Membrane bound sn-1,2-diacylglycerols explains the dissociation of hepatic insulin resistance from hepatic steatosis in MTTP knockout mice

Running Title: DAG compartmentation and hepatic IR in MTTP ko mice

Abudukadier Abulizi¹, Daniel F. Vatner¹, Zhang Ye¹, Yongliang Wang¹, Joao-Paulo Camporez¹,
Dongyan Zhang¹, Mario Kahn¹, Kun Lyu¹, Alaa Sirwi², Gary W. Cline¹, M. Mahmood Hussain²,³,
Patricia Aspichueta⁵, Varman T. Samuel¹,⁴ Gerald I. Shulman¹,⁶

¹Department of Internal Medicine, Yale School of Medicine, New Haven CT, 06520, USA
²Departments of Cell Biology and Pediatrics SUNY Downstate Medical Center, NY, 11501, USA
³Department of Foundations of Medicine, NYU Long Island School of Medicine, Mineola, NY, 11501, USA
⁴Veterans Affairs Medical Center, West Haven CT, 06516, USA
⁵Department of Physiology, Faculty of Medicine and Nursery, University of Basque Country UPV/EHU,
Leioa, Spain, ⁷Biocruces Research Institute, Barakaldo, Spain
⁶Department of Cellular & Molecular Physiology, Yale School of Medicine, New Haven CT, 06520, USA

Corresponding Author: Gerald I. Shulman, M.D., Ph.D., TAC S269, PO Box 8020, Yale School of
Medicine, New Haven, Connecticut, 06520-8020, USA Phone: (203) 785-5447; Fax: (203) 737-4059
e-mail: gerald.shulman@yale.edu
Abbreviations:

MTTP: Microsomal triglyceride transfer protein
NAFLD: Nonalcoholic fatty liver disease
T2D: Type 2 diabetes
GTT: Glucose tolerance test
EGP: Endogenous glucose production
CRMP: Controlled-release mitochondrial protonophore
TAG: Triglyceride
DAG: Diacylglycerol
Abstract

Microsomal triglyceride transfer protein (MTTP) deficiency results in a syndrome of hypolipidemia and accelerated nonalcoholic fatty liver disease (NAFLD). Animal models of decreased hepatic MTTP activity have revealed an unexplained dissociation between hepatic steatosis and hepatic insulin resistance. Here, we performed comprehensive metabolic phenotyping of liver specific MTTP knockout (L-Mttp<sup>−/−</sup>) mice and age-weight matched wild type control mice. Young (10-12 weeks old) L-Mttp<sup>−/−</sup> mice exhibited hepatic steatosis and increased diacylglycerol (DAG) content; however, the increase in hepatic DAG content was partitioned to the lipid droplet and was not increased in the plasma membrane. Young L-Mttp<sup>−/−</sup> mice also manifested normal hepatic insulin sensitivity as assessed by hyperinsulinemic-euglycemic clamps, no protein kinase Cε (PKCε) activation and normal hepatic insulin signaling from the insulin receptor through AKT Ser/Thr kinase (AKT). In contrast, aged (10 months old) L-Mttp<sup>−/−</sup> mice exhibited glucose intolerance and hepatic insulin resistance, along with increased in hepatic plasma membrane sn-1,2-DAG content and PKCε activation. Treatment with a functionally liver-targeted mitochondrial uncoupler protected the aged L-Mttp<sup>−/−</sup> mice against the development of hepatic steatosis, increased plasma membrane sn-1,2-DAG content, PKCε activation and hepatic insulin resistance. Furthermore, increased hepatic insulin sensitivity in the aged CRMP treated L-Mttp<sup>−/−</sup> mice was not associated with any reductions in hepatic ceramide content. Taken together these data demonstrate that differences in the intracellular compartmentation of sn-1,2 DAGs in the lipid droplet vs. plasma membrane explains the dissociation of NAFLD/lipid-induced hepatic insulin resistance in young L-Mttp<sup>−/−</sup> mice as well as the development of lipid-induced hepatic insulin resistance in aged L-Mttp<sup>−/−</sup> mice.

**Keywords:** lipids/liver, metabolic disease, insulin resistance, non-alcoholic fatty liver disease

drug therapy, liver microsomal triglyceride transfer protein, diabetes, steatosis, liver-targeted mitochondrial uncoupler
Introduction

Microsomal triglyceride transfer protein (MTTP) plays a critical role in the assembly and secretion of very low density lipoproteins (VLDL) in the liver and chylomicrons in the intestine (1-3). MTTP deficiency results in abetalipoproteinemia, a rare genetic disorder resulting in reduced plasma apoB containing lipoproteins and increased nonalcoholic fatty liver disease (NAFLD) (4). Pharmacological inhibition of MTTP is currently being used to treat patients with homozygous familial hypercholesterolemia, helping to significantly reduce LDL cholesterol and ApoB containing lipoprotein levels in these patients (5, 6). Of concern, pharmacologic MTTP inhibition is associated with significant toxicities, including hepatic steatosis and increased liver aminotransferase levels (7).

In mice, genetic deletion of liver MTTP induces hepatic fat accumulation; however, in spite of hepatic steatosis, these mice demonstrated normal hepatic insulin sensitivity (1, 8). In fact, chronic treatment with an MTTP inhibitor apparently improved glucose tolerance in Zucker rats (9). The metabolically neutral phenotype contrasts with the well-established relationship between hepatic lipid accumulation and hepatic insulin resistance in most humans with NAFLD and the majority of rodent models of NAFLD (10). The mechanism responsible for lipid-induced hepatic insulin resistance has been attributed to hepatic diacylglycerol, activating PKCε which promotes insulin receptor kinase threonine1160 (mouse threonine1150) phosphorylation, thereby inhibiting insulin-stimulated insulin receptor kinase tyrosine phosphorylation (11). There are important exceptions to the relationship between hepatic steatosis and hepatic insulin resistance in rodents: such as when excess hepatic lipid is limited to metabolically neutral subcellular compartments (12). Thus, while MTTP inhibition clearly lowers plasma lipids and increases hepatic lipids, it is unclear how this hepatic steatosis is associated with or dissociated from a metabolically deleterious phenotype.

To better understand the effects of MTTP ablation over time, we studied both young and aged liver specific MTTP knockout mice (L-Mttp−/−), assessing the development of lipid-induced hepatic insulin resistance. Furthermore, we assessed the content and subcellular localization of different stereochemical
isoforms of DAG in the liver of L-Mtp<sup>−/−</sup> mice. In addition, we assessed the utility of a liver-targeted mitochondrial uncoupling agent (13-17) for the treatment of hepatic steatosis in L-Mtp<sup>−/−</sup> mice, as there are no currently-available treatments for hepatic steatosis in the setting of MTTP deficiency. The orally bioavailable functionally liver-targeted controlled-release formulation of 2,4-dinitrophenol (Controlled Release Mitochondrial Protonophore: CRMP) reverses insulin resistance, hypertriglyceridemia, NASH and diabetes in rodent and nonhuman primate models of type 2 diabetes and NAFLD/NASH (14-17). Therefore, after assessing the relationship between MTTP ablation and lipid-induced hepatic insulin resistance, we administered CRMP to evaluate the potential value of liver-targeted mitochondrial uncoupling for the treatment of hepatic steatosis and hepatic insulin resistance due to reduce MTTP activity.
Material and Methods

Animal care: All experimental procedures were approved by and conducted in accordance with the Institutional Animal Care and Use Committees at Yale University. Liver specific microsomal triglyceride transfer protein knockout mice (L-\textit{Mttp}^\text{-/-}) on a C57BL/6 background were generated as described in previously (18, 19). In all studies, age-weight matched wild type (\textit{Mttp}^\text{f/f} mice) served as controls. Mice were individually housed under controlled temperature (~23°C) and lighting (12-h light/dark cycle, lights on at 7:00 am) with free access to water and food. Mice were maintained with regular chow (RC: Envigo 2108S-24% protein/58% carbohydrate/18% fat calories). Mice were fasted overnight for infusion studies, and fasted six hours for basal measurements. Body composition was assessed by $^1$H magnetic resonance spectroscopy using a Bruker BioSpin Minispec analyzer (Bruker). Energy expenditure, respiratory quotient (RQ), oxygen consumption (\textit{Vo}_2), carbon dioxide production (\textit{Vco}_2), locomotor activity, and food intake were measured using a comprehensive laboratory animal metabolic system (CLAMS; Columbus Instruments). Drinking in the metabolic cages was measured as described previously (20). CRMP was administered orally, mixed in peanut butter (2 mg/kg); control animals in the CRMP treatment experiment received peanut butter plus vehicle. In this study, “young” mice were 10-12 weeks old, while “aged” mice were 10-months old.

Glucose tolerance test: Following an overnight fast, mice were injected intraperitoneally with 1 g/kg dextrose. Blood samples were taken by tail massage for glucose and insulin measurements at 0, 15, 30, 60, 120 minutes.

Hyperinsulinemic-euglycemic clamp: Hyperinsulinemic-euglycemic clamps were performed in conscious mice as previously described (21). [3-$^3$H]glucose (Perkin-Elmer) was infused at a rate of 0.05 µCi/min for 120 min to assess basal turnover. Following the basal infusion, human insulin (Novo Nordisk) was given
as a prime [7.14 mU/(kg-min) x 3 min] then continuous [2.5 mU/(kg-min)] infusion; along with a variable infusion of 20% dextrose to maintain euglycemia (100-120 mg/dL), and [3-3H]glucose at a rate of 0.1 µCi/min. Plasma samples were obtained by tail massage at 0, 25, 45, 65, 80, 90, 100, 110, 120, 130, and 140 min. At the end of the study, mice were anesthetized with sodium pentobarbital injection (~4 mg/mouse), and tissues taken were snap frozen in liquid nitrogen and stored at −80°C for subsequent use.

**Plasma Assays:** Plasma glucose was measured using a YSI 2700D glucose analyzer (Yellow Springs Instruments). Standard kits were used to measure plasma non-esterified fatty acids (Wako) and triglycerides (Sekisui). Insulin concentrations were determined by radioimmunoassay (EMD Millipore).

**Liver lipid measurements:** Tissue triglycerides (TAGs) were extracted using the method of Bligh and Dyer (22) and measured using a standard kit (Sekisui).

For subcellular compartment-specific diacylglycerol (DAG) extraction, liver tissue was homogenized with a Doucne-type homogenizer in Buffer A (250 mM sucrose–10 mM Tris (pH 7.4)–0.5 mM EDTA). The full extraction was performed on ice or at 4°C. The homogenate was centrifuged at 17K rcf for 15 minutes, to obtain supernatant 1 and pellet 1. Supernatant 1 was centrifuged at 387K rcf for 75 minutes, the resultant pellet contains endoplasmic reticulum membrane lipid, the top layer of the supernatant contains lipid droplet lipid, the middle of the supernatant contains cytosol lipid. Pellet 1 was resuspended in Buffer A, layered on top of a 1.12 M sucrose solution, and centrifuged at 111K rcf for 20 minutes, to obtain Pellet 2 and Supernatant 2. Pellet 2 was resuspended in Buffer A and centrifuged at 17K rcf for 15 minutes; the resultant pellet contains mitochondrial membrane lipid. The interfacial layer of Supernatant 2 was taken and diluted with Buffer A, and centrifuged at 59K rcf for 9 minutes; the resultant pellet contains plasma membrane lipid.

Separation and quantitation of DAG stereoisomers were performed by LC-MS/MS using electrospray ionization on an AB Sciex Qtrap 6500 interfaced to Shimadzu UFLC, using Luna 5u Silica
(100A, 250x2.0mm) and LUX 5u Cellulose-1 (250x4.6mm) columns connected in series with an isocratic solvent of hexane:isopropanol (300:7). DAG stereoisomer standards were used to establish retention times and response relative to the internal standard (C17, C17-DAG). No additional separation steps following subcellular fractionation were done prior to LC-MS/MS analysis to avoid racemization. DAG content is expressed as the sum of individual species. Hepatic ceramide content was measured as previously described (23).

**Immunoblotting analysis:** Tissue was homogenized in ice-cold homogenization buffer with protease and phosphatase inhibitors (Complete MINI + PhosSTOP (Roche)). Protein extracts (30 µg) were separated by 4%–12% gradient SDS-PAGE (Invitrogen) and then transferred to a PVDF membrane (Millipore) using a semidry transfer cell (Bio-Rad) for two hours. After blocking with 5% nonfat dry milk in TBST (10mM Tris pH 7, 100mM NaCl, and 0.1% Tween 20), membranes were incubated overnight at 4°C with primary antibodies. Membranes were thoroughly washed, incubated with the appropriate secondary antibody (Cell Signaling Technology) and immune complexes were detected using an enhanced luminol chemiluminescence system (ECL; Thermo Scientific) and exposed to photographic film. Immunoblots were quantified by optical densitometry. For PKCε translocation, cytoplasm and plasma membrane were separated by ultracentrifugation as previously described (24, 25) prior to western blotting. Insulin receptor β, phosphorylated insulin receptor β, Akt, phosphorylated Akt (Ser473), IRE1, phosphorylated eiF2, and GAPDH antibodies were purchased from Cell Signaling Technology. Sodium potassium ATPase and phosphorylated IRE1 antibodies were purchased from Abcam Inc. PKCε antibody was purchased from BD Biosciences. Antibody against eiF2 was purchased from Santa Cruz Biotechnology. KDEL (GRP 78 and GRP 94) antibody was purchased from Enzo Life Science.

**Markers of liver inflammation:** Inflammatory cytokines were measured in liver homogenates by ELISA (Qiagen), and were normalized to total protein content measured by a standard Coomassie-based absorption assay (Thermo).
**Statistical analysis:** All data are expressed as means ± SEM. Results were assessed using a two-tailed unpaired Student t test or 2-way ANOVA followed by Tukey’s Multiple Comparison Test (Prism 7; GraphPad Software, Inc.). A P value <0.05 was considered significant.
Results

Young L-Mttp<sup>−/−</sup>mice exhibit hepatic steatosis without excess sn-1,2-diacylglycerol at the plasma membrane

Hepatic triglyceride was measured in 10-12 week old L-Mttp<sup>−/−</sup> mice and Mttp<sup>WT</sup> wild type (WT) controls. As expected, hepatic triglyceride (TAG) was significantly increased in young L-Mttp<sup>−/−</sup> mice (Fig.1A). Diacylglycerols and ceramides are two bioactive lipid metabolites that are thought to link hepatic steatosis to hepatic insulin resistance. Hepatic ceramides and diacylglycerols were both increased in young L-Mttp<sup>−/−</sup> mice (Fig.1B-C). Diacylglycerol accumulation causes hepatic insulin resistance through activation of PKCε which results in phosphorylation of insulin receptor Threonine<sup>1160</sup>, which in turn leads to inhibition of insulin receptor tyrosine kinase activity (10, 11, 26). Recently we have seen that sn-1,2-DAG at the plasma membrane is the entity that drives PKCε translocation and insulin resistance (27), thus we measured sn-1,2-DAGs as well as the other DAG stereoisomers in five intracellular compartments. Using this approach, we found that sn-1,2-DAGs were increased in the lipid droplet, cytosol, endoplasmic reticulum, and mitochondrial compartments, but not the plasma membrane compartment (Fig.1D-F) of young L-Mttp<sup>−/−</sup> mice.

Liver insulin sensitivity is preserved in young L-Mttp<sup>−/−</sup>mice

We also assessed whole body and tissue specific insulin action by hyperinsulinemic-euglycemic clamp studies in 10-12 week old L-Mttp<sup>−/−</sup> mice. Whole-body insulin sensitivity of young L-Mttp<sup>−/−</sup> mice, as reflected by the glucose infusion rate required to maintain euglycemia, was not different vs. WT (Fig.2A-B). Furthermore, insulin-stimulated peripheral glucose disposal was not changed (Fig.2C). Additionally, insulin-mediated suppression of endogenous glucose production (EGP) was also not altered in young L-Mttp<sup>−/−</sup> mice, indicating unchanged hepatic insulin sensitivity (Fig.2D-E). Thus, MTTP deficiency does not alter hepatic or peripheral insulin action in young mice despite marked hepatic steatosis and increases in
sn-1,2-DAGs in the lipid droplet fraction.

PKCε activation links hepatic DAG accumulation to hepatic insulin resistance. Young L-Mttp−/− mice displayed no change in hepatic PKCε membrane translocation, a surrogate for PKCε activation, as compared with WT mice (Figure 2F), consistent with the unchanged sn-1,2-DAG in the hepatic plasma membrane. Furthermore, insulin-stimulated protein kinase B (Akt) phosphorylation was not changed, reflecting preserved hepatic insulin signaling in young L-Mttp−/− mice, (Figure 2G).

**Aged L-Mttp−/− mice demonstrate CRMP-reversible glucose intolerance**

We evaluated 10-month-old L-Mttp−/− mice and WT controls to gauge the metabolic impact of the interaction between aging and hepatic Mttp deficiency. We first evaluated L-Mttp−/− mice by intraperitoneal glucose tolerance testing (GTT). Though the excursion in plasma glucose concentration was not altered during GTT (Fig. 3A, 3B), the plasma insulin excursions were much higher in aged L-Mttp−/− mice. These changes in plasma insulin with normal plasma glucose concentrations are a more sensitive measure of hepatic insulin resistance during a glucose tolerance test, and these insulin excursions were higher in aged L-Mttp−/− mice (Fig. 3C, 3D). To determine if insulin resistance could be reversed with a liver-targeted mitochondrial uncoupler to decrease liver fat content, we treated L-Mttp−/− mice with the controlled release form of 2,4-dinitrophenol, CRMP. CRMP-treated L-Mttp−/− mice were more glucose tolerant than untreated L-Mttp−/− mice, as reflected by 20-30% reductions in plasma glucose and insulin concentrations during the intraperitoneal glucose tolerance test. (Fig. 3A-D).

**Hepatic steatosis and PKCε activation in aged L-Mttp−/− mice are blunted by CRMP administration**

We evaluated plasma and hepatic lipid content in our aged WT, L-Mttp−/−, and CRMP-treated L-Mttp−/− mice. Fasting plasma TAG was decreased by 70% in L-Mttp−/− mice vs. WT; this lower level of plasma TAG was unaffected by intervention with CRMP (Fig. 4A). Hepatic TAG was increased in L-Mttp−/− mice vs. WT; 4 weeks CRMP treatment decreased hepatic TAG in L-Mttp−/− mice by 15% (Fig. 4B). Hepatic ceramide levels were increased in aged L-Mttp−/− mice; however, these levels were not changed by CRMP treatment.
(Fig. 4C). In contrast, total DAG was increased in aged L-Mttp⁻/⁻ mice, and this increase was dramatically blunted with CRMP treatment (Fig. 4D). We assessed DAG stereoisomers in five subcellular compartments. In the aged L-Mttp⁻/⁻ mice, sn-1,2-DAG content was increased in all subcellular compartments, contrasting with the young L-Mttp⁻/⁻ mice. The increase in DAG was attenuated by CRMP treatment (Fig. 4E-4G). PKCε membrane translocation was increased in aged L-Mttp⁻/⁻ mice; and this increase in PKCε activation was reversed by CRMP treatment (Fig. 4H). Thus, CRMP treatment effectively reduced the accumulation of hepatic TAG and DAG content in aged L-Mttp⁻/⁻ mice. Given our previous results it is likely that the decrease in plasma membrane sn-1,2-DAG accounts for the decrease in PKCε activation in these aged L-Mttp⁻/⁻ mice (27).

Whole-body energy balance was not altered by L-Mttp⁻/⁻ genotype or CRMP treatment

To examine whether the deficiency of MTTP in the liver has an effect on whole body energy balance, we performed metabolic cage studies with aged WT mice, aged L-Mttp⁻/⁻ mice, and aged L-Mttp⁻/⁻ mice treated with CRMP. Neither deficiency of MTTP nor CRMP treatment altered body weight (Fig. 5A). Consistent with the matched body weights, there were no differences in oxygen consumption, carbon dioxide production, respiratory quotient, energy expenditure, feeding and activity during metabolic cage studies in any of the three groups (Fig. 5B-G). Thus, while we observed significant differences in glucose tolerance between aged WT and L-Mttp⁻/⁻ mice, and between L-Mttp⁻/⁻ mice with and without CRMP treatment, these differences were not associated with changes in whole body energy balance, body weight or activity.

Liver inflammation and ER stress were not changed in aged L-Mttp⁻/⁻ mice

Inflammation and ER stress are major factors that promote insulin resistance (28, 29). We assessed whether inflammatory changes were associated with the alterations in insulin action observed in aged L-Mttp⁻/⁻ mice. Hepatic pro-inflammatory and anti-inflammatory cytokine content was measured: IL1α, IL1β, IL2, IL4, IL6, IL10, and IL12 were not changed in aged L-Mttp⁻/⁻ mice (Fig. 6A) as compared with WT. We also
assessed for any changes in ER stress that might also contribute to the observed changes in hepatic insulin action in aged L-Mttp<sup>−/−</sup> mice. ER stress markers were assessed by immunoblotting, and were not altered in liver tissue of aged L-Mttp<sup>−/−</sup> mice and aged L-Mttp<sup>−/−</sup> mice treated with CRMP (Fig. 6B-F). Thus, changes in liver inflammation and changes in ER stress do not appear to account for the changes in glucose tolerance we observed in aged L-Mttp<sup>−/−</sup> mice.

**CRMP reversed hepatic insulin resistance in aged L-Mttp<sup>−/−</sup> mice**

Taken together, the observed differences in hepatic DAG content and hepatic PKCe activation suggest that the improvement in glucose tolerance in aged L-Mttp<sup>−/−</sup> mice treated with CRMP is due to a reversal in lipid-induced hepatic insulin resistance. To demonstrate this definitively, we performed hyperinsulinemic-euglycemic clamps on aged L-Mttp<sup>−/−</sup> mice treated for four weeks with CRMP or vehicle. CRMP treated mice had a significant improvement in whole-body insulin sensitivity, reflected by an increased glucose infusion rate during the clamp (Fig. 7A-B). Insulin-stimulated peripheral glucose disposal was not changed (Fig. 7C). Insulin-mediated suppression of endogenous glucose production (EGP) was significantly increased in CRMP treated mice, indicating improved hepatic insulin responsiveness (Fig. 7D-E). Consistent with changes in hepatic insulin sensitivity, insulin-stimulated protein kinase B (AKT) and insulin receptor kinase (IRK) phosphorylation were significantly increased in liver tissues of L-Mttp<sup>−/−</sup> mice treated with CRMP (Fig. 7F-G). Taken together these results demonstrate that CRMP’s effect to improve glucose tolerance in aged L-Mttp<sup>−/−</sup> mice could mostly be attributed to reversal of hepatic insulin resistance.
Discussion

MTTP inhibitors have been shown to reduce circulating VLDL and LDL in animal models and human subjects (30-32), and thus are a valuable addition to the pharmacopoeia for patients with severe hypertriglyceridemia. However, the promise of MTTP-targeted therapeutics has been questioned, as hepatic MTTP inhibition or MTTP deficiency induces hepatic steatosis and transaminitis (8, 33, 34). Nonalcoholic fatty liver disease is of great clinical concern, both as a precursor to nonalcoholic steatohepatitis, and as a cause of hepatic insulin resistance (35). Prior investigations have observed that MTTP knockout mice develop hepatic steatosis, with increased diacylglycerol and ceramide content, but without the development of hepatic insulin resistance or glucose intolerance (1). Furthermore, treatment of Zucker fatty rats with an MTTP inhibitor not only improved their glucose tolerance, but also decreased fasting plasma glucose and insulin concentrations (9), consistent with reversal of insulin resistance. Until now, there has been no explanation for the discordance between hepatic bioactive lipid content and hepatic insulin action in these models. Our study both recapitulates and explains these findings. We found that L-Mttp−/− mice develop hepatic steatosis at an early age, but do not develop hepatic insulin resistance. However, we also find that with aging, L-Mttp−/− (~10 month old) mice do indeed develop hepatic insulin resistance. The discordant metabolic phenotypes of the young and aged L-Mttp−/− mice were explained well in the context of the DAG-PKCε hypothesis of lipid-induced hepatic insulin resistance (35, 36); the key to understanding the phenotype of these mice was an analysis of diacylglycerol stereoisomer content separated by subcellular compartment. Both young and aged L-Mttp−/− mice had increased total hepatic DAG content, but only the aged mice demonstrated increases in plasma membrane sn-1,2-DAG and PKCε activation.

The DAG-nPKC model helps to explain lipid-induced insulin resistance in multiple tissues (35, 36). The results from this study support this hypothesis, wherein sn-1,2-diacylglycerol accumulation in the hepatic plasma membrane drive plasma membrane PKCε recruitment and activation. Activated PKCε in turn phosphorylates Threonine1160 in the tyrosine kinase active site of the insulin receptor, destabilizing this site and disrupting insulin receptor tyrosine kinase activity (10, 11). Thus, in the setting of increased hepatic
plasma membrane sn-1,2-diacylglycerol, more insulin must bind the hepatic insulin receptor to drive the multiple downstream hepatocellular actions of insulin: i.e., the liver is insulin resistant. Of note, there are models that dissociate hepatic lipids from hepatic insulin resistance. Some, such as sobetirome (a thyroid hormone receptor-β agonist) treated rodents, demonstrate increased hepatic glucose production in spite of reduced hepatic lipids due to increased flux of gluconeogenic precursors (37). Others, such as the CGI58 knockdown mouse, demonstrate increased hepatic DAG content but no increase in PKCe activation due to storage of DAG in lipid droplets which represent a neutral compartment (12, 38). The lack of hepatic insulin resistance in the L-Mttp−/− mouse appears to be consistent with this latter mechanism. Consistent with prior studies of the L-Mttp−/− mouse (1), in the young L-Mttp−/− mouse we observed increased hepatic bioactive lipid species without an increase in hepatic insulin resistance. To explain this discrepancy between hepatic lipid and hepatic insulin resistance, we fractionated the DAGs both by subcellular compartment and by stereochemistry, as PKCs are thought to be activated by plasma membrane sn-1,2-DAG (27, 39-41), not by DAGs in other compartments or 1,3 or 2,3 DAG stereoisomers. The sn-1,2-DAG stereoisomer content was not increased in the plasma membrane from the livers of these mice, explaining the insulin sensitive hepatic steatosis phenotype. Furthermore, in contrast with both prior studies of young L-Mttp−/− mice and our study of young L-Mttp−/− mice, in aged L-Mttp−/− mice we saw increased hepatic insulin resistance, along with a concomitant increase in plasma membrane sn-1,2-DAG content. These patterns correlating plasma membrane diacylglycerol content with hepatic insulin resistance were most clearly observed with sn-1,2 diacylglycerol stereoisomers. Thus, we found hepatic insulin resistance and PKCe activation was dissociated from hepatic steatosis and total TAG/DAG content in young L-Mttp−/− mice, as the sn-1,2-DAGs were stored in neutral compartments (mostly lipid droplets) in these mice, while hepatic insulin resistance developed in the older L-Mttp−/− mice as sn-1,2-DAGs accumulated in the plasma membrane, leading to PKCe activation and decreased hepatic insulin signaling at the level of the insulin receptor.
The discrepancy between the DAG localization and the phenotypes of the young vs. old mice may provide insight into how DAGs are trafficked from the ER to the plasma membrane. Our results suggest that DAG transport from the ER to the plasma membrane may occur by multiple pathways. DAG would accumulate rapidly during VLDL secretion, and more slowly by pathway(s) independent of VLDL secretion. This model could be tested by application to other genetic variants associated with defective VLDL secretion. Mutations in APOB and TM6SF2 both lead to defective VLDL secretion, reduced LDL cholesterol, and are associated with human disease. APOB mutations cause familial hypobetalipoproteinemia, while TM6SF2 mutations are associated with NAFLD and progression to NASH (42-45). Apob mutant mice are quite phenotypically similar to the MTTP deficient mice, developing hepatic steatosis (42) without a disruption in glucose or insulin tolerance (43). While the protection against insulin resistance see in ApoB mice may be due to a reduction in delivery of triglyceride to the muscle, and reduced peripheral insulin resistance, it would be reasonable to speculate that animals with defective VLDL secretion may be slower to develop hepatic insulin resistance due to a reduction in DAG transportation from the ER to the plasma membrane. Assessment of hepatic insulin action and plasma membrane DAG content in young and older ApoB and Tm6sf2 mutant mice would help to test a multi-pathway model of DAG transport from ER to plasma membrane.

The MTTP inhibitor lomitapide has been approved to treat patients with homozygous familial hypercholesterolemia; however, this approach induces hepatic fat accumulation and increased plasma transaminases (5, 7, 46). Use of the MTTP inhibitor lomitapide is associated with increased circulating transaminases, and this medication carries a boxed warning regarding hepatotoxicity. If the hepatic fat accumulation associated with MTTP inhibition can be prevented, the hepatotoxicity of the drug class may be attenuated. In this study, we examined the effect of a functionally liver-targeted mitochondrial protonophore, CRMP (14), in the aged L-Mttp−/− mice. We found that CRMP treatment reversed hepatic fat accumulation and improved glucose tolerance in aged L-Mttp−/− mice. Consistent with these improvements in hepatic steatosis and in glucose tolerance, CRMP treatment markedly increased both whole-body insulin
responsiveness and hepatic insulin sensitivity. The improvements in hepatic insulin sensitivity were associated with reductions in plasma membrane \( sn-1,2-DAG \) content, PKC\( \varepsilon \) activation and normalized insulin signaling from the insulin receptor through Akt in aged \( L-Mttp^{--} \) mice. In contrast there were no changes in hepatic ceramide content despite the reversal of hepatic insulin resistance with CRMP treatment. Thus, while liver specific aged \( L-Mttp^{--} \) mice developed hepatic insulin resistance, this defect in hepatic insulin action was reversible by CRMP treatment. These findings offer further evidence for the role of plasma membrane \( sn-1,2-DAG \) induced PKC\( \varepsilon \) activation in causing hepatic insulin resistance and support that potential utility of CRMP and other liver-targeted mitochondrial protonophores for the treatment of NAFLD associated with reduced MTTP activity. These results also dissociate hepatic ceramide content from hepatic insulin resistance in these CRMP treated \( L-Mttp^{--} \) mice demonstrating that ceramides are involved in causing hepatic insulin resistance in this model.

Taken together these data demonstrate that differences in the intracellular compartmentation of \( sn-1,2-DAGs \) in the lipid droplet vs. plasma membrane explain the dissociation of NAFLD and lipid-induced hepatic insulin resistance in young \( L-Mttp^{--} \) mice as well as the development of lipid-induced hepatic insulin resistance in aged \( L-Mttp^{--} \) mice. Consistent with the key role for plasma membrane \( sn-1,2 \) DAG induced PKC\( \varepsilon \) activation in causing hepatic insulin resistance in the aged \( L-Mttp^{--} \) mice, we show that liver-targeted mitochondrial uncoupling with CRMP reverses hepatic steatosis, plasma membrane \( sn-1,2 \) DAG accumulation, hepatic PKC\( \varepsilon \) activation, and hepatic insulin resistance in these mice.
Acknowledgments

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

*Statements of Assistance*- We would like to thank Gina Butrico, Ali Nasiri, Xiaoxian Ma, Codruta Todeasa, and Maria Batsu and the Yale Diabetes Research Core facility for excellent technical support.

*Guarantor*- G.I.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

*Funding*- We would like to thank our funding sources: The National Institutes of Health (R01 DK116774, R01 DK119668, R01 DK114793, R01 DK113984, K23 DK10287, P30 DK045735, DK121490, HL137202) and the Veterans Health Administration (Merit Review Award I01 BX000901, BX004113).

*Duality of Interest*- Dr. Shulman is an inventor on the Yale University Patent for CRMP and scientific co-founder of TLC. Inc. which is developing liver-targeted mitochondrial agents (including CRMP) for the treatment of NAFLD/NASH and associated metabolic diseases. There are no other potential conflicts of interest relevant to this article.
References

1. Minehira, K., S. G. Young, C. J. Villanueva, L. Yetukuri, M. Oresic, M. K. Hellerstein, R. V. Farese, Jr., J. D. Horton, F. Preitner, B. Thorens, and L. Tappy. 2008. Blocking VLDL secretion causes hepatic steatosis but does not affect peripheral lipid stores or insulin sensitivity in mice. *J Lipid Res* **49**: 2038-2044.
2. Hussain, M. M., J. Shi, and Dreizen. P. 2003. Microsomal triglyceride transfer protein and its role in apolipoprotein B-lipoprotein assembly. *J. Lipid Res* **44**: 22-32.
3. Sirwi, A., and M. M. Hussain. 2018. Lipid transfer proteins in the assembly of apoB-containing lipoproteins. *J. Lipid Res* **59**: 1094-1102.
4. Wetterau, J. R., L. P. Aggerbeck, M. E. Bouma, C. Eisenberg, A. Munck, M. Hermier, J. Schmitz, G. Gay, D. J. Rader, and R. E. Gregg. 1992. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. *Science* **258**: 999-1001.
5. Berberich, A. J., and R. A. Hegele. 2017. Lomitapide for the treatment of hypercholesterolemia. *Expert Opin Pharmacother* **18**: 1261-1268.
6. Walsh, M. T., and M. M. Hussain. 2017. Targeting microsomal triglyceride transfer protein and lipoprotein assembly to treat homozygous familial hypercholesterolemia. *Crit Rev Clin Lab Sci* **54**: 26-48.
7. Cuchel, M., L. T. Bloedon, P. O. Szapary, D. M. Kolansky, M. L. Wolfe, A. Sarkis, J. S. Millar, K. Ikewaki, E. S. Siegelman, R. E. Gregg, and D. J. Rader. 2007. Inhibition of microsomal triglyceride transfer protein in familial hypercholesterolemia. *N Engl J Med* **356**: 148-156.
8. Raabe, M., M. M. Veniant, M. A. Sullivan, C. H. Zlot, J. Bjorkegren, L. B. Nielsen, J. S. Wong, R. L. Hamilton, and S. G. Young. 1999. Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. *J Clin Invest* **103**: 1287-1298.
9. Dhote, V., A. Joharapurkar, S. Kshirsagar, N. Dhanesha, V. Patel, A. Patel, S. Raval, and M. Jain. 2011. Inhibition of microsomal triglyceride transfer protein improves insulin sensitivity and reduces atherogenic risk in Zucker fatty rats. *Clin Exp Pharmacol Physiol* **38**: 338-344.
10. Samuel, V. T., and G. I. Shulman. 2016. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. *J Clin Invest* **126**: 12-22.
11. Petersen, M. C., A. K. Madiraju, B. M. Gassaway, M. Marcel, A. R. Nasiri, G. Butrico, M. J. Marcucci, D. Zhang, A. Abulizi, X. M. Zhang, W. Philbrick, S. R. Hubbard, M. J. Jurczak, V. T. Samuel, J. Rinehart, and G. I. Shulman. 2016. Insulin receptor Thr1160 phosphorylation mediates lipid-induced hepatic insulin resistance. *The Journal of clinical investigation* **126**: 4361-4371.
12. Cantley, J. L., T. Yoshimura, J. P. Camporez, D. Zhang, F. R. Jornayvaz, N. Kumashiro, F. Guebre-Egziabher, M. J. Jurczak, M. Kahn, B. A. Guigni, J. Serr, J. Hankin, R. C. Murphy, G. W. Cline, S. Bhunot, V. P. Manchem, J. M. Brown, V. T. Samuel, and G. I. Shulman. 2013. CGI-58 knockdown sequesters diacylglycerols in lipid droplets/ER-preventing diacylglycerol-mediated hepatic insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 1869-1874.
13. Perry, R. J., T. Kim, X. M. Zhang, H. Y. Lee, D. Pesta, V. B. Popov, D. Zhang, Y. Rahimi, M. J. Jurczak, G. W. Cline, D. A. Spiegel, and G. I. Shulman. 2013. Reversal of hypertriglyceridemia, fatty liver disease, and insulin resistance by a liver-targeted mitochondrial uncoupler. *Cell metabolism* **18**: 740-748.
14. Perry, R. J., D. Zhang, X. M. Zhang, J. L. Boyer, and G. I. Shulman. 2015. Controlled-release mitochondrial protonophore reverses diabetes and steatohepatitis in rats. *Science* **347**: 1253-1256.
15. Goedeke, L., L. Peng, V. Montalvo-Romeral, G. M. Butrico, S. Dufour, X. M. Zhang, R. J. Perry, G. W. Cline, P. Kievit, K. Chng, K. F. Petersen, and G. I. Shulman. 2019. Controlled-release mitochondrial protonophore (CRMP) reverses dyslipidemia and hepatic steatosis in dysmetabolic nonhuman primates. *Sci Transl Med* **11**.
16. Abulizi, A., R. J. Perry, J. P. G. Camporez, M. J. Jurczak, K. F. Petersen, P. Aspichueta, and G. I. Shulman. 2017. A controlled-release mitochondrial protonophore reverses hypertriglyceridemia, nonalcoholic steatohepatitis, and diabetes in lipodystrophic mice. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* **31**: 2916-2924.
17. Abulizi, A., J. P. Camporez, D. Zhang, V. T. Samuel, G. I. Shulman, and D. F. Vatner. 2019. Ectopic lipid deposition mediates insulin resistance in adipose specific 11beta-hydroxysteroid dehydrogenase type 1 transgenic mice. *Metabolism: clinical and experimental* **93**: 1-9.
18. Dikkers, A., W. Annema, J. F. de Boer, J. Iqbal, M. M. Hussain, and U. J. Tietge. 2014. Differential impact of hepatic deficiency and total body inhibition of MTP on cholesterol metabolism and RCT in mice. *J Lipid Res* **55**: 816-825.
19. Iqbal, J., M. T. Walsh, S. M. Hammad, M. Cuchel, P. Tarugi, R. A. Hegele, N. O. Davidson, D. J. Rader, R. L. Klein, and M. M. Hussain. 2015. Microsomal triglyceride transfer protein transfers and determines plasma concentrations of ceramide and sphingomyelin but not glycosylceramide. *J. Biol. Chem* **290**: 25863-25875.
20. Birkenfeld, A. L., H. Y. Lee, F. Guebre-Egziabher, T. C. Alves, M. J. Jurczak, F. R. Jornayvaz, D. Zhang, J. J. Hsiao, A. Martin-Montalvo, A. Fischer-Rosinsky, J. Spranger, A. F. Pfeiffer, J. Jordan, M. F. Fromm, J. Konig, S. Lieske, C. M. Carmean, D. W. Frederick, D. Weismann, F. Knauf, P. M. Irusta, R. De Cabo, S. L. Helfand, V. T. Samuel, and G. I. Shulman. 2011. Deletion of the mammalian INDY homolog mimics aspects of dietary restriction and protects against adiposity and insulin resistance in mice. *Cell metabolism* **14**: 184-195.
21. Jornayvaz, F. R., A. L. Birkenfeld, M. J. Jurczak, S. Kanda, B. A. Guigni, D. C. Jiang, D. Zhang, H. Y. Lee, V. T. Samuel, and G. I. Shulman. 2011. Hepatic insulin resistance in mice with hepatic overexpression of diacylglycerol acyltransferase 2. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 5748-5752.
22. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911-917.
23. Yu, C., Y. Chen, G. W. Cline, D. Zhang, H. Zong, Y. Wang, R. Bergeron, J. K. Kim, S. W. Cushman, G. J. Cooney, B. Atcheson, M. F. White, E. W. Kraegen, and G. I. Shulman. 2002. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *The Journal of biological chemistry* **277**: 50230-50236.
24. Kumashiro, N., D. M. Erion, D. Zhang, M. Kahn, S. A. Beddow, X. Chu, C. D. Still, G. S. Gerhard, X. Han, J. Dziura, K. F. Petersen, V. T. Samuel, and G. I. Shulman. 2011. Cellular mechanism of insulin resistance in nonalcoholic fatty liver disease. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 16381-16385.
25. Qu, X., J. P. Seale, and R. Donnelly. 1999. Tissue and isoform-selective activation of protein kinase C in insulin-resistant obese Zucker rats - effects of feeding. *The Journal of endocrinology* 162: 207-214.
26. Samuel, V. T., K. F. Petersen, and G. I. Shulman. 2010. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet* 375: 2267-2277.
27. Lyu, K., Y. Zhang, D. Zhang, M. Khan, K. W. ter Horst, M. R. S. Rodrigues, R. C. Gaspar, S. M. Hirabara, P. K. Luukkonen, S. Lee, S. Bhanot, J. Rinehart, N. Blume, M. G. Rasch, M. J. Serlie, J. S. Bogan, G. W. Cline, V. T. Samuel, and G. I. Shulman. 2020. A Membrane-Bound Diacylglycerol Species Induces PKCe-Mediated Hepatic Insulin Resistance. *Cell metabolism*.
28. Dowman, J. K., J. W. Tomlinson, and P. N. Newsome. 2010. Pathogenesis of non-alcoholic fatty liver disease. *QJM* 103: 71-83.
29. Tilg, H., and A. R. Moschen. 2008. Inflammatory mechanisms in the regulation of insulin resistance. *Mol Med* 14: 222-231.
30. Wetterau, J. R., R. E. Gregg, T. W. Harrity, C. Arbeeny, M. Cap, F. Connolly, C. H. Chu, R. J. George, D. A. Gordon, H. Jamil, K. G. Jolibois, L. K. Kunselman, S. J. Lan, T. J. Maccagnan, B. Ricci, M. Yan, D. Young, Y. Chen, O. M. Frysman, J. V. Logan, C. L. Musial, M. A. Poss, J. A. Robl, L. M. Simpkins, W. A. Slusarchyk, R. Sulsky, P. Taunk, D. R. Magnin, J. A. Tino, R. M. Lawrence, J. K. Dickson, Jr., and S. A. Biller. 1998. An MTP inhibitor that normalizes atherogenic lipoprotein levels in WHHL rabbits. *Science* 282: 751-754.
31. Hussain, M. M., J. Shi, and P. Dreizen. 2003. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J Lipid Res* 44: 22-32.
32. Chandler, C. E., D. E. Wilder, J. L. Pettini, Y. E. Savoy, S. F. Petras, G. Chang, J. Vincent, and H. J. Harwood, Jr. 2003. CP-346086: an MTP inhibitor that lowers plasma cholesterol and triglycerides in experimental animals and in humans. *J Lipid Res* 44: 1887-1901.
33. Davidson, M. H. 2009. Novel nonstatin strategies to lower low-density lipoprotein cholesterol. *Curr Atheroscler Rep* 11: 67-70.
34. Rizzo, M., and A. S. Wierzbicki. 2011. New lipid modulating drugs: the role of microsomal transport protein inhibitors. *Curr Pharm Des* 17: 943-949.
35. Samuel, V. T., and G. I. Shulman. 2018. Nonalcoholic Fatty Liver Disease as a Nexus of Metabolic and Hepatic Diseases. *Cell metabolism* 27: 22-41.
36. Petersen, M. C., and G. I. Shulman. 2018. Mechanisms of Insulin Action and Insulin Resistance. *Physiol Rev* 98: 2133-2223.
37. Vatner, D. F., D. Weismann, S. A. Beddow, N. Kumashiro, D. M. Erion, X. H. Liao, G. J. Grover, P. Webb, K. J. Phillips, R. E. Weiss, J. S. Bogan, J. Baxter, G. I. Shulman, and V. T. Samuel. 2013. Thyroid hormone receptor-beta agonists prevent hepatic steatosis in fat-fed rats but impair insulin sensitivity via discrete pathways. *American journal of physiology. Endocrinology and metabolism* 305: E89-100.
38. Brown, J. M., J. L. Betters, C. Lord, Y. Ma, X. Han, K. Yang, H. M. Alger, J. Melchior, J. Sawyer, R. Shah, M. D. Wilson, X. Liu, M. J. Graham, R. Lee, R. Crooke, G. I. Shulman, B. Xue, H. Shi, and L. Yu. 2010. CGI-58 knockdown in mice causes hepatic steatosis but prevents diet-induced obesity and glucose intolerance. *J Lipid Res* 51: 3306-3315.
39. Boni, L. T., and R. R. Rando. 1985. The nature of protein kinase C activation by physically defined phospholipid vesicles and diacylglycerols. *J Biol Chem* 260: 10819-10825.
40. Nomura, H., K. Ase, K. Sekiguchi, U. Kikkawa, Y. Nishizuka, Y. Nakano, and T. Satoh. 1986. Stereospecificity of diacylglycerol for stimulus-response coupling in platelets. *Biochemical and biophysical research communications* **140**: 1143-1151.

41. Rando, R. R., and N. Young. 1984. The stereospecific activation of protein kinase C. *Biochemical and biophysical research communications* **122**: 818-823.

42. Kim, E., P. Ambroziak, M. M. Veniant, R. L. Hamilton, and S. G. Young. 1998. A gene-targeted mouse model for familial hypobetalipoproteinemia. Low levels of apolipoprotein B mRNA in association with a nonsense mutation in exon 26 of the apolipoprotein B gene. *J Biol Chem* **273**: 33977-33984.

43. Schonfeld, G., P. Yue, X. Lin, and Z. Chen. 2008. Fatty liver and insulin resistance: not always linked. *Trans Am Clin Climatol Assoc* **119**: 217-223; discussion 223-214.

44. Smagris, E., S. Gilyard, S. BasuRay, J. C. Cohen, and H. H. Hobbs. 2016. Inactivation of Tm6sf2, a Gene Defective in Fatty Liver Disease, Impairs Lipidation but Not Secretion of Very Low Density Lipoproteins. *J Biol Chem* **291**: 10659-10676.

45. Ehrhardt, N., M. E. Doche, S. Chen, H. Z. Mao, M. T. Walsh, C. Bedoya, M. Guindi, W. Xiong, J. Ignatius Irudayam, J. Iqbal, S. Fuchs, S. W. French, M. Mahmood Hussain, M. Arditi, V. Arumugaswami, and M. Peterfy. 2017. Hepatic Tm6sf2 overexpression affects cellular ApoB-trafficking, plasma lipid levels, hepatic steatosis and atherosclerosis. *Hum Mol Genet* **26**: 2719-2731.

46. Vuorio, A., M. J. Tikkanen, and P. T. Kovanen. 2014. Inhibition of hepatic microsomal triglyceride transfer protein - a novel therapeutic option for treatment of homozygous familial hypercholesterolemia. *Vasc Health Risk Manag* **10**: 263-270.
Fig. 1. Young L-Mttp⁻/⁻ mice develop hepatic steatosis without accumulation of plasma membrane sn-1,2-DAG (A) Liver triglyceride (TAG) content. (B) Liver ceramide content. (C) Total DAG. (D) Sn-1,2 DAG, (E) Sn-1,3 DAG, and (F) Sn-2,3 DAG content in five compartments of liver from young WT and L-Mttp⁻/⁻ mice: Plasma membrane (PM), Mitochondrial (Mito), Endoplasmic reticulum (ER), Lipid droplet (LD) and Cytosol (Cyto). Individual statistical comparisons evaluated by student's 2 tailed t-test. Data are mean ± S.E.M. of n=6-8 per group.
Figure 2. Normal whole-body insulin sensitivity in young L-Mttp−/− mice.

(A) Plasma glucose concentrations and (B) Glucose Infusion Rate (GIR) during the hyperinsulinemic portion of the clamp study. (C) Whole body insulin stimulated peripheral glucose disposal ("Glucose Uptake"). (D) Endogenous glucose production. (E) Insulin mediated suppression of endogenous glucose production. (F) PKCε translocation in liver. (G) Liver AKT phosphorylation, comparing basal and insulin stimulated liver.

Statistical comparisons made by unpaired 2-way students’ t-test. Data are mean ± S.E.M. of n=7-8 per group.
Fig. 3. Aged L-MtTP−/− mice demonstrated glucose intolerance which improved with CRMP. 

(A) Plasma glucose concentrations vs time and (B) area under the glucose vs time curve (AUC) during intraperitoneal glucose tolerance tests. (C) Plasma insulin concentration vs time and (D) AUC of the insulin vs time curve during intraperitoneal glucose tolerance tests. Mice were overnight fasted before the glucose tolerance test. Data are represented as mean ± SEM. Statistical comparisons made by 2-way ANOVA Data are mean ± S.E.M. of n=7-8 per group.
Fig. 4. Hepatic steatosis, hepatic plasma membrane DAG accumulation, and PKCε membrane translocation were all observed in aged L-Mttp−/− mice.

(A) Plasma triglyceride concentration. (B) Liver triglyceride content. (C) Liver ceramide content. (D) Liver total DAG. (E) Sn-1,2 DAG, (F) Sn-1,3 DAG, and (G) Sn-2,3 DAG content in hepatic subcellular compartments from aged WT mice, aged L-Mttp−/− mice, and aged L-Mttp−/− mice treated with CRMP. Plasma membrane (PM), Mitochondrial (Mito), Endoplasmic reticulum (ER), lipid droplet (LD) and cytosol (Cyto). (H) Hepatic PKCε translocation. Statistical comparisons made by 2-way ANOVA. Data are mean ± S.E.M. of n=6-8 per group.
Fig. 5. Whole-body energy balance was not different between aged WT, aged L-Mtp^-/-, aged L-Mtp^-/- mice treated with CRMP. (A) Body weight. (B) Oxygen consumption (V_O2) and (C) Carbon dioxide production (V_CO2). (D) Respiratory exchange ratio (RER). (E) Energy expenditure throughout the day. (F) Food intake. (G) Daily activity. Statistical comparisons made by 2-way ANOVA. Data are mean ± S.E.M. of n=6 per group.
Fig. 6. Liver inflammatory markers and activation of the unfolded protein response were unaltered by genotype or drug treatment in aged L-\textit{Mttp}\textsuperscript{c} and L-\textit{Mttp}\textsuperscript{c} treated with CRMP

(A) Hepatic cytokine concentrations. (B) ER stress/unfolded protein response markers assessed by immunoblot. (C) GRP78. (D) GRP94. (E) Phosphorylation of eIF2 (F) Phosphorylation of IRE1.

Statistical comparisons made by 2-way ANOVA. Data are mean ± S.E.M. of n=3 per group.
Figure 7. CRMP treatment improved hepatic insulin sensitivity of aged L-Mtp<sup>−/−</sup> mice as assessed by hyperinsulinemic-euglycemic clamps. (A) Plasma glucose during the clamp. (B) Glucose infusion rate (GIR) during the clamp. (C) Insulin stimulated peripheral glucose disposal (“glucose uptake”). (D) Endogenous glucose production (EGP) in both the basal and hyperinsulinemic clamped state. (E) Insulin mediated suppression of EGP, represented as percent suppression. (F) Liver AKT phosphorylation under basal and insulin stimulated conditions. (G) Liver insulin receptor β (insulin receptor kinase: “IRK”) phosphorylation under basal and insulin stimulated conditions. Statistical comparisons made by t-test. Data are mean ± S.E.M. of n=6-7 per group.