Evidence for Regulated Monoacylglycerol Acyltransferase Expression and Activity in Human Liver

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Running title: MGAT activity in human liver

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Abstract

Intrahepatic lipid accumulation is extremely common in obese subjects and is associated with the development of insulin resistance and diabetes. Hepatic diacylglycerol and triacylglycerol synthesis predominantly occurs through acylation of glycerol-3-phosphate. However, an alternative pathway for synthesizing diacylglycerol from monoacylglycerol (monoacylglycerol acyltransferases (MGATs)) could also contribute to hepatic glyceride pools. MGAT activity and the expression of the three genes encoding MGAT enzymes (MOGAT1, MOGAT2, and MOGAT3) were determined in liver biopsies from obese human subjects before and after gastric bypass surgery. MOGAT expression was also assessed in liver of subjects with non-alcoholic fatty liver disease (NAFLD) or control livers. All MOGAT genes were expressed in liver and hepatic MGAT activity was readily detectable in liver lysates. The hepatic expression of MOGAT3 was highly correlated with MGAT activity, whereas MOGAT1 and MOGAT2 expression was not and knockdown of MOGAT3 expression attenuated MGAT activity in a liver-derived cell line. Marked weight loss following gastric bypass surgery was associated with a significant reduction in MOGAT2 and MOGAT3 expression, which were also overexpressed in NAFLD subjects. In conclusion, these data suggest that the MGAT pathway is active and dynamically regulated in human liver and could be an important target for pharmacologic intervention for the treatment of obesity-related insulin resistance and NAFLD.

Keywords: hepatic steatosis, triacylglycerol, diacylglycerol, NAFLD
Introduction

Obesity is associated with nonalcoholic fatty liver disease (NAFLD), which represents a spectrum of liver abnormalities that are characterized by an increase in intrahepatic triacylglycerol (TAG) content (i.e. steatosis) with or without inflammation and fibrosis (i.e. steatohepatitis). The presence of steatosis is an important marker of metabolic dysfunction and correlates closely with multi-organ insulin resistance and dyslipidemia (1), which are key risk factors for diabetes and coronary artery disease. However, the mechanisms responsible for the link between increased intrahepatic TAG content and metabolic disease are not clear.

It has been hypothesized that the hepatocellular accumulation of specific lipid intermediates, including diacylglycerol (DAG), acyl-CoA, and ceramide, in people with NAFLD is the molecular mechanism driving insulin resistance (2, 3). Accordingly, an understanding of the metabolic interrelationships among hepatic glycerolipid synthesis and lipid intermediates has important physiological and clinical implications. There are two pathways for diacylglycerol synthesis. In the liver, most DAG and TAG is synthesized from the sequential acylation of glycerol-3-phosphate. However, these lipids can also be synthesized from monoacylglycerol through the monoacylglycerol acyltransferase (MGAT) pathway (Figure 1) (4, 5). The MGAT and glycerol-3-phosphate pathways are convergent and each result in the synthesis of DAG, which is then acylated to form TAG by the DAG acyltransferase (DGAT) enzymes (Figure 1). The MGAT pathway is an important TAG biogenesis pathway in intestinal epithelial cells, which re-esterify dietary fat into chylomicron particles. There is evidence that the MGAT pathway is active in neonatal and diabetic rodent liver (6, 7) and may also be present in liver of adult humans (8), but the relevance of MGAT activity to intrahepatic lipid metabolism is unclear.
In the last decade, the genes that encode MGAT activity (9-11), which are known as MOGATs to avoid confusion with the genes encoding mannosyl (α-1,3)-glycoprotein beta-1,2-N-acetylglucosaminyltransferases, have been cloned. In humans, there are three MOGAT family genes (MOGAT1, MOGAT2, and MOGAT3). Rodents also have three Mogat genes (Mogat1, Mogat2, and Mogat3). However, the human and murine MOGAT3/Mogat3 genes are not analogs and evolved separately in post-speciation duplication events. The murine Mogat3 genes arose from duplication of Mogat2, while human MOGAT3 gene may have arisen from duplication of the gene encoding DGAT2 (12). Human MGAT3 also possesses significant DGAT activity and can catalyze both reactions required to convert monoacylglycerol to TAG (13). In fact, the amino acid sequence of all the MGATs exhibits homology to that of DGAT enzymes, and the two principal DGATs also possess modest MGAT activity (4, 14, 15).

Given the potential implications of MGAT activity in the pathogenesis of hepatic insulin resistance and NAFLD, we sought to evaluate MGAT activity and to characterize the expression of the MOGAT family genes in liver of human subjects. The novel findings of this study are that [1] the expression of MOGATs and MGAT activity is readily detectible in liver of human subjects, [2] hepatic MGAT activity is strongly correlated with MOGAT3 expression in obese human subjects, [3] the hepatic expression of MOGAT2 and MOGAT3 is significantly down-regulated following gastric bypass surgery-induced weight loss, and [4] hepatic MOGAT2 and MOGAT3 expression is increased in subjects with NAFLD. Collectively, these data suggest that the MGAT pathway could be a therapeutic target for pharmacologic intervention for obesity-related hepatic insulin resistance and NAFLD.
Research Design and Methods

Study Subjects

In Cohort 1, liver biopsies were obtained from 43 women and 11 men (BMI of 57.4 ± 11.9 kg/m2) during gastric bypass surgery (GBS), and MOGAT expression and MGAT activity were determined as described below. After subjects fasted overnight (12 h), blood samples were obtained to determine plasma glucose and insulin concentrations. GBS was performed the day after blood samples were collected. Liver tissue was obtained by needle biopsy during the GBS procedure, before gastric stapling and intestinal resection were performed. Hepatic tissue was rinsed in sterile saline, immediately frozen in liquid nitrogen, and then stored at -80°C until subsequent analyses.

Nine subjects from Cohort 1 (Cohort 2; Table 1) underwent a stable-isotopically labeled glucose tracer infusion the day before GBS and then again 1 year after surgery, in order to measure endogenous glucose production and the Hepatic Insulin Sensitivity Index (HISI). Subjects were admitted to the GCRC at Washington University School of Medicine on the evening before the infusion study. At 1800 h, subjects consumed a standard meal, which provided ~ 8 kcal/kg body weight and contained 55% of total energy as carbohydrate, 30% as fat and 15% as protein. At 0600 h the following morning a 3-hour primed, continuous infusion of $[6,6^{-2}H_2]$glucose (priming dose: 22.5 umol·kg$^{-1}$; infusion rate: 0.25 umol·kg$^{-1}$ min$^{-1}$), dissolved in 0.9% NaCl solution, was started. Blood samples were obtained before beginning the tracer infusion to determine background plasma glucose tracer-to-tracee ratio (TTR), and every 10 min during the final 30 min of the infusion period to determine insulin concentrations and glucose kinetics. These blood samples were collected in chilled tubes containing sodium EDTA. Samples were placed on ice, plasma was separated by using refrigerated centrifugation within 30
min of collection and then stored at -80 °C until final analyses were performed. In this Cohort, liver biopsies were obtained twice, by needle biopsy during GBS and percutaneously 1-year after surgery during the infusion study.

Cohort 3 (Table 2) was comprised of subjects who had a liver biopsy to evaluate elevated serum aminotransferases. Biopsy specimens were divided for histopathologic examination and RNA isolation/gene expression. The diagnosis NAFLD was based on histologic scoring of the liver biopsies. To obtain tissue from non-NAFLD livers, hepatic biopsies from cadaveric donors were obtained at time of transplant.

All subjects gave their written, informed consent before participating in this study, which was approved by the Human Research Protection Office of Washington University School of Medicine in St Louis, MO.

Methods

RNA isolation and Quantitative Real-time RT-PCR. For tissue profiling studies (Figure 2), human MOGAT gene expression profiling was performed on total RNA purchased from Clontech (Mountain View, CA). Real-time PCR (RT-PCR) was performed using the TaqMan RNA-to-C\text{T} kit (Applied Biosystems, Foster City, CA) and normalized to the expression of peptidylprolyl isomerase A (cyclophilin A, PPIA). The sequences of the MOGAT1 and PPIA primer and probes are described in Supplemental Table 1 and were prepared by Integrated DNA Technologies (Coralville, IA). The MOGAT2 and MOGAT3 Taqman assays (Hs00228268_m1 and Hs00698325_m1, respectively) were purchased from Applied Biosystems.

For studies presented in Table 3, total RNA was isolated from liver tissues using RNA-Bee (Tel-Test, Friendswood, TX) according the manufacturer’s instructions. Real-time RT-PCR was performed using Applied Biosystems reverse transcription (RT) kit, PowerSYBR PCR mix,
and the ABI PRISM 7500 sequence detection system. Arbitrary units of target gene mRNA were corrected to 36B4 RNA content to control for loading. The primers used in these analyses can be found in Supplemental Table 1.

Sources of human liver and intestine microsome and Western blotting: XTreme 200 human liver microsome and S9 fractions (lot numbers 0810413 and 0810405, respectively) and pooled human intestine microsome and combined cytosol and microsome (S9) fractions (lot numbers 0710352 and 0710351, respectively) were purchased from XenoTech (Lenexa, KS). Membranes over-expressing human MGAT proteins were generated from baculovirus infected Sf9 cells using standard techniques. For Western blotting, 50 μg of human liver or intestine microsome and S9 fractions were load to 4-12% Novex NuPAGE Bis-Tris precast gels (Invitrogen, Carlsbad, CA). Membranes were blotted in 3% dried milk in TBS-Tween 20 (Sigma, St. Louis, MO) and probed with an MGAT2 or MGAT3 antibody (Santa Cruz, Santa Cruz, CA) at 4°C overnight.

Isolation of Human Liver Microsomes. Human liver biopsy samples were processed in FastPrep Lysing Matrix tubes (MP Biomedicals, Solon, OH) containing 1 ml of ice-cold microsome preparation buffer (50 mM Tris-HCl, pH7.4, 1 mM EDTA, 0.25 M sucrose) supplemented with complete, EDTA-free protease inhibitor cocktail according to the manufacturer’s instructions (Roche, Indianapolis, IN). The lysate was centrifuged at 400-500 X g for 10 min at 4°C, the supernatant removed and recentrifuged at 12,000-13,000 X g for 10 min at 4°C. The supernatant from the 12,000-13,000 X g spin was centrifuged at 104,000-109,000 X g for 60 min at 4°C and the pellet resuspended in a final volume of 1 ml of microsome preparation buffer and centrifuged
under the same conditions. The resultant pellet was resuspended in 20 µl microsome preparation buffer without proteinase inhibitors and protein concentration determined prior to storage at -80°C.

Determination of MGAT activity. To determine MGAT activity, various amount of microsomes were incubated with 20 µM [14C]decanoyl-CoA (PerkinElmer, Waltham, MA) and 200 µM 2-oleoylglycerol in 50 mM HEPES, pH7.4, 10 mM MgCl2, 0.001% Triton X100, 2.5% v/v acetone. A non-selective lipase inhibitor (methyl arachidonyl fluorophosphonate; MAFP) was added to the reaction to block hydrolysis of both the product and substrate (16). The reaction was incubated at room temperature for 5 – 60 min and terminated by the addition of 50 µl 1% phosphoric acid. Lipids were extracted with 300 µl of CHCl3/MeOH (2:1, v/v) and separated by thin-layer chromatography (TLC) with hexane/ethyl ether/acetic acid (80:20:1, v/v/v). The TLC plates were exposed to phosphor screen (GE Healthcare, Piscataway, NJ) and scanned by Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). For determination of MGAT activity in human liver biopsies, a standard curve was generated using the XTreme200 pooled human liver microsomes and the measured activity confirmed to be on the linear portion of the standard curve.

Adenoviral-mediated over-expression of human MGAT3. Adenovirus expressing human MGAT3 was generated by ViraQuest (North Liberty, IA). Human hepatocellular carcinoma cells, HepG2, were plated into 96-well plates (40,000/well) and transduced with hMGAT3 adenovirus (MOI = 75) for 48 hours. Cells were assayed for lipogenic activity using [14C]glycerol (American Radio Chemicals, St. Louis, MO) (0.2 µCi per well, final concentration 22.6 µM) in
the presence of 400 µM dodecanoate for 3.5 h. When included, a selective DGAT1 inhibitor (Example 3 (17)) was added to a final concentration of 3 µM in DMSO (0.1% v/v final concentration). Lipids were extracted and analyzed as described above.

**siRNA-mediated knockdown of MGATs in human hepatocytes.** HepG2 cells were transfected in 24-well plates with either scrambled (Ambion Silencer Select Negative Control No.1, Life Technologies, Grand Island, NY) or MGAT3-targeting siRNA (Sigma-Aldrich, St. Louis, MO) using SilentFect Lipid Reagent according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA). Following transfection, cells were allowed to recover for 48 h prior to MGAT assay. Cellular MGAT activity was determined as outlined above except [¹⁴C]monooleoyl glycerol (0.5 μCi/well, final concentration 18 μM, American Radio Chemicals, St. Louis, MO) was used as the acyl acceptor to more directly assess MGAT activity. Sodium dodecanoate was added to a final concentration of 400 µM and the assay was allowed to proceed for 3.5 h prior to analysis as outlined above.

**Sample analyses and calculations.** Plasma insulin was determined by a radioimmunoassay, plasma glucose concentrations were measured by using an automated glucose analyzer (Yellow Springs Instruments Inc., Yellow Springs, OH), and plasma glucose TTR was determined by using gas chromatography-mass spectroscopy (GC-MS; MSD 5973 system with capillary column; Hewlett-Packard; Palo Alto, CA), as previously described (18). Isotopic steady state conditions were achieved during the final 30 min of the infusion period, so Steele’s equation for steady-state conditions was used to calculate glucose kinetics (19). Endogenous glucose
production in plasma was calculated by dividing the glucose tracer infusion rate by the average plasma glucose TTR during the last 30 min of the infusion period. The HOMA-IR was calculated from fasting plasma glucose (in μmol/L) and insulin (in mU/L) concentrations. Hepatic insulin sensitivity was determined by the reciprocal of the Hepatic Insulin Resistance Index, which was calculated as the product of the basal endogenous glucose production rate (in μmol·kg·min⁻¹) and fasting plasma insulin concentration (in mU/L) (20).

Statistical Analyses. The relationships between tissue gene expression and enzymatic activity were determined by using Pearson Correlation Coefficients analyses. Paired t-test was used to evaluate the statistical significance of the difference in values before and one year after GBS. Non-parametric Mann-Whitney test was used to evaluate the statistical significance of the difference between normal and NAFLD subjects. A p-value ≤0.05 was considered statistically significant.

Results

MOGAT family gene expression. Human MOGAT1, MOGAT2, and MOGAT3 mRNAs were all highly expressed in pooled liver RNA and MOGAT2 and MOGAT3 mRNAs were highly expressed in small intestinal RNA (Figure 2). In line with earlier observations (21), the human MOGAT1 transcript was found to be subject to extensive alternative splicing and, surprisingly, and we were unable to amplify the putative full-length MOGAT1 transcript from reverse-transcribed cDNA from liver, gall bladder, adipose or intestine (data not shown). Western blot analysis of pooled human intestine (18 pooled donors) and liver samples (200 pooled donors) revealed robust expression of MGAT2 protein in human intestinal S9 and microsomal fraction
while MGAT2 protein expression was relatively much lower in human liver lysates compared to intestinal lysates (Figure 2). This suggests that MGAT2 content of liver is influenced by post-transcriptional mechanisms since MGAT2 protein content was low relative to MOGAT2 mRNA levels. In contrast, MGAT3 protein was detected in liver and intestinal extracts (Figure 2), indicating that this MGAT family protein is expressed at detectable levels in human liver.

**MGAT activity in human liver microsomes.** We next sought to quantify MGAT activity in human liver. Although MGAT3 can utilize both sn-1- monoacylglycerol (MAG) and sn-2-MAG as substrate, in the presence of sn-1-MAG it also efficiently functions as a DGAT (11, 13). To specifically examine MGAT activity, sn-2-monooleoylglycerol (200 μM) was used as the acyl acceptor and [14C]decanoyl CoA (20 μM) as the acyl donor. In addition, previous work has suggested that MGAT activity is very low in hepatic lysates (22, 23). We postulated that lipase activity, which is reported to be very high in adult liver (24), might be obscuring MGAT activity by depleting the substrate (MAG) or product (DAG) of MGAT enzymes (25). After profiling several lipase inhibitors (data not shown), it was determined that inclusion of a non-specific lipase inhibitor (MAFP) in the reaction mixture increased the incorporation of [14C]decanoyl-CoA into DAG and TAG by human liver microsomes (Figure 3A). In contrast, MAFP had no effect on intestinal MGAT activity. These data suggest that high lipase activity in the liver may mask MGAT activity in hepatic microsomes and, henceforth, MAFP was utilized in all MGAT activity assays.

To directly compare MGAT activity in human liver and intestinal isolates, a microsome titration was performed (Figure 3B). As determined by the production of [14C]DAG or TAG, approximately 10-fold more liver microsomal protein was required to elicit MGAT activity
comparable to that observed in the intestine. Collectively, these data indicate that the overall MGAT activity in human liver, though markedly lower than in intestine, is readily detectable.

**MOGAT3 expression is strongly related to hepatic MGAT activity in obese human subjects.**

MGAT activity of hepatic lysates from 57 obese subjects (Cohort 1) was determined and correlated with the expression of MOGAT family genes using parallel biopsies. Prior to assaying the individual human tissue samples, the linearity of the liver microsomal MGAT reaction (20 μM [14C]decanoyl-CoA, 200 μM sn-2-monooleoylglycerol) was carefully examined and final assay conditions determined (Figure 4). All subsequent MGAT assays were performed at room temperature for 60 min using 5 μg of microsomal protein.

The expression of MOGAT3 was strongly related to MGAT activity (p = 0.01; Figure 5) as assessed by incorporation of [14C]decanoyl CoA into DAG, TAG, or total incorporation into both DAG and TAG. This is consistent with MGAT3 acting as both an MGAT and DGAT enzyme (13). In contrast, MOGAT2 expression was not correlated with MGAT activity (Figure 5). However, given the disconnect between MOGAT2 mRNA and MGAT2 protein in liver (Figure 2), we cannot exclude the possibility that MGAT2 protein levels are regulated independent of the RNA and thus may correlate with MGAT activity. As expected, MOGAT1 mRNA levels were also not related to MGAT activity (Table 3). Hepatic expression of lysophosphatidylglycerol acyltransferase 1 (LPGAT1) and DGAT family genes, which also possess MGAT activity in vitro, was also not related to MGAT activity (Table 3).

**MGAT3 functions as both an MGAT and DGAT enzyme in HepG2 cells.** Previous work has suggested that MOGAT3, which may have arisen from duplication of the gene encoding DGAT2, encodes an enzyme with both MGAT and DGAT activity. We sought to evaluate this further
using a cell-based assay. HepG2 cells were infected with adenovirus expressing MGAT3 and/or GFP and incorporation of \([^{14}C]\)glycerol into glycerides was assessed. Overexpression of MGAT3 led to a decrease in the incorporation of \([^{14}C]\)glycerol into MAG and a modest increase in \([^{14}C]\)TAG accumulation, but did not affect \([^{14}C]\)DAG content of the cells (Figure 6A). Treatment with a selective DGAT1 inhibitor (DGAT1i; Example 3 reference 15) markedly reduced the cellular \([^{14}C]\)TAG content and caused an increase in \([^{14}C]\)DAG levels. The effects of the DGAT1i on DAG and TAG were reversed when MGAT3 was overexpressed.

To determine whether loss of MGAT3 affected MGAT activity, HepG2 cells were transfected with siRNA oligonucleotides to knockdown MOGAT3 (Figure 6B). Loss of MGAT3 by itself did not impair the incorporation of \([^{14}C]\)mono-oleoyl glycerol into DAG or TAG. However, in the presence of the DGAT1i, the MAG incorporation into TAG was diminished. Collectively, these data are consistent with the idea that most of the DAG synthesized by MGAT3 is rapidly converted to TAG and that MGAT3 functions as both an MGAT and DGAT enzyme in cultured cells.

MOGAT gene expression is diminished following marked weight loss and is increased in subjects with NAFLD. Finally, we sought to determine whether MOGAT expression was regulated in liver by physiologic or pathologic stimuli. MOGAT2 and MOGAT3 expression was assessed in 9 extremely obese subjects undergoing RYGB (Cohort 2; Table 1). One year after RYGB, subjects had lost 38% of the initial BMI. RYGB led to a significant improvement in systemic and hepatic insulin sensitivity, as measured by the HOMA-IR value and the HISI, and decreased serum TAG concentrations (Table 1). The hepatic expression of MOGAT2 and MOGAT3 was significantly down-regulated in biopsies obtained 1 year after RYGB compared to samples obtained during surgery (Figure 7A).
The expression of MOGATs was also assessed in a distinct cohort of subjects with NAFLD (Cohort 3; Table 2). These subjects were obese, but had an average BMI that was similar to the control group that did not have NAFLD. NAFLD subjects were hypertriglyceridemic (p<0.04), tended to be hyperinsulinemic (p<0.08) and had elevated alanine aminotransferase levels compared to controls (Table 2). MOGAT2 and MOGAT3 expression was significantly increased in subjects with NAFLD compared to control subjects (Figure 7B). Collectively, these data suggest that MOGAT2 and MOGAT3 expression is up-regulated in subjects with NAFLD and that marked weight loss leads to a correction in the expression of these glyceride-synthesizing enzymes in extremely obese subjects.

Discussion

An understanding of the factors that control hepatic intermediary glycerolipid metabolism has important implications for the potential treatment of obesity-related metabolic diseases. In this study, we evaluated the expression and activity of the MGAT enzymes in liver of obese human subjects. Our data demonstrate that MGAT activity is present in human liver and that expression of the gene encoding MGAT3 is directly related to hepatic MGAT activity. Furthermore, in a liver-derived cell line, siRNA-mediated knockdown of MOGAT3 led to reduced MGAT activity. Our data suggest that the expression of the MOGAT genes is dynamically regulated in liver of obese human subjects and is increased in subjects with NAFLD. The findings presented herein could have significant clinical implications, given the established link between DAG and activation of intracellular signaling pathways that negatively impact hepatic insulin sensitivity (2). Drugs that specifically target MGAT3 could have
therapeutic potential for treating hepatic insulin resistance, dyslipidemias, and other metabolic abnormalities associated with hepatic steatosis.

Work conducted in rodent models has suggested that intestinal MGAT activity is important for absorption of dietary fat (26) and that hepatic MGAT activity is important for controlling glycerolipid metabolism in neonatal liver (6, 7). However, data from other studies suggest that the physiological importance of the MGAT enzymes in fatty acid metabolism in adult liver is minimal. For example, some reports show little MGAT activity in hepatic lysates from human subjects (23). Similarly, Cortés et al. reported that, despite strong induction of Mogat1 and Mogat2 mRNA and MGAT1 protein in the livers of Agpat2-deficient mice, they were unable to detect MGAT activity (22). It was postulated this might be due to abundant lipase activity in the liver homogenates assayed. With this in mind, we profiled a number of known lipase inhibitors and found that, in the presence of MAFP, a non-specific inhibitor of the serine hydrolase superfamily of enzymes which includes multiple lipases, MGAT activity was readily detectable in human liver fractions. The carboxyl ester lipase activity is reported to be high in adult liver (24) and hepatic carboxyl ester lipases are known to be active against both MAG and DAG (25). The inhibition of this lipase may unmask hepatic MGAT activity by preventing the rapid hydrolysis of the MAG substrate and/or newly synthesized DAG or TAG.

The primary metabolic source of MAG, the substrate of MGATs needed for glyceride synthesis in liver, is not known. It is possible that one of the principal functions of MGATs in liver is to recycle MAG derived from lipolysis of intrahepatic TAG and DAG. Recycling of MAG intermediates during periods of rapid TAG turnover may be energetically favorable to new synthesis of glycerol-3-phosphate. Alternatively, the dephosphorylation of lysophosphatidic acid by lipid phosphate phosphatases can directly generate MAG (27, 28). There is also one report of
direct acylation of glycerol (29). The other significant source of MAG in hepatocytes could be derived from hepatic reuptake of lipoprotein/chylomicron particle remnants containing MAG as lipolytic products.

The results from our study underscore the importance of studying human liver tissue to understand the metabolic function and clinical relevance of the MGATs. For example, whereas the rodent Mogat1 gene encodes a protein that is expressed in stomach and kidney, the human MOGAT1 gene is primarily non-coding due to extensive alternative splicing (data not shown) (21). The present study also suggests the importance of MOGAT3, which is not analogous to murine Mogat3, to hepatic MGAT activity. In fact, the mouse Mogat3 gene is a pseudogene (12).

We also detected high expression of MOGATs in human liver, which is in close agreement with other reports (8-11), but contrast the expression profile in murine species. None of the Mogat genes is well-expressed in normal mouse liver, suggesting species-specific differences in expression profiles.

Recent work has suggested the potential utility of targeting MGAT activity in treatment of obesity-related diseases of the liver and other organs. It was recently demonstrated that Mogat2 knockout mice are protected from diet-induced obesity and hepatic steatosis (26). There are also a few reports that the hepatic expression of Mogat1 (22, 30) and Lpgat1 (31) is up-regulated in mouse models of hepatic steatosis and that the targeted knockdown of Lpgat1 in liver of diet-induced obese mice reduced hepatic steatosis and insulin resistance (31). These studies conducted in mouse models are consistent with the present findings that MOGAT2 and MOGAT3 expression is robustly increased in liver of subjects with NAFLD and was significantly down-regulated by marked weight loss in extremely obese individuals. It is difficult to determine the molecular or physiologic factors that influence the expression of MOGAT2 and MOGAT3 in
these obese subjects. Gastric bypass surgery, in addition to causing marked weight loss, leads to a striking improvement in hepatic steatosis, insulin sensitivity, and other metabolic parameters (Table 1 and (32-34)). Future studies will be needed to clarify the factors that regulate MOGAT expression in liver.

In summary, we found that the MGAT enzymes, are active in human liver and that the expression of the genes encoding these enzymes is dynamically regulated. The present data are also consistent with the idea that MGAT enzyme activity could represent a viable target for pharmacologic intervention to treat hepatic insulin resistance and NAFLD.
Acknowledgments

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References


Figure Legends.

**Figure 1.** Two convergent pathways for triacylglycerol biosynthesis. The stepwise acylation of glycerol through the two pathways for triacylglycerol synthesis are shown. Abbreviations: fatty acid (FA), glycerol-3-phosphate (G-3-P), G-3-P acyltransferase (GPAT), lysophosphatidic acid (LPA), acylglycerol-3-phosphate acyltransferase (AGPAT), phosphatidic acid (PA), monoacylglycerol (MAG), MAG acyltransferase (MGAT), diacylglycerol (DAG), DAG acyltransferase (DGAT), and triacylglycerol (TAG).

**Figure 2.** Tissue-specific expression of human MOGATs. The tissue specific expression profiles of human MOGAT1 [A], MOGAT2 [B], and MOGAT3 [C] were determined using RT-QPCR. MOGAT mRNA levels were normalized to the expression of peptidylprolyl isomerase A (cyclophilin A, PPIA). Cycle threshold (CT) in RNA from liver for each gene is shown for reference. Data are mean ± standard deviation from technical replicates. * indicates undetectable expression (CT > 35). [D] Western blot analyses for MGAT2 and MGAT3 proteins in S9 and microsomal fractions from human intestine and liver are shown. S9 and microsomal fractions are pools from derived from multiple individual donors, n = 200 for liver and n = 18 for intestine. 50 μg of human liver or intestine microsome protein was probed with either MGAT2 or MGAT3 antibodies. Membranes derived with Sf9 cells infected with either human MGAT2 or MGAT3 are used as controls.

**Figure 3.** Expression of human MOGAT2 and MOGAT3 protein and MGAT activity in liver and intestine. [A] The effect of the addition of a non-specific lipase inhibitor was determined by adding MAFP to reactions containing either pooled human intestinal or liver
microsomes and determining the incorporation of $[^{14}\text{C}]$decanoyl-CoA into $sn$-1,2-DAG and TAG. [B]. Direct comparison of MGAT activity in human liver ($squares$) and intestine microsomes ($circles$). MGAT activity was assessed as outlined above and the incorporation of $[^{14}\text{C}]$decanoyl-CoA into TAG and $sn$-1,2-DAG quantified following TLC.

**Figure 4. Characterization of MGAT assay.** The effects of time (0 – 60 min) and protein (1 – 20 µg) concentration on MGAT activity were analyzed. MGAT activity was assessed as described in Figure 4 using $[^{14}\text{C}]$decanoyl-CoA as the acyl donor and $sn$-2-monooleoylglycerol as the acyl acceptor. Quantification of the $sn$-1,2-DAG is shown below the TLC image.

**Figure 5. Hepatic MOGAT3 expression is related to MGAT activity.** Scatter plots depict expression of hepatic MOGAT3 or MOGAT2 in relation to hepatic MGAT activity in individual subjects.

**Figure 6. Cell-based activity of human MGAT3.** [A] HepG2 cells were transduced with an adenovirus expressing human MGAT3 (Ad-MGAT3) for 48 h. Cells were assayed for lipogenic activity using $[^{14}\text{C}]$glycerol in the presence of 400 µM dodecanoate. A selective DGAT1 inhibitor (DGAT1i) was added to the cells as a 1000X stock in DMSO to a final concentration of 3 µM in DMSO. Lipids were extracted and analyzed as described above. A graphical representation of the TLC plate is shown at right. [B] HepG2 cells were transfected with siRNA to MOGAT3 (or scrambled control) and the expression of MOGAT3 (graph at left) or incorporation of $[^{14}\text{C}]$mono-oleyl glycerol into TAG (graph at right) was quantified.
Figure 7. *MOGAT2* and *MOGAT3* expression in obese subjects is diminished following marked weight loss and is induced in subjects with NAFLD. [A] The graphs depict the expression of *MOGAT2* and *MOGAT3* in liver of obese subjects at time of GBS and 1 year later. [B] The box and whisker plots depict the expression of *MOGAT2* and *MOGAT3* in liver of subjects with NAFLD or control subjects.
Table 1. RYGB Cohort 2

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<td>18.3±3.1 (7.4-31.5)</td>
<td>4.7±0.8 (2.5-9.8)</td>
<td>0.0011</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>102.9±4.9 (93.4-136.3)</td>
<td>82.5±1.7 (75.2-90.0)</td>
<td>0.0019</td>
</tr>
<tr>
<td>HOMA-IR score</td>
<td>4.8±1.0 (2.4-10.6)</td>
<td>0.9±0.1 (0.5-1.8)</td>
<td>0.0025</td>
</tr>
<tr>
<td>Hepatic Insulin Sensitivity Index</td>
<td>98.8±18.7 (32-198)</td>
<td>240.8±30.4 (131-337)</td>
<td>0.0013</td>
</tr>
<tr>
<td>TAG (mg/dl)</td>
<td>144±15.3 (79-220)</td>
<td>72.0±7.7 (52-126)</td>
<td>0.0006</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>47.1±3.4 (36-62)</td>
<td>67.6±4.9 (48-99)</td>
<td>0.0034</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>101.4±9.9 (64-143)</td>
<td>88.7±4.8 (73-113)</td>
<td>0.2625</td>
</tr>
<tr>
<td>AST (IU/I)</td>
<td>26.3±5.6 (14-52)</td>
<td>23.6±4.0 (14-52)</td>
<td>0.7120</td>
</tr>
<tr>
<td>ALT (IU/I)</td>
<td>42.2±19.0 (10-158)</td>
<td>29.8±6.1 (11-72)</td>
<td>0.5489</td>
</tr>
</tbody>
</table>

BMI, Body Mass Index; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; TAG, Triglycerides; HDL, High-density lipoprotein; LDL, Low-density lipoproteins; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
Table 2. Cohort 3

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=7)</th>
<th>NAFLD (n=15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±S.E.M. (range)</td>
<td>Mean±S.E.M.(range)</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>Males 4; Females 3</td>
<td>Males 4; Females 10</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>50±5 (32-71)</td>
<td>42±3 (21-53)</td>
<td>0.1008</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>90±7 (66-111)</td>
<td>97±6 (72-122)</td>
<td>0.4786</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.2±4.9 (22.8-44.3)</td>
<td>34.6±1.7 (24.2-43.3)</td>
<td>0.4679</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>7.7±2.7 (4.5-13)</td>
<td>23.5±8. (7.4-125.4)</td>
<td><strong>0.0840</strong></td>
</tr>
<tr>
<td>TAG (mg/dl)</td>
<td>90±230 (58-149)</td>
<td>196±25 (82-358)</td>
<td><strong>0.0361</strong></td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>50±5 (42-58)</td>
<td>44±3 (32-67)</td>
<td>0.2978</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>99±19 (63-127)</td>
<td>126±8 (73-164)</td>
<td>0.2765</td>
</tr>
<tr>
<td>AST (IU/I)</td>
<td>43±9 (26-76)</td>
<td>67±10 (13-132)</td>
<td>0.1042</td>
</tr>
<tr>
<td>ALT (IU/I)</td>
<td>56±19 (16-113)</td>
<td>131±28 (17-352)</td>
<td><strong>0.0375</strong></td>
</tr>
</tbody>
</table>

*All Subjects were hep pan negative, tested negative for all strains of hepatitis. BMI, Body Mass Index; TAG, Triglycerides; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
Table 3. Correlation coefficients (r) of gene expression to enzyme activity.

<table>
<thead>
<tr>
<th></th>
<th>DAG</th>
<th>TAG</th>
<th>DAG + TAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOGAT1</td>
<td>-0.29</td>
<td>-0.28</td>
<td>-0.29</td>
</tr>
<tr>
<td>LPGAT1</td>
<td>0.15</td>
<td>0.18</td>
<td>0.17</td>
</tr>
<tr>
<td>DGAT1</td>
<td>0.14</td>
<td>0.14</td>
<td>0.15</td>
</tr>
<tr>
<td>DGAT2</td>
<td>0.07</td>
<td>0.10</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*p<0.01
Figure 1

Glycerol Phosphate Pathway

G-3-P \( \rightarrow \) LPA \( \rightarrow \) PA \( \rightarrow \) lipins \( \rightarrow \) DAG \( \rightarrow \) TAG

Acyl-CoA

AGPATs

MGATs

Monoacylglycerol Pathway

Acyl-CoA

Acyl-CoA

MAG

Acyl-CoA
**Figure 2**

**A.** Relative mRNA abundance of MOGAT1 in various tissues.

**B.** Relative mRNA abundance of MOGAT2 in various tissues.

**C.** Relative mRNA abundance of MOGAT3 in various tissues.

**D.** Western Blot analysis showing the expression of different MGATs in liver and small intestine.
Figure 3

A. Intestine

<table>
<thead>
<tr>
<th>Orlistat (µM)</th>
<th>MAFP (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Liver

<table>
<thead>
<tr>
<th>Orlistat (µM)</th>
<th>MAFP (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B. [14C]Dodecanoyl CoA Incorporation (DPM/µg protein)

Liver

Intestine

<table>
<thead>
<tr>
<th>Orlistat (µM)</th>
<th>MAFP (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

[14C]Dodecanoyl CoA Incorporation (DPM/µg protein)

Liver

Intestine

Log Microsomal Protein (µg)

-1 0 1 2
Figure 4

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes (μg)</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>1</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

- TAG
- Fatty acid
- DAG

**Time course**

- DAG (DPM)
- Time (min)
- Microsome (μg)

**Microsome titration**

- DAG (DPM)
- Microsome (μg)
Figure 5

- **DAG**
  - MOGAT3 vs. DAG activity (DPM) with an R-value of 0.43.
  - MAOGAT2 vs. DAG activity (DPM) with an R-value of -0.21.

- **TAG**
  - MOGAT3 vs. TAG activity (DPM) with an R-value of 0.39.
  - MOGAT2 vs. TAG activity (DPM) with an R-value of -0.20.
Figure 6

A. 

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DGAT1i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-MGAT3</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

TAG | DAG | MAG | [14C]Glycerol Incorporation (DPM)

B. 

<table>
<thead>
<tr>
<th></th>
<th>DGAT1i</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Relative mRNA Abundance

[14C]Triacylglycerol Formation (DPM/mg protein)
Figure 7

A

Normalized AU

MOGAT2  MOGAT3

pre post

B

Normalized AU

3Q 2Q Mean

Control NAFLD Control NAFLD

MOGAT2 MOGAT3