Roles of Acyl-CoA:Diacylglycerol Acyltransferases 1 and 2 in Triacylglycerol Synthesis and Secretion in Primary Hepatocytes

Chen Li, Lena Li, Jihong Lian, Russell Watts, Randal Nelson, Bryan Goodwin, Richard Lehner

Objective—Very low–density lipoprotein assembly and secretion are regulated by the availability of triacylglycerol. Although compelling evidence indicates that the majority of triacylglycerol in very low–density lipoprotein is derived from re-esterification of lipolytic products released by endoplasmic reticulum–associated lipases, little is known about roles of acyl-CoA:diacylglycerol acyltransferases (DGATs) in this process. We aimed to investigate the contribution of DGAT1 and DGAT2 in lipid metabolism and lipoprotein secretion in primary mouse and human hepatocytes.

Approach and Results—we used highly selective small-molecule inhibitors of DGAT1 and DGAT2, and we tracked secretion of lipids synthesized de novo from [3H]acetic acid and from exogenously supplied [3H]oleic acid. Inactivation of individual DGAT activity did not affect incorporation of either radiolabeled precursor into intracellular triacylglycerol, whereas combined inactivation of both DGATs severely attenuated triacylglycerol synthesis. However, inhibition of DGAT2 augmented fatty acid oxidation, whereas inhibition of DGAT1 increased triacylglycerol secretion, suggesting preferential channeling of separate DGAT-derived triacylglycerol pools to distinct metabolic pathways. Inactivation of DGAT2 impaired cytosolic lipid droplet expansion, whereas DGAT1 inactivation promoted large lipid droplet formation. Moreover, inactivation of DGAT2 attenuated expression of lipogenic genes. Finally, triacylglycerol secretion was significantly reduced on DGAT2 inhibition without altering extracellular apolipoprotein B levels.

Conclusions—Our data suggest that DGAT1 and DGAT2 can compensate for each other to synthesize triacylglycerol, but triacylglycerol synthesized by DGAT1 is preferentially channeled to oxidation, whereas DGAT2 synthesizes triacylglycerol destined for very low–density lipoprotein assembly.

Key Words: diacylglycerol o-acyltransferase ■ hepatocytes ■ triglycerides

Hepatic steatosis, the clinical manifestation of ectopic accumulation of triacylglycerol in the liver, is associated with cardiovascular diseases through increased production of proatherogenic very low–density lipoprotein (VLDL), insulin resistance, and diabetes mellitus.1,2 The assembly of secretory competent VLDL is dependent on the provision of lipids, triacylglycerol in particular. Acyl-CoA:diacylglycerol acyltransferase (DGAT) 1 and DGAT2 catalyze the final and only committed step in mammalian triacylglycerol biosynthesis.3 Although both DGATs esterify diacylglycerol to triacylglycerol, the enzymes share no homology in primary amino acid sequences and are encoded by genes belonging to distinct gene families. The 2 DGATs seem to perform non-redundant physiological functions, reflected by different phenotypes of Dgat1−/− and Dgat2−/− mice.4,5 Dgat2−/− mice die within a few hours after birth because of a drastic reduction of triacylglycerol content and a severely impaired epidermal barrier protection.5 Diminished Dgat2 expression in adult mice by antisense oligonucleotide markedly decreases hepatic triacylglycerol content and improves steatosis in obese mice.6,7 In contrast, Dgat1−/− mice are viable, possibly because of a more moderate reduction in tissue triacylglycerol and a mild decrease in dietary triacylglycerol absorption.8,9 Moreover, plasma triacylglycerol concentration in DGAT1-deficient mice remains unchanged after a 4-hour fasting, suggesting a relatively minor role of DGAT1 in hepatic lipoprotein production.4,10 Although extensive investigation of DGATs has been undertaken in tissues such as adipose, small intestine, and skin, less is known about their roles in hepatic triacylglycerol synthesis and secretion.

Differences in fatty acid (FA) compositions of hepatic intracellular triacylglycerol and plasma VLDL triacylglycerol revealed...
an indirect transfer of triacylglycerol for VLDL maturation.\textsuperscript{11} It has been established that the majority (60%–80%) of triacylglycerol in VLDL is derived from re-esterification of lipolytic products in hepatocytes.\textsuperscript{12–14} This sinusous supply of triacylglycerol for VLDL maturation overcomes the inability of triacylglycerol stored in cytosolic lipid droplets (LDs) to cross the lipid bilayer of the endoplasmic reticulum (ER) and provides a mechanism for the regulation of VLDL secretion independently of plasma FA and intracellular triacylglycerol concentration.\textsuperscript{15} We have previously shown that lipases involved in the hydrolysis of pre-formed triacylglycerol include ER-localized carboxylesterase 1 and triacylglycerol hydrolase, and arylacetamide deacetylase\textsuperscript{16–18}, however, it has not been determined which DGAT catalyzes the re-esterification of diacylglycerol to support VLDL maturation.

Topological studies separated DGAT activities in hepatic microsomes into overt, cytosolic side-localized, and latent, luminal side-localized.\textsuperscript{19–21} This led to a hypothesis that the overt DGAT activity might be responsible for the synthesis of triacylglycerol stored in cytosolic LDs, whereas the latent DGAT activity would mainly contribute to the formation of luminal LDs, lipidation of primordial apolipoprotein B (ApoB) particles, and maturation/expansion of VLDL.\textsuperscript{22,23} DGAT1 contains multiple transmembrane domains\textsuperscript{24} and has been suggested to assume a dual topology within the ER of HepG2 cells, exhibiting comparable activities on both cytosolic and luminal sides of the ER membrane.\textsuperscript{25} In contrast, DGAT2 was postulated to be intercalated into the ER bilayer through 1 or 2 transmembrane domains, with both the N and C termini oriented toward the cytosol.\textsuperscript{20} In addition to its ER localization, DGAT2 was found on the surface of LDs in the Caenorhabditis elegans intestinal segments and Drosophila melanogaster Schneider 2 cells.\textsuperscript{19,26,27} Thus, DGAT2 would be predicted to contribute to the overt activity. However, divergent DGAT1 and DGAT2 topologies might not be sufficient to assign specific roles these enzymes might play in channeling triacylglycerol for distinct metabolic functions because there seems to be a functional crosstalk between triacylglycerol residing in cytosolic LDs and ER luminal LDs.\textsuperscript{12,28}

To investigate distinct contributions of the 2 DGATs to hepatic triacylglycerol synthesis, deposition in LDs, and triacylglycerol secretion in VLDL, we pharmacologically inhibited their activities in primary mouse and human hepatocytes with potent, highly selective small-molecule inhibitors.\textsuperscript{29,30} Moreover, because some studies showed that DGAT2 preferentially used endogenously synthesized FA to form triacylglycerol in HepG2 hepatoma cell line, whereas DGAT1 was primarily responsible for exogenous FA esterification,\textsuperscript{25,31} we tracked lipids synthesized from FA made de novo or from exogenously supplied oleic acid.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**DGAT1 and DGAT2 Can Compensate for Each Other to Synthesize Triacylglycerol**

To investigate individual DGAT function in triacylglycerol synthesis in primary mouse hepatocytes, we inhibited DGAT1 or DGAT2 activity individually or together by small-molecule inhibitors; then, we assessed de novo synthesis of triacylglycerol by incubations with \[^{14}C\]acetate and incorporation of exogenously supplied \[^{14}C\]oleic acid into triacylglycerol. Cells were incubated with 50 \(\mu\)mol/L \[^{14}C\]acetate or 0.4 mmol/L \[^{14}C\]oleic acid, respectively. Cellular triacylglycerol mass did not change after 4-hour incubation with acetate, whereas it was augmented after 0.4 mmol/L olate supplementation (Figure 1 in the online-only Data Supplement), indicating that excess exogenous olate contributed to a significant enhancement of hepatic triacylglycerol pool, whereas FA synthesized de novo from acetate did not significantly increase intracellular triacylglycerol content. Triacylglycerol synthesis from de novo synthesized FAs (acetate precursor) was not affected by the inhibition of either DGAT1 or DGAT2 alone, and it was slightly decreased in DGAT2-inactivated cells incubated with olate, whereas inactivation of both DGAT1 and DGAT2 dramatically reduced triacylglycerol synthesis (>80%) irrespective of whether FA was endogenously synthesized or supplied exogenously (Figures 1A and 2A). Moreover, DGAT inhibitors did not affect mRNA expression of either Dgat1 or Dgat2 (Figure II in the online-only Data Supplement). Therefore, DGAT1 and DGAT2 can compensate for each other to synthesize triacylglycerol in mouse hepatocytes, although DGAT2 seems to be more efficient in esterifying exogenous FA.

### Inhibition of DGATs Influences sn-1,2-Diacylglycerol and Glycerophospholipid Synthesis

Sn-1,2-diacylglycerol is at the pivotal junction of the Kennedy pathway of glycerolipid synthesis and can be converted to either phosphatidylcholine, phosphatidylethanolamine, or triacylglycerol. Destinations of sn-1,2-diacylglycerol for membrane-forming phosphatidylcholine and phosphatidylethanolamine or for energy storage as triacylglycerol are controlled by enzymes, cytidine 5'-diphosphocholine-choline/ethanolamine:sn-1,2-diacylglycerol phosphocholine/phosphatidyl ethanolamine transferases, and DGATs, respectively.\textsuperscript{32,33} We postulated that inhibition of DGAT1 or DGAT2 would reduce esterification of sn-1,2-diacylglycerol to triacylglycerol and increase channeling of sn-1,2-diacylglycerol to glycerophospholipid synthesis. Indeed, combined inhibition of DGAT1 and DGAT2 resulted in accumulation of sn-1,2-diacylglycerol, which also occurred to a lesser but significant degree on inhibition of DGAT2 but not DGAT1 alone (Figures 1B and 2B). These results suggest a more active role of DGAT2 in hepatic triacylglycerol synthesis, in agreement with previous reports showing that reduction of Dgat2, but not Dgat1, expression by antisense oligonucleotide decreased hepatic triacylglycerol levels in high-fat diet–induced obese mice.\textsuperscript{6,7}

### Nonstandard Abbreviations and Acronyms

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ApoB</td>
<td>apolipoprotein B</td>
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<tr>
<td>DGAT</td>
<td>diacylglycerol acyltransferase</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FA</td>
<td>fatty acid</td>
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<tr>
<td>LD</td>
<td>lipid droplet</td>
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<td>VLDL</td>
<td>very-low density lipoprotein</td>
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\[ER = \text{endoplasmic reticulum}; \text{VLDL} = \text{very-low density lipoprotein}\]

\[^{14}C\]acetate or 0.4 mmol/L \[^{14}C\]oleic acid, respectively. Cellular triacylglycerol mass did not change after 4-hour incubation with acetate, whereas it was augmented after 0.4 mmol/L olate supplementation (Figure 1 in the online-only Data Supplement), indicating that excess exogenous olate contributed to a significant enhancement of hepatic triacylglycerol pool, whereas FA synthesized de novo from acetate did not significantly increase intracellular triacylglycerol content. Triacylglycerol synthesis from de novo synthesized FAs (acetate precursor) was not affected by the inhibition of either DGAT1 or DGAT2 alone, and it was slightly decreased in DGAT2-inactivated cells incubated with olate, whereas inactivation of both DGAT1 and DGAT2 dramatically reduced triacylglycerol synthesis (>80%) irrespective of whether FA was endogenously synthesized or supplied exogenously (Figures 1A and 2A). Moreover, DGAT inhibitors did not affect mRNA expression of either Dgat1 or Dgat2 (Figure II in the online-only Data Supplement). Therefore, DGAT1 and DGAT2 can compensate for each other to synthesize triacylglycerol in mouse hepatocytes, although DGAT2 seems to be more efficient in esterifying exogenous FA.

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Although increased sn-1,2-diacylglycerol availability from de novo lipogenesis (acetate) did not lead to enhanced phosphatidylcholine or phosphatidylethanolamine synthesis (Figure 1C and 1D), sn-1,2-diacylglycerol produced from exogenous oleic acid was channeled to glycerophospholipid synthesis (Figure 2C and 2D). Moreover, inactivation of both DGATs augmented the formation of phosphatidylinositol and phosphatidylserine, regardless of the acyl donor (de novo synthesized FA or exogenous FA; Figures 1E and 2E).

**DGAT2 Promotes Whereas DGAT1 Restricts Cytosolic LD Growth in Mouse Hepatocytes**

Phosphatidylcholine acts as a surfactant to prevent LD coalescence, and genetic ablation of CTP:phosphocholine cytidylyltransferase α, the rate-limiting enzyme in phosphatidylcholine synthesis, results in giant LDs. We therefore hypothesized that the observed increased phosphatidylcholine synthesis from exogenous oleate (Figure 2C) would alter LD morphology. To test this hypothesis, LD biogenesis was stimulated by incubation of hepatocytes with oleate together with a trace of Bodipy FA analogue. The experimental protocol was laid out as presented in the schematic diagram (Figure 3A, (1)), and representative images from this experiment are shown in Figure 3B (top). Thirty cells were analyzed in each group. The number of LDs per cell did not change unless both of the DGATs were inactivated. The mean area of individual LD and the percentage of large LDs (area, >3 μm²) were decreased, and the frequency of small LDs (area, <1 μm²) was increased when DGAT2 or both DGATs were inhibited, whereas DGAT1 inhibition exerted opposite effects (Figure 3C).

Next, we investigated whether any morphological changes would occur in LDs containing preformed triacylglycerol, in other words, when DGAT activity was inhibited during triacylglycerol turnover. The experimental protocol is shown in the schematic diagram (Figure 3A, (2)), and representative images from this experiment are shown in Figure 3B (bottom). Thirty cells from each condition were analyzed. When DGAT2 or

**Figure 1.** Influence of diacylglycerol acyl transferase (DGAT) inhibition on various lipid species generated from de novo synthesized fatty acid. Mouse hepatocytes were incubated in DMEM containing 10 μCi [3H]acetic acid and 50 μmol/L acetate for 4 hours, in the presence or absence of DGAT1 or DGAT2 inhibitors alone or in combination. Incorporation of [3H]acetate into intracellular triacylglycerol (TG; A), sn-1,2-DG (B), phosphatidylcholine (PC; C), phosphatidylethanolamine (PE; D), combined phosphatidylinositol (PI) and phosphatidylserine (PS; E); fatty acid (FA; F); normalized to total cellular protein. Data are presented as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 vs control (DMSO) based on 3 independent biological replicates.
both DGATs were inhibited, the frequency of small LDs (area, <1 μm²) was increased, accompanied with a slight increase of the number of LDs per cell, whereas the mean area and the percentage of large LDs (area, >5 μm²) were decreased. DGAT1 inhibition alone had no significant influence on the size and number of preformed LDs (Figure 3D). The mean area of individual LD containing preformed triacylglycerol as opposed to newly synthesized triacylglycerol was larger, although the intracellular triacylglycerol content did not change (data not shown), suggesting that LD coalescence occurred during triacylglycerol turnover.

Inhibition of DGAT2, but Not DGAT1, Reduces Lipogenic Gene Expression

Because Dgat2-/- mice exhibited markedly diminished FA levels in the liver and plasma, we examined the potential modulatory role of DGATs on de novo synthesis of FA by analyzing lipogenic gene expression and SREBP1c maturation. Messenger RNA expression of genes involved in de novo lipogenesis (Acaca, Fasn, and Srebf1c) and desaturation (Scd1) was significantly downregulated on DGAT2 inhibition (Figure 4A). Nuclear localization of the master lipogenic transcriptional activator SREBP1c was diminished on DGAT2 inactivation (Figure 4B). Because of the short-time treatment with DGAT inhibitors (4 hours), changes in protein expression and metabolism may not be evident, and therefore, it was not surprising that the total amount of full-length SREBP1 precursor did not alter and nor was de novo triacylglycerol synthesis on singular DGAT2 inactivation (Figure 1A). Nonetheless, metabolic changes might occur during prolonged treatment with DGAT inhibitors.
Triacylglycerol Pool Generated by DGAT1 Is Preferentially Used for Supplying Substrates for Oxidation, Whereas DGAT2-Derived triacylglycerol Is More Favored for Secretion

Although DGAT1 and DGAT2 can compensate for each other to synthesize triacylglycerol, their distinct membrane topologies, interacting protein partners, and subcellular localizations might imply different destinations and uses of their products. Neutral lipids stored in the cytosol or ER lumen of hepatocytes are destined for distinct uses. ER lumen triacylglycerol is deposited into primordial ApoB particles or resident ER lumenal LDs, both closely correlated with VLDL maturation, whereas triacylglycerol stored in cytosolic LDs provides FA for oxidation in mitochondria. Therefore, we first
Inhibition of DGAT2, but Not DGAT1, Reduces Re-Esterification of Lipolytic Products and Triacylglycerol Secretion Without an Effect on Extracellular ApoB Levels in Mouse Hepatocytes

To distinguish potentially different functions of DGAT1 and DGAT2 in provision of triacylglycerol for VLDL assembly by re-esterification of lipolytic products from preformed lipids, we introduced the DGAT inhibitors immediately before the chase period, that is, after lipids were synthesized from radiolabeled precursors as depicted in Figure 6A. Inhibition of DGAT2 dramatically reduced triacylglycerol secretion, suggesting a prominent role of DGAT2 in the re-esterification of diacylglycerol to form VLDL-destined triacylglycerol, although DGAT1 might have a minor additive effect because inhibition of both DGATs further dampened triacylglycerol secretion (Figure 6B and 6C). DGAT2 inhibition–mediated attenuation of VLDL triacylglycerol secretion was independent of microsomal triglyceride transfer protein expression (Figure V1A and V1B in the online-only Data Supplement). In addition, there was no preference for FA origin used for the resynthesis of VLDL triacylglycerol because similar attenuation of VLDL secretion was observed when preformed triacylglycerol was made de novo from acetate or from exogenously supplied oleate (Figure 6B and 6C).

More triacylglycerol undergoes lipolysis than is required to support VLDL assembly, and excess re-esterified triacylglycerol re-enters the intracellular triacylglycerol storage pool. However, it is technically challenging to segregate recycled triacylglycerol from the pre-existing triacylglycerol. To distinguish the pre-existing triacylglycerol pool from the newly synthesized triacylglycerol pool, a double-labeling experiment was carried out, in which the preformed triacylglycerol was labeled with [14C]oleic acid during an overnight incubation (pulse), followed by a 4-hour chase period, during which the newly synthesized triacylglycerol pool was labeled with [3H]oleic acid. DGAT inhibitors were only present in the chase period, during which pre-existing [14C] triacylglycerol was undergoing lipolysis–re-esterification cycle, whereas the newly synthesized triacylglycerol was [3H]-labeled. DGAT2 inactivation only decreased the secretion of [14C] triacylglycerol, but not [3H] triacylglycerol (Figure VII in the online-only Data Supplement), further demonstrating a critical role of DGAT2 for VLDL secretion through re-esterification of lipolytic products of preformed triacylglycerol.

Figure 4. Inhibition of diacylglycerol acyltransferase 2 (DGAT2), but not DGAT1, reduces lipogenic gene expression. Mouse hepatocytes were incubated in DMEM containing 50 μmol/L acetate for 4 hours, in the presence or absence of 5 μmol/L DGAT1 or DGAT2 inhibitors alone or in combination. A, Quantitative polymerase chain reaction analysis of lipogenic gene expression. Data are presented as the ratio of expression of a given gene to cyclophilin (Cyc). Values are normalized to control (DMSO) where the gene/Cyc ratio was set as 1. B, Immunoblotting of mature form of SREBP1c in the nuclei (nSREBP1) and of full-length SREBP1c precursor in the membrane (memSREBP1), lamin A/C and calnexin served as loading controls for nuclei and the endoplasmic reticulum membranes, respectively.

Table 1. Effects of DGAT inhibitors on lipogenic gene expression in mouse hepatocytes

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<th>DGAT Inhibitor</th>
<th>Acaca</th>
<th>Fasn</th>
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Examination whether DGAT1 or DGAT2 inactivation would affect intracellular triacylglycerol distribution by analyzing the proportion of newly synthesized triacylglycerol dispersed into cytosol and membranes. However, no change in the subcellular localization of synthesized triacylglycerol was observed when either DGAT was inhibited (Figure V in the online-only Data Supplement). This is perhaps not surprising because the capacity of triacylglycerol storage within the ER bilayer is limited, and triacylglycerol stored in cytosolic and ER lumenal depots is intimately linked.

We next addressed whether inactivation of DGATs directly influences triacylglycerol use using an experimental protocol shown in Figure 5A. After the chase period, radioactivity in acid soluble metabolites and medium triacylglycerol was measured, which indicated FA oxidation and VLDL secretion, respectively. Inhibition of DGAT2 (triacylglycerol synthesized by DGAT1) augmented FA oxidation, whereas DGAT1 inhibition (triacylglycerol synthesized by DGAT2) had no effect on FA oxidation (Figure 5B), suggesting that DGAT1-derived triacylglycerol pool was preferentially used for FA oxidation. This observation is in agreement with previous studies in rats with diet-induced nonalcoholic fatty liver disease, where increased FA oxidation was also observed on antisense oligonucleotide–mediated decrease of DGat2 expression. Notably, reduced de novo lipogenic gene expression (Figure 4A) and augmented FA oxidation (Figure 5B) in DGAT2-inactivated hepatocytes were not accompanied by significant changes in malonyl-CoA mass during the 4-hour incubation (results not shown). On the contrary, inhibition of DGAT1 (triacylglycerol synthesized by DGAT2) increased triacylglycerol secretion, on which DGAT2 inhibition had no effect (Figure 5C), thus suggesting that DGAT2-derived triacylglycerol pool was more efficiently channeled to VLDL assembly. In separate experiments, to validate that DGAT inhibitors were completely removed before the chase period, cells were incubated with [3H]acetic acid or [3H]oleic acid during the chase period. Triacylglycerol synthesis was nearly completely recovered, suggesting that DGAT inhibitors were washed out (Figure IV in the online-only Data Supplement).
Finally, we analyzed the effect of DGAT1 and DGAT2 inactivation on ApoB secretion. The extracellular ApoB48 and ApoB100 levels were significantly decreased after simultaneous inhibition of both DGATs but not when either of the 2 DGATs was inhibited alone (Figure 6D), and the lack of effect on ApoB secretion was persistent even when neither acetate nor oleate was supplemented (Figure V in the online-only Data Supplement). This suggested that DGAT2 inactivation leads to secretion of less lipidated thus denser ApoB. To test this inference, we determined the lipoprotein densities after DGAT inhibition by density gradient centrifugation of secreted \[^{35}S\]-labeled ApoB. ApoB100 and ApoB48 shifted to fractions of higher densities when DGAT2 or both of the DGATs were inhibited (Figure 6E). However, this was more obvious in cells supplemented with oleate than acetate, possibly because incubation with acetate did not stimulate substantial triacylglycerol synthesis and therefore VLDL secretion.

In conclusion, in mouse hepatocytes, DGAT2 plays the dominant role in the re-esterification process and acts as a driving force in facilitation of VLDL maturation, without altering extracellular ApoB levels.

DGAT1 and DGAT2 Regulate VLDL Secretion in a Similar Pattern in Humans as in Mouse Hepatocytes

Although mouse hepatocytes secrete ApoB48 and ApoB100 lipoproteins, human hepatocytes secrete lipoproteins only containing ApoB100, because the latter do not express APOBEC-1, the catalytic component of the apoB mRNA-editing complex. Studies in the HepG2 hepatoma cell line showed that the length of the ApoB molecule was proportional to nascent lipoprotein core circumference, thus determining lipoprotein sizes and compositions, which may also correlate to different metabolic fates and rates. Therefore, the coexistence of ApoB48 and ApoB100 on VLDL particles secreted from mouse hepatocytes adds a layer of complexity to the interpretation of lipoprotein metabolism. In view of this knowledge, we used human primary hepatocytes to further investigate DGAT1 and DGAT2 functions in VLDL secretion. We found that similar to mouse hepatocytes, DGAT2 in primary human hepatocytes was more important for VLDL maturation than DGAT1 because inhibition of DGAT2 alone significantly diminished triacylglycerol secretion (Figure 7A). Moreover, a combination of both DGAT inhibitors nearly...
Figure 6. Inhibition of diacylglycerol acyltransferase 2 (DGAT2), but not DGAT1, reduces re-esterification of lipolytic products and triacylglycerol (TG) secretion without an effect on extracellular ApoB levels in mouse hepatocytes. Mouse hepatocytes were incubated in DMEM containing 10 μCi [3H]acetate and 50 μmol/L acetate, or 5 μCi [3H]oleic acid and 0.4 mmol/L oleate/0.5% BSA for 4 hours, and cells were then washed and incubated in DMEM for additional 4 hours in the presence or absence of DGAT1 or DGAT2 inhibitors alone or in combination. Media were harvested and analyzed for TG and ApoB secretion. Cells were collected and analyzed for intracellular lipids levels. A, Schematic diagram of chronological order of [3H]-labeling and drug treatments. Secreted and intracellular TG, intracellular sn-1,2-DG, and phospholipid (PL) synthesized from [3H]acetic acid (B), [3H]oleic acid (C). D, Immunoblot analysis of extracellular ApoB48 and ApoB100 levels. Extracellular ApoB100 levels were quantified by GeneTools (Syngene Inc), and normalized to the total protein load quantified by Ponceau S Red staining. E, [35S]Met/Cys-labeled extracellular ApoB100 and ApoB48 of lipoproteins with densities ranging from 1.006 g/mL (fraction 10) to 1.21 g/mL (fraction 1), 10 fractions/treatment. Statistical data are presented as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 vs control (DMSO) based on 3 independent biological replicates.
abrogated secretion of triacylglycerol synthesized de novo from acetic acid (Figure 7A). We could not analyze the secretion of oleic acid–derived triacylglycerol in human hepatocytes on simultaneous inhibition of both DGATs because of a massive cell death under this particular circumstance, likely due to toxic effects of excess free FA. Interestingly, different from mouse hepatocytes, extracellular ApoB100 levels were invariable in acetate-supplemented human hepatocytes even when both DGATs were inhibited (Figure 7B). In conclusion, in human hepatocytes, we observed equivalent phenomena as in mouse hepatocytes with respect to contribution of the 2 DGATs to VLDL triacylglycerol secretion: DGAT2 was required for triacylglycerol secretion, on which DGAT1 had a small additive effect. However, extracellular ApoB100 levels remained constant even when both DGATs were inactivated.

### Discussion

In this study, we investigated the roles of DGAT1 and DGAT2 in lipid metabolism and VLDL secretion in primary mouse and human hepatocytes by inactivation of DGAT1 and DGAT2 with specific small-molecule inhibitors alone or in combination. We found that in mouse hepatocytes, DGAT1 and DGAT2 can compensate for each other to synthesize triacylglycerol, but the triacylglycerol pool generated by DGAT1 was preferably used for supplying substrates for oxidation, whereas triacylglycerol synthesized by DGAT2 was preferentially used for secretion as in mouse hepatocytes with respect to contribution of the 2 DGATs to VLDL triacylglycerol secretion: DGAT2 was required for triacylglycerol secretion, on which DGAT1 had a small additive effect. However, extracellular ApoB100 levels remained constant even when both DGATs were inactivated.

**Figure 7.** Diacylglycerol acyltransferase-1 (DGAT1) and DGAT2 regulate very low-density lipoprotein (VLDL) secretion in a similar pattern in human as in mouse hepatocytes. Human hepatocytes were incubated in DMEM containing 10 μCi [3H]oleic acid and 50 μmol/L acetate, or 5 μCi [3H]oleic acid and 0.4 mmol/L oleate/0.5% BSA for 4 hours (pulse), and cells were then washed and incubated in DMEM for additional 4 hours (chase). DGAT1 and DGAT2 inhibitors were added to human hepatocytes at both pulse and chase periods. Media were harvested and analyzed for triacylglycerol (TG) and ApoB secretion.

**A.** Secretion of TG synthesized from [3H]oleic acid (left) or [3H]oleic acid (right).

**B.** Immunoblot analysis of extracellular ApoB100 levels. Statistical data are presented as mean±SEM. *P<0.05, ***P<0.001 vs control (DMSO) based on 3 independent biological replicates.
affected. Notably, an important role of DGAT1 was reported in the re-esterification of partial glycerides with exogenously derived FA, whereas DGAT2 was proposed to have limited effects on re-esterification. However, these studies used the HepG2 hepatoma cell line, which has been shown to be deficient in the use of preformed triacylglycerol for VLDL assembly, possibly because of the absence of lipases, such as carboxylesterase 1d/triacylglycerol hydrolase and arylacetamide deacetylase. Second, DGAT1-deficient mice fed high-fat diet presented with much lower hepatic triacylglycerol stores but normal plasma triacylglycerol concentration when compared with wild-type control mice, and DGAT1 was found not to be essential for chylomicron formation in mice. On the other hand, DGAT2-deficient mice had low plasma triacylglycerol concentration immediately after birth, which suggests impairment in VLDL secretion; however, a liver-specific Dgat2−/− mouse model would be required to further test the role of DGAT2 in hepatic triacylglycerol metabolism.

Another important question is the substrate preference of the 2 DGATs. Oleic acid supplementation mimics FA released from adipose tissue or circulating lipoproteins, whereas de novo synthesized FA from acetate represents carbohydrate metabolism. Both sources of FA can be esterified into triacylglycerol for storage or packaged into VLDL particles for secretion. Although previous studies showed preference of DGAT2 for de novo synthesized FA as a substrate for triacylglycerol production, we did not observe any significant differences between DGAT1 and DGAT2 with respect to using endogenously (acetate) or exogenously (oleate) derived FA for triacylglycerol synthesis or secretion.

Interestingly, inhibition of DGAT2 reduced the mature form of SREBP1 in the nucleus, which led to decreased transcription of several lipogenic genes including Acaca, Fasn, Scd1, and Srebf1c itself, whereas inhibition of DGAT1 had no effect on lipogenesis. A role for DGAT2 in the regulation of de novo FA synthesis was reported in HeLa cells where SCD1 was found to physically interact with DGAT2, thereby providing a pool of monounsaturated FAs for triacylglycerol biosynthesis.

Increased FA oxidation was observed after DGAT2 inhibition but not after DGAT1 inhibition. One possible explanation is that FA not targeted for secretion is channeled for oxidation. Another possibility is that DGAT1-generated LDs cannot undergo expansion in the absence of DGAT2, and these smaller LDs are substrates for adipose triglyceride lipase leading to release of FA targeted for oxidation.
Inhibition of both DGAT1 and DGAT2 activities resulted in extensive cellular death of primary human hepatocytes supplemented with oleic acid followed by prolonged (>12 hours) chase. The cell death could be possibly because of excess free FA that might have accumulated in the ER as a result of not being able to be (re)esterified to triacylglycerol for storage or secretion. Excess free FA elicits profound changes in ER membrane phospholipids, which is followed by ER deformation and leakage of protein-folding chaperones to the cytosol. In addition, alterations in mitochondrial membrane phospholipids and activation of nicotinamide adenine dinucleotide phosphate oxidase by diacylglycerol through protein kinase C–dependent pathways produce reactive oxygen species, release cytochrome c, and result in mitochondrial dysfunction.

Currently, it is not clear how the cytosolic topology of DGAT2 active site contributes to VLDL assembly. ER-localized DGAT2 should only contribute to the overt DGAT activity. However, pharmacological inhibition of DGAT2 by niacin decreased both overt and latent DGAT activities by 30%. Therefore, integration of DGAT1 and DGAT2 function with their topologies requires further experimentation. A working model of DGAT1 and DGAT2 contribution to lipid metabolism in hepatocytes is presented in Figure 8. DGAT1 and DGAT2 can compensate for each other to synthesize triacylglycerol in hepatocytes, but the triacylglycerol pool synthesized by DGAT1 and stored in small LDs is more effectively used for supplying substrates for oxidation, whereas DGAT2 generates triacylglycerol for storage in large LDs and VLDL lipiddation via re-esterification of diacylglycerol derived from lipolysis of preformed triacylglycerol stores.

Acknowledgments
We thank Jamie Lewis and Dr Kneteman for primary human hepatocytes. We also thank Jianfeng Huang and Wesam Bahihim for critical discussion throughout this research.

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Disclosures
Dr Goodwin is an employee and shareholder of Pfizer Inc.

References
DGAT2 Regulates VLDL Secretion

Our research dissected separate functions of 2 forms of acyl-CoA:diacylglycerol acyltransferase in triacylglycerol synthesis and secretion in primary mouse and human hepatocytes and implicated acyl-CoA:diacylglycerol acyltransferases 2 as a potential novel target for therapeutic intervention against cardiovascular disease.


Significance

Our research dissected separate functions of 2 forms of acyl-CoA:diacylglycerol acyltransferase in triacylglycerol synthesis and secretion in primary mouse and human hepatocytes and implicated acyl-CoA:diacylglycerol acyltransferases 2 as a potential novel target for therapeutic intervention against cardiovascular disease.
Roles of Acyl-CoA:Diacylglycerol Acyltransferases 1 and 2 in Triacylglycerol Synthesis and Secretion in Primary Hepatocytes
Chen Li, Lena Li, Jihong Lian, Russell Watts, Randal Nelson, Bryan Goodwin and Richard Lehner

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Materials and Methods

Animals
All animal procedures were approved by the University of Alberta’s Animal Care and Use Committee and were in accordance with guidelines of the Canadian Council on Animal Care. All mice used in this study were 2-3 months old C57BL/6J females maintained on chow diet (PicoLab Rodent Diet 20; LabDiet, Richmond, IN). Unless otherwise stated, all mice were fasted overnight before experiments.

Materials
Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and horse serum were purchased from Invitrogen Canada (Burlington, ON, Canada). RPMI-1640 Medium was from Sigma-Aldrich Canada (Oakville, ON, Canada). Essentially fatty acid free bovine serum albumin (BSA) and Complete protease inhibitor cocktail tablets were from Roche Diagnostics (Laval, QC, Canada). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-calnexin polyclonal antibodies were from AKELA Pharma (Montreal, QC, Canada). Anti-apoB polyclonal antibodies were purchased from Chemicon International (Temecula, CA). Horseradish peroxidase-conjugated secondary antibodies were from Pierce Chemical (Rockford, IL). Prestained SDS-PAGE standards, low/high Range, and Bradford protein assay reagent were from Bio-Rad Laboratories Canada (Mississauga, ON, Canada). Bodipy FL C12 was purchased from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade or higher. [9,10(n)-3H]oleic acid (OA) (54.6 Ci/mmol) was from Amersham (Oakville, ON, Canada); [3H]acetate acid (0.1 Ci/mmol) and EasyTag™ EXPRESS35S Protein Labeling Mix, [35S]-, 14mCi (518MBq) were from Perkin Elmer (Woodbridge, ON, Canada). Inhibitors of DGAT1 (PF-04620110), and DGAT2 (Example 109B) were synthesized by Pfizer Inc. (Groton CT). Unless otherwise stated, DGAT1 and DGAT2 inhibitors were used at a concentration of 5 µM.

Preparation of Primary Mouse Hepatocytes
Primary mouse hepatocytes were isolated by collagenase perfusion of livers from wild type C57BL/6J mice and plated on 60 mm collagen-coated dishes at a confluence of 2.0 × 10^6 cells/dish, or on #1 coverslips (BD BioCoat™) in six-well plates at 2.0 × 10^5 cells/well. Hepatocytes were allowed to attach in DMEM supplemented with 15% FBS at 37°C in humidified air containing 5% CO_2 for 4 h, followed by incubations with experimental media.

Preparation of Primary Human Hepatocytes
Primary human hepatocytes were isolated and purified by collagenase-based perfusion of liver fragments (~20 g) obtained from resection specimens far away from the tumor margin. Human liver samples used for hepatocyte isolation were obtained from patients undergoing operations for therapeutic purposes at the Service of Digestive Tract Surgery, University of Alberta. Ethical approval was obtained from the University of Alberta's Faculty of Medicine Research Ethics
Board and all patients consented to participate in the study. Isolated primary hepatocytes were plated on 60 mm collagen-coated dishes (BD BioCoat™) at a density of 1.5×10^6 cells/dish and kept at 37°C in humidified air containing 5% CO₂. The cells were incubated in modified Roswell Park Memorial Institute (RPMI)-1640 culture medium (GIBCO) containing 15% FBS for 4 h before changing to experimental media.

**Metabolic Labeling**

Primary mouse or human hepatocytes were incubated for 4 h to attach to the collagen-coated dishes in DMEM or RPMI supplemented with 15% FBS, respectively. Then they were washed three times with serum-free medium, 5 min per wash, and incubated for 4 h in 2 mL of serum-free medium containing 5 μCi [3H]OA and 0.4 mM OA/0.5% BSA, or 10 μCi [3H]acetic acid and 50 μM acetate. Some media and cells (Pulse incubations) were harvested, lipids were extracted using chloroform-methanol (2:1, v/v) and resolved by thin-layer chromatography (TLC). Radioactivity in various lipid classes was determined by liquid scintillation counting. Remaining cells were washed three times with serum-free medium, 5 min per wash, then incubated with 2 ml of serum-free medium for another 4 h or overnight (Chase incubations), cells and media were collected and lipids analyzed as described above. DGAT inhibitors alone or in combination were introduced an hour before Pulse or Chase experiment as indicated in figure legends.

[14C] and [3H] Double Labeling of Lipids

Primary mouse hepatocytes were plated to attach to collagen-coated dishes for 4 h in DMEM supplemented with 15% FBS. Then cells were washed three times with serum-free DMEM, 5 min per wash, and incubated overnight (12-16 h) in 2 mL of DMEM containing 1 μCi [14C]OA and 0.4 mM OA/0.5% BSA ([14C] labeling). Cells were then washed three times with 0.5%BSA DMEM, 5 min per wash, and incubated for 4 h in 2 mL of DMEM containing 5 μCi [3H]OA and 0.4 mM OA/0.5% BSA ([3H] labeling). Hepatocytes were preincubated with DGATs inhibitors for one hour before incubation with [3H]OA for 4 h. Media were collected, and cells were washed with PBS twice before harvest. Intracellular and secreted lipids were extracted using chloroform-methanol (2:1, v/v) and resolved by TLC. Radioactivity in [3H]TG and [14C]TG was determined by liquid scintillation counting, and normalized to cellular protein mass.

**Visualization of Thin Layer Chromatography (TLC) Plates by Charring (TG Mass Semi-Quantification)**

TLC plates were thoroughly infiltrated with 3% cupric sulfate, followed by 8% phosphoric acid; then heated at 180°C for 10 min. Lipid spots were captured by G:BOX Chemi XX6 (Syngene, MD) with upper white light on, then quantified by GeneTools (Version 4.03.00). TG spots were normalized to total phospholipids (PL).
**Fatty Acid β-Oxidation Measurements**

Chase media obtained from the metabolic labeling studies were analyzed for the content of acid-soluble metabolites (ASM) resulting from FA β-oxidation. 30 µl of 20% BSA and 16 µl of 70% perchloric acid were added to 200 µl of culture medium from each sample. Samples were then centrifuged at 25,000g for 5 min before an aliquot of the supernatant was counted for radioactivity.

**Analysis of VLDL Density**

Hepatocytes were incubated for 4 h to attach to the collagen-coated dishes in DMEM supplemented with 15% FBS, then washed three times with DMEM, 5 min per wash, and incubated for 4 h in 2 ml of DMEM containing 0.4 mM OA/0.5% BSA, or 50 µM acetate. Next, cells were washed three times with DMEM, 5 min per wash, and incubated with DGATs inhibitors for 1 h, then washed with Methionine/Cysteine-free DMEM, 5 min per wash, and incubated for one hour with [³⁵S]-Protein Labeling Mix (200 µCi/ml) in the Methionine/Cysteine-free DMEM (2 ml/dish). Media were then removed, and cells were briefly washed with DMEM before incubation with DGATs inhibitors overnight (12-16 h). Finally media were collected for VLDL density analysis as described as follows. 1.8 ml medium of each sample was combined with 100 µl of plasma from an overnight-fasted mouse, 100 µl of Complete protease inhibitor cocktail (X20), and 0.97 g desiccated KBr. The mixture was gently placed into a 5.0 ml Quick-Seal tube, and overlaid with 3 ml of 0.9% NaCl. The samples were centrifuged at 416,000 x g for 1 h in a VTi 65.2 rotor. Ten fractions, 0.5 ml each, with densities ranging from 1.006 g/ml (fraction 10) to 1.21 g/ml (fraction 1), were collected from the bottom of the tube. ApoB was precipitated by Cab-O-Sil from each fraction and analyzed by electrophoresis on SDS 4% polyacrylamide gels. The gels were transferred to PVDF membranes, which were exposed to Kodak BioMax MR films (Carestream Health Canada Company, Vaughan, ON, Canada) for 48 h before developing.

**Detection of Cytosolic LDs by Confocal Fluorescence Scanning Microscopy**

Mouse hepatocytes were incubated with DMEM and 6 µM Bodipy FL C12 combined with 0.4 mM OA/0.5% BSA for 4 h. Some cells were fixed at this point with 4% paraformaldehyde (Pulse), while other cells were washed three times with serum-free DMEM, and incubated for additional 4 h in serum-free DMEM before fixation (Chase). Images were collected with a confocal laser scanning microscope (Leica TCS SP5, LAS AF Lite 3.2 software; Leica Microsystems Inc., Wetzlar, Germany ) equipped with a Leica PLAN APO 100X/1.44 oil M25 objective. The 488 nm laser line was used to image Bodipy FL C12, and the emission signals were collected with a band pass 500–530 nm filter-IR blocking. Images of representative cells were taken at middle planes. For quantification of LD numbers and sizes, Z stacks covering entire cells were projected, and analyzed with ImageJ 1.48V.
mRNA Expression Analysis
Total RNA was isolated by the Trizol reagent (Life Technologies, Carlsbad, CA). cDNA were synthesized with the Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time qPCR was performed with the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen™, Burlington, ON, Canada) in the Rotor-Gene 3000 instrument (Montreal Biotech, Quebec, Canada). Primers for tested genes were listed in Supplemental Table 1.

Isolation of Nuclear Extracts
Primary mouse hepatocytes, 1X10^7 cells for each treatment, were collected in 2 ml of ice-cold IM buffer (250 mM sucrose, 50 mM Tris, 1 mM EDTA, pH7.4), and homogenized using a Dounce Tissue Grinder. Nuclei were pelleted by centrifugation at 500 x g for 20 min at 4 °C, and sequentially washed with IM buffer and Hypertonic Buffer (10 mM HEPES, 420 mM NaCl, 1.5 mM MgCl2, 2.5% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, pH7.4). Nuclear pellets were resuspended in 100 µl of Hypertonic Buffer by vigorous vortexing, and incubated on a shaking rack on ice for 2 h. The mixtures were then centrifuged at 100,000 rpm at 4 °C for 1 h in a TLA-100 rotor. Supernatants (nuclear extracts) were collected for immunoblotting analysis.

Subcellular Fractionation
Mouse hepatocytes suspensions in ice-cold IM buffer (250 mM sucrose, 50 mM Tris, 1 mM EDTA, pH7.4) containing the Complete protease inhibitor cocktail (1 tablet/20 ml) were homogenized with a homogenizer bearing a 15.4 μm clearance ball (Isobiotec, Heidelberg, Germany). Cellular debris and nuclei were removed by centrifugation at 600 x g for 15 min, and the supernatants were centrifuged at 200,000 x g for 1 h. Supernatants (cytosol) were collected, and pellets (membranes) were resuspended in IM buffer containing protease inhibitors.

Immunoblotting
Hepatocytes were sonicated and an aliquot of each sample was resuspended in SDS-PAGE loading buffer before proteins were resolved and transferred to Immobilon-P transfer membrane (PVDF) membranes (Millipore Canada Ltd, Etobicoke, ON, Canada). Membranes were blocked with 5% skim milk in TBS containing 0.1% Tween 20 (T-TBS) and then incubated with primary antibodies followed by HRP-conjugated secondary antibodies at appropriate concentrations. Immunoreactivity was detected by G:BOX Chemi XX6 (Syngene, MD) using Supersignal West Dura or Pierce ECL Western Blotting Detection Reagents (Thermo Scientific™, IL). Quantitative analyses were performed with GeneTools (Version 4.03.00). ApoB-containing lipoproteins were pelleted from media by Cab-O-Sil and analyzed by immunoblotting as described above.

Statistical Analysis
Data are presented as the mean±SEM. Analysis was performed using GraphPad PRISM 4 software. Significant differences between groups were determined by
One-way ANOVA followed by Bonferroni posttests. P<0.05 was interpreted as significantly different.

References
Supplemental Figure Legends

Supp. Figure I: Influence of DGAT inhibition on intracellular TG mass under different treatments. Mouse hepatocytes were incubated in DMEM without any other additions (Blank), or with 0.4 mM oleate/0.5% BSA or with 50 μM acetate for 4 h, in the presence or absence of DGAT1 or DGAT2 inhibitors alone or in combination. TG levels were normalized to total PL. Data are presented as mean±SEM. **P<0.01, ***P<0.001 versus control (DMSO) based on three independent biological replicates. Abbreviations: TG, triacylglycerol; PL, phospholipid.

Supp. Figure II: Inhibition of DGAT1 or DGAT2 does not affect their mRNA expression. Mouse hepatocytes were incubated in DMEM containing 50 μM acetate for 4 h, in the presence or absence of DGAT1 or DGAT2 inhibitors alone or in combination. A) The abundance of Dgat1 and Dgat2 mRNA was determined by real-time qPCR. Data are presented as the ratio of Dgat1 or Dgat2 expression to cyclophilin (Cyc) expression. Values are normalized to DMSO (control) where the gene/Cyc ratio was set as 1. N.S. indicates no significant difference from the control based on three independent biological replicates. B) The C_T values are shown as C_T Mean ± C_T SD, 3 biological replicates in each experimental condition.

Supp. Figure III: Influence of DGAT inhibition on intracellular TG synthesis at different doses. Mouse hepatocytes were preincubated with DGAT1 and 2 inhibitors at 0 µM, 1 µM, 5 µM and 10 µM, alone or in combination, and then added A) C) E) 10 µCi [^3]H]acetic acid and 50 μM acetate or B) D) F) 5 μCi[^3]H]oleic acid combined with 0.4 mM oleate/0.5% BSA, incubated for 4 h. Data are presented as mean±SEM. **P<0.01, ***P<0.001 versus control (DMSO) based on three independent biological replicates.

Supp. Figure IV: Validation of complete removal of DGAT inhibitors before analysis undertaken in Figure 5. A) Schematic diagram of chronological orders of drug treatments and[^3]H] labeling. B) Incorporation of[^3]H]oleic acid or[^3]H]acetic acid into intracellular TG, normalized to total cellular protein. Data are presented as mean±SEM. P values versus control (DMSO), determined by unpaired two-tailed t test, were indicated on top of the black bars.

Supp. Figure V: Inhibition of either DGAT1 or DGAT2 alone does not affect intracellular TG distribution. (A) Percentage of TG synthesized from[^3]H]oleic acid in cytosol. (B) Purity of subcellular fractions. Immunoblotting with anti-mouse calnexin (microsomal marker) and GAPDH (cytosolic marker) polyclonal antibodies were performed in total homogenate, cytosol and membrane fractions.
Supp. Figure VI: Effect of DGAT inhibition on TG secretion is not due to differential MTP expression. Mouse hepatocytes were treated in the same way as illustrated in Figure 6A. MTP expression levels were detected by immunoblot analysis in cells incubated with A) 50 µM acetate or B) 0.4 mM oleate/0.5% BSA for 4 h in DMEM (Pulse), followed by an overnight (12-16 h) Chase. C) Extracellular ApoB levels in cells pulsed in the absence of DGAT inhibitors and chased overnight in the presence or absence of DGATs inhibitors alone or in combination. Ponceau S Red staining served as protein loading control.

Supp. Figure VII: DGAT2 catalyzes re-esterification of lipolytic products from preformed TG destined for VLDL secretion. A) [\textsuperscript{14}C]OA labeled TG secretion. B) [\textsuperscript{3}H]OA labeled TG secretion. Secreted [\textsuperscript{14}C]TG was derived from preformed TG pools, whereas [\textsuperscript{3}H]TG was derived from newly synthesized TG.
Supplemental Figure II

A

B

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Supplemental Figure III

A. DGAT1inh

B. DGAT1inh

C. DGAT2inh

D. DGAT2inh

E. DGAT1/2inhs

F. DGAT1/2inhs
Supplemental Figure IV

A

DGAT inhibitors

\[ \text{Incubation (Acetate or Oleate in DMEM)} \]

\[ \text{Wash (DMEM)} \]

\[ \text{Incubation (Acetate+[H]acetate) OR (Oleate/BSA+[H]oleate)} \]

4 h 15 min 4 h

Pulse

B

![Graph showing dpm/mg cell protein for Oleate and Acetate with DMSO and DGAT1/2inhs treatments, with P-values of 0.31 and 0.30 respectively.]

- DMSO
- DGAT1/2inhs

Oleate

Acetate
Supplemental Figure V

A

Incorporation of \(^{1}H\)-oleic acid into TG in cytosol/cytosol and microsome\%.

DMSO
DGAT1inh
DGAT2inh

B

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GAPDH
Calnexin
Supplemental Figure VII

A

$^{14}$C-TG

DMSO

DGAT1inh

DGAT2inh

DGAT1/2inh

B

$^{3}$H-TG

dpnm/mg cell protein

**

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*