAAGC-3' (reverse) AND 5'-TTCCCAGT-3' (probe).

Purification and analysis of heparan disaccharides from liver
Heparan sulphate (HS) disaccharides from murine liver tissue were prepared and measured as described previously. Briefly, 50 mg of liver tissue was homogenized in 300 µl NH₄Ac/Ca(Ac)₂, pH 7, and digested by a mixture of recombinant heparinas I, II and III (5 IU each; kind gifts from Dr. Jian Liu, University of North Carolina, Chapel Hill, USA) for two hours at 37°C. Samples were heat inactivated and centrifuged (16,000 g for 5 min). The supernatant was transferred to an Amicon ultracentrifugal filter (Millipore) with a 5-kDa cut-off. The filtered samples containing heparan disaccharides were applied to an LC/MS/MS (Acquity UPLC®, Waters Inc.) and Quattro Premiere XE (Micromass) using multiple reactions monitoring in negative ion mode. Separation of HS disaccharides by LC was performed using a Hypercarb column (2.1 mm i.d. ×100 mm, 5 μm, Thermo Scientific) with a gradient elution (10 mM NH₄HCO₃, pH10, to 100% acetonitrile). HS disaccharides standards were purchased from Iduron (Manchester, UK). SULF2 activity in vivo was analysed as the ratio of trisulfated (D2S6) vs disulfated (D0S6 and D2S0) heparan disaccharides (see 31 for nomenclature). The two disulfated disaccharides could not be separated.

Isolation and DyLight labelling of TRL fraction
Human TRLs (d < 1.006 g/ml) were isolated by density gradient ultracentrifugation (SW41 rotor; 19 h, 36,000 rpm, 10ºC) from serum obtained from fasting healthy volunteers. The TRL fraction was labelled with Dy-Light fluorophore (Amine-Reactive Fluors 488, Thermo-Scientific), which allows a high dye-to-protein ratio. The labelling was performed according to the manufacturer’s protocol.

Primary hepatocyte isolation and lipoprotein binding
Following administration of ASO or PBS to db/db and db/m mice, primary hepatocytes were isolated using collagenase perfusion as described previously. Isolated hepatocytes were plated on Primaria multiwell plates (Becton Dickinson) using Williams’ Medium E 1x (GIBCO ® Invitrogen). After 3 hours,
the original culture medium was replaced by serum-free Williams’ Medium E containing 1% BSA, followed by 6-hrs incubation at 37°C. Several minutes before the binding experiments, cells were pre-chilled on ice followed by a wash with Medium E/1% BSA at 4°C. Cells were incubated with a combination of DyLight-TRL (50 µg/ml) and bovine lipoprotein lipase (LPL 5 ug/ml; L2254, Sigma) for 30 minutes at 4°C. Cells were rinsed once with cold PBS and lysed in 200 µl RIPA buffer supplemented with protease inhibitors (Roche, Basel Switzerland). Cell lysates were collected and transferred into a black 384-well plate, and fluorescence was measured using the Cytofluor Multiwell plate Reader 4000 (Biosystems, USA).

Postprandial fat tolerance testing
A stock preparation of 1 ml corn oil (Sigma #C8267) was supplemented with 27 µCi of [11.12-3H] retinol (44.4 Ci/mmol; Perkin Elmer Life Sciences) in ethanol. Mice were fasted for 4 hours, after which each mouse received 10 µl of the corn oil/[3H]retinol mixture per gram of body weight by gastric gavage. Blood was sampled at the indicated times by submandibular bleeding. Triglycerides were measured on an Olympus Clinical Analyzer (Beckman Coulter) and [3H] was quantified by scintillation counting.

Statistical Analyses
Normally distributed data are presented as mean ± SEM unless otherwise stated. For comparisons between a single treatment group and a control, the unpaired, two-tailed Student’s t test was used. For comparisons amongst several groups, analysis of variance (ANOVA) was initially used, followed by pairwise comparisons using the Student-Newman-Keuls q statistics. P-values less than 0.05 were considered significant. Data and graphics were analysed and constructed by GraphPad Prism, Version 5 for Windows.

RESULTS
Treatment of db/db mice with Sulf2 ASO specifically restores hepatic expression of Sulf2 to normal
By the end of the five-week treatment period, body weights, random non-fasting plasma glucose and insulin levels, and HOMA-IR values were significantly higher in PBS-treated db/db mice compared to PBS-treated db/m mice (Table 1). These parameters were not corrected by the non-target ASO or by either dose of the Sulf2 ASO in db/db mice. Markers of liver function were mildly elevated following non-target and Sulf2 ASO (Table 1). Liver, kidney and spleen histology did not show remarkable differences between saline and oligo-treated animals (data not shown).

Hepatic Sulf2 mRNA expression was strongly induced in PBS-treated db/db mice, to five times the levels in db/m mice (Figure. 1A, PBS-treated db/db vs. PBS-treated db/m, P<0.0001), consistent with our prior report. Importantly, administration of Sulf2 ASO to db/db mice suppressed hepatic Sulf2 mRNA levels by 70-80%, and the higher dose restored hepatic Sulf2 mRNA to levels indistinguishable from db/m (Figure. 1A). The Sulf2 ASO had no effect on hepatic Sulf1 mRNA levels (Figure. 1B),
Seven-week-old male db/db and db/m mice were given PBS, Sulf2 ASO, or non-target (NT) ASO for five weeks at indicated weekly doses. Two days after the final dose, body weight and random plasma levels of glucose, insulin, ALT and AST were measured. Displayed are means ± SEM, n=5–8 animals/group. * P-value by ANOVA; columns labelled with different lowercase letters (a,b,c) are statistically different from each other by the Student-Newman-Keuls test (p<0.05).

indicating specificity. These data show that Sulf2 ASO effectively and selectively normalizes hepatic Sulf2 mRNA expression in db/db mice.

Administration of Sulf2 ASO in vivo to db/db mice increases trisulfated heparan disaccharides in liver and completely restores the ability of primary hepatocytes to bind triglyceride-rich lipoproteins.

To assess the effects of Sulf2 inhibition in vivo, we began by analyzing heparan sulfation in liver homogenates from db/db mice treated with Sulf2 vs. non-target ASO. Consistent with the pattern of hepatic Sulf2 expression in Figure 1, administration of low and high dose Sulf2 ASO to db/db mice significantly raised the ratio of tri- to di-sulfated heparan disaccharides in their livers, whereas the non-target ASO had no effect over PBS (0.73 ± 0.04 and 0.72 ± 0.06 vs 0.56 ± 0.08 respectively; p<0.05, Figure 2A). Next, we analysed the binding of DyLight-labeled VLDL to primary hepatocytes isolated from db/db mice following treatment with Sulf2 or non-target ASOs and from db/m mice following treatment with PBS. Compared to db/m hepatocytes, hepatocytes isolated from db/db mice after administration of the non-target ASO exhibited a significant impairment in VLDL binding that was completely corrected in hepatocytes from db/db mice following treatment with Sulf2 ASO (Figure 2B). These data collectively show that Sulf2 inhibition in vivo in db/db mice increases hepatic HSPG sulfation and restores hepatocyte binding of TRLs.

Treatment of db/db mice with Sulf2 ASO corrects their random non-fasting hypertriglyceridemia.

Consistent with previous reports 25, PBS-treated db/db mice exhibited a significant non-fasting hypertriglyceridemia (Figure. 3A, PBS-treated db/db vs. PBS-treated db/m, P<0.05). Administration of the non-target ASO to db/db mice had no detectable effect on their non-fasting TG levels. In
Figure 1 - Treatment of db/db mice with Sulf2 ASO specifically restores hepatic expression of Sulf2 to normal. Seven-week-old male db/m and db/db mice were given PBS, non-target (NT) ASO, or Sulf2 ASO for five weeks at indicated weekly doses (n=5-8 animals per group). Two days after the final dose, we harvested livers for RNA isolation. Levels of Sulf2 (panel A) and Sulf1 (panel B) mRNA were assessed by way of qRT-PCR, normalized to 18S RNA, and expressed relative to PBS-treated db/m mice. In panel A p < 0.0001 by ANOVA; columns labelled with different lowercase letters (a,b,c) are statistically significant different from each other by the Student-Newman-Keuls test (p< 0.05). In panel B, the p-value was not significant by ANOVA.
Administration of Sulf2 ASO in vivo to db/db mice increases trisulfated heparan disaccharides in liver and restores the ability of primary hepatocytes to bind triglyceride-rich lipoproteins. Panel A: db/db mice were given PBS or ASO as indicated for five weeks (n=4 per group). Sulfation of heparan disaccharides in liver homogenates was measured and expressed as the ratio of tri- to di-sulfated disaccharides (D2S6 vs D2S0 and D0S6 combined). P < 0.0001 by ANOVA; columns labelled with different lowercase letters (a,b) are statistically significant different from each other by the Student-Newman-Keuls test (p< 0.05). Panel B: Mice were given PBS or ASO as indicated for five weeks. Primary hepatocytes were isolated two days after the final dose (n=4 animals per group). Hepatocytes were cultured overnight at 37°C and then incubated for 30 minutes at 4°C with DyLight-labelled VLDL (50 µg/ml) plus LPL (5 µg/ml). VLDL binding was assessed by measuring cell-associated fluorescence (RFU: relative fluorescence units). * p < 0.05 compared to PBS-treated db/m.
Figure 3 - Treatment of db/db mice with Sulf2 ASO corrects their random non-fasting hypertriglyceridemia. Plasma lipids were measured in the same mice as in Figure 1, two days after the final dose of PBS or ASO (n=5-8 per group). (A) Random, non-fasting plasma triglyceride levels. (B) Random, non-fasting plasma total cholesterol concentrations. P < 0.0001 by ANOVA; columns labelled with different lowercase letters (a,b) are statistically significant different from each other by the Student-Newman-Keuls test (p< 0.05).

In contrast, the Sulf2 ASO caused a dose-dependent improvement in non-fasting hypertriglyceridemia, reaching a 50% reduction in non-fasting TG levels at the higher dose (Figure 3A, 102 ± 8 mg/dl in db/db Sulf2 ASO 50 mg/kg vs. 171 ± 23 mg/dl in db/db non-target ASO and 212 ± 18 mg/dl in db/db PBS, p<0.05), thereby restoring this parameter to a level indistinguishable from PBS-treated db/m mice (125 ± 7 mg/dl. Fasting plasma TG levels (not shown) and non-fasting plasma total cholesterol
concentrations (Figure. 3B) were significantly higher in PBS-treated db/db mice compared to the db/m mice and were not corrected by either dose of Sulf2 ASO.

Treatment of db/db mice with Sulf2 ASO completely abolishes their postprandial dyslipidemia. After five weeks of treatment, db/db animals were fasted for 4h, then given a gavage of corn oil enriched with [³H]retinol. As shown in Figure 4A-B, Sulf2 ASO administration to db/db mice flattened their postprandial TG excursions. The iAUC was 1500 ± 470 (mg/dL)·h in db/db mice given the non-target ASO, which fell to just 160 ± 40 (mg/dL)·h in mice treated with the higher dose of Sulf2 ASO (Figure. 4A-B). Likewise, Sulf2 ASO lowered plasma [³H]retinol excursions by >50%, indicating a profound improvement in the clearance of chylomicron remnant particles (Figure. 4C-D).

Figure 4 - Treatment of db/db mice with Sulf2 ASO completely abolishes their postprandial dyslipidemia. db/db mice were given PBS or ASO as indicated for five weeks (n=4-6 animals per group). Two days after the final dose, the mice were fasted for 4h, then given a gastric gavage of corn oil enriched with [³H] retinol (10 µl corn oil per gram of body weight). ▼ = PBS; ◼ = Non-target ASO; ♦ = Sulf2 ASO (20 mg/kg); ▲ = Sulf2 ASO 50 (mg/kg) (A) Postprandial excursions and (B) incremental Area Under the Curves (iAUC) of plasma triglycerides. (C) Postprandial excursions and (D) iAUC of plasma [³H]retinol concentrations. * p<0.05 compared to Sulf2 ASO (50mg/kg).
DISCUSSION

In the present study, we show that Sulf2 inhibition in T2DM db/db mice increases heparan sulfation, normalizes the ability of their hepatocytes to bind TRLs, substantially decreases non-fasting plasma TG levels, and abolishes postprandial hypertriglyceridemia. Thus, despite extensive, persistent metabolic derangements in these animals (Table 1), inhibition of a single overexpressed molecule, Sulf2, down to control levels normalizes the hepatic metabolism of atherogenic remnant lipoproteins. These findings provide the first proof-of-concept in vivo to support Sulf2 inhibition as an attractive strategy to improve metabolic dyslipoproteinemia. Moreover, our current results bolster the concept that diabetes dysregulates a surprisingly small number of key molecules involved in the function of hepatic syndecan-1 as a receptor for TRL remnants.

Following Sulf2 ASO administration, non-fasting plasma TG levels were decreased by 50%. Non-fasting TG levels closely reflect persistent postprandial TRL particles. Likewise, by examining plasma TG excursions following corn-oil gavage, we found robust improvement following Sulf2 ASO administration to db/db mice (>90% reduction in iAUC). The magnitude of this improvement vastly exceeds the effects on postprandial TG excursions of conventional lipid-lowering interventions, such as statins (10-15% reduction in iAUC) and fibrates (10-20% reduction in iAUC). Unlike Sulf2 ASO, these conventional interventions fail to specifically target the key molecular derangement in T2DM liver. Although NT-ASO also produce a mild, non-specific effect, the effects of Sulf2 ASOs are most consistent, greater in magnitude and hence clearly directly related to SULF2 inhibition.

Clinical implications

Residual atherosclerotic cardiovascular risk in T2DM patients remains substantial, even during maximal conventional treatment with currently available therapies. Recent work has implicated non-fasting TG levels, a marker of persistent remnant lipoproteins, as an independent risk factor for atherosclerotic cardiovascular disease, but there have been no therapeutic strategies that selectively target persistent postprandial remnants. Our present findings demonstrate that hepatic Sulf2 inhibition in vivo corrects postprandial dyslipidemia in T2DM mice. Translation of these findings to the clinic will benefit from the relative maturity of ASO technology. In other circumstances, ASO administration has been selective and effective against hepatic targets. Importantly, ASOs have been reported to be safe and effective during short-term administration to humans. In our system, the Sulf2 ASO lowered abnormally high levels of Sulf2 mRNA in T2DM mouse livers to normal, but not below normal, which is highly desirable from the standpoint of safety. In conclusion, our work provides a key proof-of-concept in vivo for a novel therapeutic approach to improve metabolic dyslipidemia through restoration of hepatic HSPG function in diabetes.
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CHAPTER 9

REFERENCE LIST


