Evidence for Triacylglycerol Synthesis in the Lumen of Microsomes via a Lipolysis-Esterification Pathway Involving Carnitine Acyltransferases*

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In this study a pathway for the synthesis of triacylglycerol (TAG) within the lumen of the endoplasmic reticulum has been identified, using microsomes that had been preconditioned by depleting their endogenous substrates and then fusing them with biotinylated phosphatidylserine liposomes containing CoASH and Mg²⁺. Incubating these fused microsomes with tri[³H]oleoylglycerol and [¹⁴C]oleoyl-CoA yielded microsomal associated triacylglycerol, which resisted extensive washing and had a [³H]:[¹⁴C] ratio close to 2:1. The data suggest that the precursor tri[³H]oleoylglycerol was hydrolyzed by microsomal lipase to membrane-bound di[¹⁴C]oleoylglycerol and subsequently re-esterified with luminal [¹⁴C]oleoyl-CoA. The accumulation of TAG within the microsomes, even when overt dicylglycerol acyltransferase (DGAT I) was inactive, is consistent with the existence of a latent dicylglycerol acyltransferase (DGAT II) within the microsomal lumen. Moreover, because luminal synthesis of TAG was carnitine-dependent and markedly reduced by glybenclamide, a potent carnitine acyltransferase inhibitor, microsomal carnitine acyltransferase appears to be essential for trafficking the [¹⁴C]oleoyl-CoA into the microsomal lumen for subsequent incorporation into newly synthesized TAG. This study thus provides the first direct demonstration of an enzymatic process leading to the synthesis of luminal triacylglycerol, which is a major component of very low density lipoproteins.

Overt and latent forms of carnitine acyltransferase (CAT I and CAT II, respectively) have been shown to exist in mitochondria, peroxisomes, and microsomes (1), suggesting a parallel occurrence of the two isozymic forms of this enzyme in these organelles. Although these two enzymes are known to facilitate the uptake of acyl-CoAs (via interconversion with acylcarnitine) into mitochondria and peroxisomes and CAT II, respectively, microsomes were first incubated with carboxylase (1, 12) but with the inclusion of 50 mM EDTA to remove the unreacted reagents.

The microsomes were resuspended and incubated with tri[³H]oleoylglycerol (tri[³H]TAG) to generate membrane-bound di[¹⁴C]oleoylglycerol (di[¹⁴C]DAG) through the action of microsomal lipase, followed by a further centrifugation of the microsomes through dibutyl phthalate, an oil immiscible with water, to remove the unreacted reagents.

III. Because CoASH is depleted from microsomes during their isolation (10) and cannot be taken up by simple diffusion (8, 11), the CoASH was replenished by fusing them with biotinylated phosphatidylserine liposomes encapsulating CoASH and Mg²⁺.

IV. Microsomes were then incubated with [¹⁴C]oleoyl-CoA in the presence and absence of carnitine to prove that the pathway of internal, but not external, TAG synthesis from the membrane-bound DAG is carnitine-dependent. Both pools of newly synthesized TAG were subsequently quantified. TAG was extracted and purified by thin-layer chromatography before determining the relative amounts of [³H] and [¹⁴C] incorporated into the TAG.

Isolation of Microsomes—Rough microsomes were isolated from the livers of 10-week old male Wistar rats using a previously described method (1, 12) but with the inclusion of 50 mM EDTA to remove ribosomes (13). Removal of ribosomes exposes the highly positively charged N-terminal membrane-anchoring domain (pl = 11) (14), thus

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§§The abbreviations used are: CAT I and II, carnitine acyltransferase I (overt) and II (latent); VLDL, very low density lipoproteins; DAG, diacylglycerol; TAG, triacylglycerol; DGAT I and II, dicylglycerol acyltransferase I (overt) and II (latent); BSA, bovine serum albumin; FS, phosphatidylserine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); MES, 2-(N-morpholino)-ethanesulfonic acid.
regions corresponding to TAG and DAG were identified by comparison of di[3H]DAG, thus preventing further hydrolysis of di[3H]DAG by DAG lipase. The assay was carried out in a final volume of 1 ml containing ~150 μg of microsomal protein, 175 mM morpholinoethanosulfonic acid (pH 6.0), 400 μg of BSA, and 50 μg of a 1:1 (w/w) mixture of phosphatidylcholine and phosphatidylserine, previously sonicated for 2 min in 10 mM Tris-HCl (pH 7.0) containing 10 mM MnCl₂. The reaction was started by the addition of 50 μg tri[3H]oleoylglycerol in 25 μl of acetone (final concentration in the assay mixture was 2.5%). Note that the assay components employed above have previously been shown to have no effect on the latency of glucose-6-phosphatase (17). After 40 min of incubation at 37 °C, 3 ml of cold homogenization buffer was added to rapidly cool the reaction mixture, which was then put on ice. The ice-cold mixture was layered over 3 ml of dibutyl phthalate oil (top) and 1 ml of 50 mM triethanolamine, pH 7.3, containing 0.5 mM succinate and 1 mM dithiothreitol (bottom) in a 10-ml polycarbonate tube and centrifuged at 115,000 × g at 4 °C for 60 min. The aqueous solution above the oil layer was carefully aspirated with a Pasteur pipette, and the wall of the tube was gently washed with water and wiped with a tissue before removing the oil and the buffer underneath. For the determination of microsomal-bound di[3H]DAG, 35578 mCi/mmol) was added to the microsomal pellet followed by vigorous mixing. After mixing, 1 ml of heptane and 0.5 ml of water were added. The reaction was then vortexed vigorously and centrifuged (700 × g for 10 min). After phase separation, an aliquot (400 μl) was taken from the upper heptane phase (total volume approximately 1.3 ml) and evaporated to dryness under nitrogen. The dried material was solubilized in ≈50 μl of diethyl ether and applied to a thin-layer chromatography plate (250 μm silica gel LK6DF), which was developed using hexanediethyl ether:acetic acid (80:20:1) as the solvent. The regions corresponding to TAG and DAG were identified by comparison with known standards and scraped into scintillation vials to which 3 ml of Omnifluor scintillation fluid was added and the radioactivity measured in a Beckman LS 3802 liquid scintillation β-counter (Beckman Instruments). Greater than 98% of TAG and 94% of DAG was extracted and recovered as judged by the application of standard radiolabeled TAG and DAG. The yield of di[3H]DAG synthesized is described under Results and Discussion.

Preparation of Liposomes and Their Subsequent Fusion with Microsomes—Biotinylated phosphatidylserine (PS) was prepared from phosphatidylserine according to a previously described method (18). The generation of biotinylated PS liposomes (encapsulating a buffer containing 0.32 mM succrose, 2 mM HEPES (pH 7.0), 2 mM dithiothreitol, 2 mM NaCl, with and without 16 mM CaCl₂ and 2 mM MgCl₂) and their subsequent fusion with microsomes were carried out as described previously (19, 20). Fused microsomes, which as a consequence of fusion had biotinylated PS on their surface, were separated from unfused microsomes by allowing them to bind to streptavidin-coated Dynabeads, a step which exploits the remarkably high affinity of streptavidin for biotin (Kₐ = 10⁻¹⁵ M) (21). The fused microsomes bound to the Dynabeads were then isolated using a Magnetic Particle Concentrator. Dynabeads were applied to only one-tenth of the mixture of fused and unfused microsomes after the removal of unfused liposomes, which would otherwise have interfered with the fused microsomes binding to the Dynabeads. Unfused liposomes were removed by layering an aliquot of the microsome/liposome mixture (5 ml) over 3 ml of dibutyl phthalate (top) and 1 ml of 50 mM triethanolamine, pH 7.3, containing 0.5 mM succinate and 1 mM dithiothreitol (bottom) in a 10-ml polycarbonate tube and centrifuged at 115,000 × g at 4 °C for 60 min. The supernatant was then used for the determination of microsomal-bound di[3H]DAG precursor was facilitated by providing the optimum conditions for CAT I and DGAT. Because DGAT (22, 23) and CAT I (24) are both active at neutral pH when phosphate is present, potassium phosphate was included as described previously (25). Additions were made to the microsomal-liposomal suspension to give final concentrations of 220 mM sucrose, 40 mM KCl, 1 mM EGTA, 4 mM MgCl₂, 4 mM ATP, 100 mM potassium phosphate, and 40 mM Tris-HCl (pH 7.4), with or without 0.5 mM carnitine. The reaction was initiated by the addition of [14C]oleoyl-CoA (5 mM) and 0.8, 1, 2, 5, 25, and 50 μM, and included fatty acid-free bovine serum albumin (BSA) at an oleoyl-CoA/BSA molar ratio of 9:1. The role of BSA as a binder and delivery system for acyl-CoA was partially substituted for by the presence of liposomes in the system (26, 27). The mixtures were incubated at 37 °C for 40 min before being rapidly cooled on ice. The microsomes were then washed by centrifugation through dibutyl phthalate oil (top), which maintained the biotinylated PS carboxylate group in a negatively charged state (57). The buffer was 20 mM MES, pH 5.0, containing 0.32 mM succinate. The glucose-6-phosphatase activity was determined in fused microsomes that bound to the indicated volume of a 10 mg/ml suspension of streptavidin-coated Dynabeads (A) and in the mixture of fused and unfused (total) microsomes (B). The extent of fusion was calculated from the enzyme activity (nmol P i/min) that bound to a saturating volume of Dynabeads, expressed as a percentage of the total activity.

RESULTS AND DISCUSSION

Fusion Experiments—In this study, microsomes were fused with liposomes encapsulating various components to investigate the proposed involvement of latent activities of DGAT and CAT in the luminal synthesis of TAG. Fused microsomes were magnetically separated from the unfused microsomes by being bound to 115,000 × g for 60 min to Dynabeads. The extent of fusion was 54.2 ± 5.0, as determined by expressing the glucose-6-phosphatase activity in microsomes bound to a saturating amount (300 μl) of Dynabeads (Fig. 1A) as a percentage of the total activity of the enzyme in the original mixture of fused and unfused microsomes (Fig. 1B). This extent of fusion was higher than the 39% recorded by Pistolesi et al. (19) using PS lipo-
omes, confirming our assumption that biotinylation of the PS would enhance subsequent fusion by increasing the electro-negativity of the liposomes (18) and thus their attraction for the positively charged microsomes (33). Production of Microsomal-bound Di[^3H]Oleoyl Glycerol by Microsomal Lipase—When microsomes were incubated with 50 μM tri[^3H]TAG and washed free of unreacted tri[^3H]TAG by centrifuging the microsomes through dibutyl phthalate, 14.6 ± 2.9 nmol of di[^3H]DAG/mg of microsomal protein was shown to be bound to the microsomal membrane. Microsomal lipase has been shown to hydrolyze TAG to DAG and 2-monoacylglycerols (34). However, under our assay conditions the concentration of TAG was vastly in excess of newly formed DAG and would thus have prevented further breakdown of DAG to 2-monoacylglycerol by preferentially binding to the active site of the lipase (35). Distinction between TAGProduced External and Internal to the Microsomal Membrane—When microsomes containing membrane-bound di[^3H]DAG and loaded with CoASH were further incubated with [14C]oleoyl-CoA in the absence of carnitine, 98% of the resultant radiolabeled TAG could be removed by centrifuging the microsomes through dibutyl phthalate. Only 0.08 ± 0.01 nmol of TAG/mg of microsomal protein remained associated with the microsomes, implying that the TAG synthesized in the absence of carnitine must have been predominantly external to the microsomes. In contrast, if microsomes were incubated with [14C]oleoyl-CoA in the presence of carnitine, the amount of TAG remaining associated with the washed microsomes was 11.58 ± 2.36 nmol of TAG/mg of fused microsomal protein, corresponding to a ~140-fold increase. Because dibutyl phthalate was very effective in removing the externally synthesized TAG, it is concluded that the majority of TAG generated in the presence of carnitine, and remaining associated with the microsomes after passage through dibutyl phthalate, must have been synthesized inside the microsomes. Because only trace amounts (~2%) of externally synthesized TAG remain associated with dibutyl phthalate-washed microsomes, the TAG synthesized by fused microsomes in the presence of carnitine must have been predominantly free in the lumen and/or bound to the inner surface of the microsomal membrane.

The [H]14-C molar ratios in the external and internal pools of TAG were 2.02 ± 0.03 and 2.13 ± 0.04, respectively, confirming that, for both pools of TAG, the tri[^3H]TAG was initially hydrolyzed to di[^3H]DAG, which is membrane-diffusible (36), and that this DAG was subsequently re-esterified with the introduced [14C]oleoyl-CoA. It has been previously shown that DGAT has a stereospecific preference for sn-1,2-DAG over sn-2,3-DAG or sn-1,3-DAG (23, 37–39), suggesting that the acyla-tion of the DAG is likely to have occurred in the sn-3 position. This demonstration of the existence of a lipolysis/esterification pathway is consistent with the observation of Yang et al. (40) that the fatty acid composition of the TAG of liver and of VLDLs were homologous in the sn-1 and sn-2 positions but distinctly different in the sn-3 position. The fact that the [H]14-C molar ratio in the TAG produced within the microsomal lumen was slightly higher than the expected ratio of 2.0 suggests that there may have been some endogenous acyl-CoA within the lumen, which would have competed with the [14C]oleoyl-CoA during the re-esterification of DAG. Furthermore, if the newly synthesized TAG had been derived from monoacylglycerol then the [H]14-C ratio would be expected to be 1:2. Clearly this was not the case.

Dependence of DGAT I and DGAT II Activities on Acyl-CoA—The extent of external and internal synthesis of TAG by the carnitine-independent and carnitine-dependent pathways, respectively, at varying concentrations of oleoyl-CoA and at a fixed amount of membrane-bound DAG, is compared in Fig. 2. When these data were analyzed using double reciprocal plots, the amounts of extraluminal and internal TAG that were synthesized at a saturating concentration of [14C]oleoyl-CoA, were calculated to be 5.6 ± 0.9 and 10.9 ± 2.9 nmol of TAG/mg of microsomal protein, respectively. That is, the extent of synthesis of internal TAG (the carnitine-dependent pathway) was 1.95-fold higher than that of external TAG (the carnitine-independent pathway) shown in this study. The total TAG synthesized at this saturating concentration of oleoyl-CoA was 16.5 ± 3.8 nmol of TAG/mg of microsomal protein, which is not significantly different (p > 0.1) from the original amount of DAG bound to the microsomal membrane (see above). This study used microsomes with an essentially outside-out orientation, i.e. having a similar sidedness to intact endoplasmic reticulum, as judged by the latency of glucose-6-phosphatase (data not shown). Thus, TAG synthesized within the lumen and in the extraluminal space correspond to the distinct microsomal and cytoplasmic pools of TAG, respectively.

The luminal synthesis of TAG within a mixture of fused and unfused microsomes (6.21 ± 0.96 nmol/mg of total microsomal protein) was shown to be predominantly associated with those microsomes that had fused with biotinylated PS liposomes, previously loaded with 16 mM CoASH and 2 mM Mg2+ (Fig. 3). This is evident from the fact that only the fused microsomes, i.e. those bound to streptavidin-coated Dynabeads, had appreciable amounts of internal radiolabeled TAG (11.58 ± 2.36 nmol/mg of fused microsomal protein). Unfused microsomes, i.e. those not bound to the streptavidin-coated Dynabeads, contained negligible amounts of internal radiolabeled TAG. There was also negligible TAG produced within the lumen of microsomes that had been fused with either empty liposomes (data not shown) or with liposomes containing Mg2+ but no CoASH (Fig. 4). Therefore, it is concluded that it was not simply the act of fusion but rather was the consequence of CoASH being delivered to the microsomal lumen, thereby facilitating intraluminal acyl-CoA synthesis, that promoted the production of luminal TAG.

DGAT II Activity When DGAT I Is Specifically Inactivated—The conclusion proposed earlier that the TAG generated in the presence of carnitine, which survived washing through dibutyl
The amount of newly synthesized luminal TAG was determined for total (fused + unfused) microsomes, fused microsomes, and unfused microsomes, i.e. those not bound to Dynabeads. The data represent the means ± S.E. of three separate experiments.

FIG. 3. Internal synthesis of TAG in fused and unfused microsomes. The amount of newly synthesized luminal TAG was determined under different experimental conditions. In a typical complete assay, DAG-bound microsomes were loaded with CoASH by fusing them with liposomes encapsulating 16 mM CoASH. These microsomes were incubated with 50 μM [14C]oleoyl-CoA and 0.5 mM carnitine. The extent of incorporation of the [14C]oleoyl moiety into luminal TAG was quantitated in the absence (+) or presence (−) of CoASH in the microsomal lumen (via liposomes) and carnitine in the incubation media. When both CoASH and carnitine were absent, [14C] oleoyl-CoA was directly loaded into microsomes by fusing them with liposomes encapsulating 50 μM of the labeled acyl-CoA. The data represent the means ± S.E. of triplicate experiments.

FIG. 4. Incorporation of the acyl moiety of exogenously added [14C]oleoyl-CoA into newly formed luminal TAG. The amount of exogenous [14C]oleoyl-CoA incorporated into newly synthesized luminal TAG was determined under different experimental conditions. In a typical complete assay, DAG-bound microsomes were loaded with CoASH by fusing them with liposomes encapsulating 16 mM CoASH. These microsomes were incubated with 50 μM [14C]oleoyl-CoA and 0.5 mM carnitine. The extent of incorporation of the [14C]oleoyl moiety into luminal TAG was quantitated in the absence (+) or presence (−) of CoASH in the microsomal lumen (via liposomes) and carnitine in the incubation media. When both CoASH and carnitine were absent, [14C] oleoyl-CoA was directly loaded into microsomes by fusing them with liposomes encapsulating 50 μM of the labeled acyl-CoA. The data represent the means ± S.E. of triplicate experiments.

Delivery of Acyl Moieties across the Microsomal Membrane to DGAT II—Distinct overt and latent forms of microsomal CAT have been independently isolated and purified in two different laboratories (1, 46). This suggests that carnitine-mediated transfer of acyl moieties across the microsomal membrane might occur. However, whereas mitochondria and peroxisomes have been shown to possess a carnitine-acylcarnitine translocase system (8, 47) such a system has not been detected in microsomes (47). Nevertheless, acylcarnitine (but not acyl-CoA) transfer across the endoplasmic reticulum membrane does occur, perhaps by a gated channel mechanism (47). In such a system, acyl-CoA (which is impermeable to microsomal membranes (45, 47)) is converted by CAT I into its corresponding acylcarnitine. This crosses the membrane via a gated channel and is re-esterified to acyl-CoA by a CAT II-catalyzed reaction. The experiment represented in Fig. 4 supports the existence of such a system. When all of the assay components were present, the amount of the [14C]acyl moiety incorporated into the newly synthesized luminal TAG by DGAT II was 11.58 ± 2.36 nmol/mg of fused microsomal protein. This amount was ~140-fold higher than that produced when carnitine was omitted from the medium and ~236-fold higher than that produced when microsomes were devoid of CoASH (Fig. 4). In the former case, acylcarnitine would not have formed and in the latter case, luminal acylcarnitine would not have been reconverted to acyl-CoA. To prove that the availability of acyl-CoA within the microsomal lumen is critical for the DGAT II reaction, the system was bypassed by directly loading acyl-CoA into the microsomes by fusing them with liposomes encapsulating 50 μM [14C]oleoyl-CoA. In these microsomes, despite being devoid of CoASH and deprived of exogenously added carnitine, appreciable amounts of the [14C]acyl moiety (8.84 ± 1.30 nmol/mg of fused microsomal protein) were incorporated into newly synthesized luminal TAG (Fig. 4). When microsomes were fused with liposomes containing 50 μM [14C] oleoyl-CoA and 8 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), there was a high level of inhibition (71.0 ± 8.3%) of luminal synthesis of TAG (data not shown). Because DTNB is unable to penetrate the membrane (48), this suggests that one or more thiol groups are at or sufficiently near to the active center of DGAT II such that the enzyme is inhibited when they are reacted with DTNB. DTNB has been previously shown to strongly inhibit acyl-CoA:cholesterol acyltransferase (49), which is genetically and structurally related to DGAT (50), but to have no effect on microsomal lipase activity (51).

Effect of Glybenclamide on the Production of Luminal TAG—When the sulfonylurea drug glybenclamide (220 μM), a potent inhibitor of CAT I (4), was included during the incubation of microsomes with tri[3H]TAG but removed by washing the mi-
In conclusion, a proposed pathway, by which intraluminal microsomal TAG is derived from cytoplasmic TAG and acyl-CoA, is suggested to be as follows. First, microsomal TAG lipase generates membrane-bound DAG from TAG. Second, CAT I and CAT II work sequentially to generate acyl-CoA inside the microsomes. Third, DGAT II utilizes this intraluminal acyl-CoA to synthesize TAG from the membrane-bound DAG, which is membrane-diffusible and thus exists on both sides of the membrane. The pathway is depicted in Fig. 6, which shows the radiolabeled precursors and products used experimentally to confirm this pathway.

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