Differential Effects of Fenofibrate or Simvastatin Treatment of Rats on Hepatic Microsomal Overt and Latent Diacylglycerol Acyltransferase Activities

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Hepatic triacylglycerol secretion is elevated in insulin-resistant states. Microsomal diacylglycerol acyltransferase (DGAT) catalyzes the final reaction in the synthesis of triacylglycerol (TAG). We have previously described two DGAT activities in rat liver microsomes, one overt (cytosol-facing) and one latent (endoplasmic reticulum lumen-facing) (Owen MR, Corstorphine CG, Zammit VA: Overt and latent activities of diacylglycerol acyltransferase in rat liver microsomes: possible roles in very-low-density lipoprotein triacylglycerol secretion. Biochem J 323:17–21, 1977). It was suggested that they are involved in the synthesis of TAG for the cytosolic droplet and VLDL lipidation, respectively. In the present study, we measured the overt and latent DGAT activities in rats fed diets containing one of two hypolipidemic drugs: fenofibrate (a peroxisome proliferator–activated receptor α [PPARα] agonist) and simvastatin (a 3-hydroxy-3-methylglutaryl [HMG]-CoA reductase inhibitor). We found that the activities of the two DGATs could be varied independently by these treatments. Fenofibrate raised overt DGAT activity but lowered that of latent DGAT. In contrast, simvastatin markedly lowered overt DGAT activity without affecting that of latent DGAT. The increase in overt DGAT activity induced by fenofibrate could not be mimicked by feeding a diet enriched in n-3 polyunsaturated fatty acids (PUFA), which lowered overt DGAT activity but did not affect latent DGAT, suggesting that n-3 PUFA act through a mechanism independent of PPARα activation. The fibrate-induced increase in overt DGAT activity and the inhibition of latent DGAT may provide a mechanism through which acyl moieties are retained within the liver for oxidation through the pathways concomitantly upregulated by PPARα activation. Diabetes 51:1708–1713, 2002

In insulin-resistant states in general, and type 2 diabetes in particular, the plasma concentration of VLDL-derived triacylglycerol (TAG) is increased, partly due to the increased secretion of VLDL by the liver. Such hypertriglyceridemia is associated with increased incidence of coronary artery disease and stroke and has been suggested to contribute directly to the induction of the insulin-resistant state in the preobese phase (1). Two types of hypolipidemic drugs are widely used, commonly in combination, to treat hyperlipemia, namely, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors (statins) and peroxisome proliferator–activated receptor α (PPARα) agonists (fibrates). Statins inhibit the synthesis of cholesterol and the secretion of apolipoprotein B (apoB) by the liver (2). Fibrates are ligands for the nuclear receptor PPARα, and in rodent liver, they increase the expression of enzymes involved in peroxisomal and mitochondrial fatty acid oxidation (e.g., peroxisomal acyl-CoA oxidase, malonyl-CoA-sensitive carnitine palmitoyltransferase I, and mitochondrial HMG-CoA synthase) (3). In addition, they upregulate Δ5- and Δ6-desaturases (4) such that, through the formation of polyunsaturated fatty acids (PUFA), they may indirectly inhibit the expression of enzymes involved in the lipogenic and TAG-secretion pathways. However, PUFA are also able to regulate these genes independently of PPARα activation (5).

The rate of TAG secretion by the liver depends on both number and TAG content of the VLDL particles secreted. Therefore, the regulation of the synthesis and partitioning of TAG within the hepatocyte between retention in cytosolic droplets and secretion (6,7) is of primary importance in determining the rate of hepatic VLDL-TAG production and the degree of hypertriglyceridemia achieved in insulin-resistant conditions. Diacylglycerol acyltransferases (DGATs) are important enzymes in the control of the rate of TAG synthesis, as their activity commits diacylglycerol (DG) to the synthesis of TAG. We have shown previously that DGAT activity is expressed on both aspects of the endoplasmic reticular (ER) membrane (8). A putative role for latent DGAT was suggested to be the synthesis of TAG on the luminal aspect of the ER membrane (9,10) from DAG formed on the cytosolic aspect, after diffusion of the latter across the ER membrane (11). Formation of DAG on the cytosolic aspect of the ER occurs through the operation of the phosphatidate pathway and/or through the cycle of hydrolysis and re-esterification of cytosolic drop-
let TAG (12). For TAG synthesis to occur on the luminal aspect of the ER, it is necessary for acyl moieties also to be transferred across the membrane, as acylcarnitine esters (13). This is made possible by the expression of overt and latent carnitine long-chain acyltransferases in the ER membrane (14,15). The ability of such transfer to result in the incorporation of cytosolic acyl moieties into ER luminal TAG has been demonstrated experimentally in cell-free reconstructed systems in vitro (16,17). A role for latent DGAT in the synthesis of a secretion-dedicated pool of TAG on the luminal aspect of the ER would explain why cytosolic TAG is not incorporated en bloc into VLDL-TAG but requires a degree of prior hydrolysis to partial glycerides and acyl-CoA. It may also account for the difference of the acyl chain composition between intrahepatic (cytosolic) and secreted TAG (18). Since our initial description of separate overt and latent microsomal DGAT activities, the cDNAs coding for the expression of two unrelated proteins exhibiting DGAT activity have been cloned (19,20).

We tested whether overt and latent DGAT activities are affected differently by drugs that lower VLDL-TAG secretion through different mechanisms of action. We measured overt and latent DGAT activities in the microsomes of livers of rats that were fed either the PPAR agonist fenofoibrate or the HMG-CoA reductase inhibitor simvastatin. The data show that overt and latent DGAT activities can be affected independently and, in the case of fenofibrate, in opposite directions, supporting the hypothesis that the two activities have distinct functions with respect to VLDL-TAG secretion.

**RESEARCH DESIGN AND METHODS**

**Materials.** Radiolabeled 1-[14C]palmitoyl-CoA (50 μCi/μmol) was obtained from Amersham Life Sciences. [9,10-3H(N)]triolein (1 μCi) was obtained from Dupont NEN Research Products (Hounslow, U.K.). Mannose-6-phosphate, fenofibrate, alamethacin, phosphatidylglycerol, and the Inhibitor of Active Lipid Transfer (IALT) were from Sigma (Poole, U.K.). Silica-gel 60 thin-layer chromatography (TLC) plates were purchased from Merck. Rosiglitazone and pioglitazone were from Aventis (Strasbourg, France). Ethanol, in which alamethacin was dissolved, acted as substrate for measurement of AEAT activity. The final concentration of ethanol (15 μmol/l) in the assay mixture was the same for all experiments. 

**Preparation of liver microsomes and DGAT assays.** Microsomes from rat liver were prepared as described previously (8,16). Rat livers were homogenized in ice-cold medium containing 300 mmol/l sucrose, 1 mmol/l EGTA, and 5 mmol/l Tris·HCl, pH 7.4. Microsomal membranes were then prepared by differential centrifugation (8). The final microsomal pellet was suspended into aliquots and stored at −70°C until used. Permeabilization of microsomes with alamethacin was carried out as previously described, immediately before the assay of overt and total DGAT activities in intact and permeabilized microsomes, respectively (8). DGAT and acyl-CoA:ethanol acyltransferase (AEAT) activities were measured simultaneously in the same assay tubes, as previously described (8). The liposomal substrate mixture for assay of DGAT activities was prepared as described in Owen et al. (8). Briefly, 3.33 mmol/l dipalmitoylglycerol and 2.67 mmol/l phosphatidylglycerol were added to assay medium containing 300 mmol/l sucrose, 10 mmol/l Tris·HCl (pH 7.4), 1 mmol/l EGTA, 10 mmol/l MgCl2, and 1 mmol/l MgCl2/0.05). As a result, the ratio between the two activities was increased more than fourfold by fenofibrate treatment, from 0.68 to 2.8. These opposing effects on the two activities were accompanied by a 50% increase in the TAG content of the liver (Table 1).
Effects of simvastatin feeding on hepatic overt and latent DGAT activities. In contrast to fenofibrate, the feeding of simvastatin for 4 days resulted in a 66% decrease in overt DGAT activity (P < 0.01) (Fig. 2). In addition, simvastatin feeding had no effect on the expression of latent DGAT activity (Fig. 2), again in contrast to the effect of fenofibrate feeding. Simvastatin also tended to decrease the TAG content of the liver, although this did not reach statistical significance (Table 1).

Feeding of n-3 PUFA–rich diet does not mimic the effects of fenofibrate on DGAT activities. We wanted to test whether the effects of fenofibrate on hepatic overt and latent DGAT activities could be mimicked by feeding a diet containing fish oil, which is enriched in n-3 PUFA. The data are given in Fig. 3. In contrast to fenofibrate, the fish-oil diet lowered the activity of overt DGAT. Moreover, feeding of the fish-oil diet had no effect on latent DGAT activity, again in contrast to fenofibrate feeding.

The feeding of a corn-oil diet (enriched in n-6 PUFA) had no effect on either overt or latent DGAT activities (not shown).

DISCUSSION
This study shows that overt and latent DGAT activities of the rat hepatic microsomal membranes can be altered independently by pharmacological or dietary treatments in vivo. Of particular interest is the observation that the PPARα agonist fenofibrate affected the two activities in opposite directions, doubling overt DGAT activity and halving that of latent DGAT. In view of the well-established lowering of the hepatic VLDL-TAG secretion rate by fibrates, this observation strengthens the evidence for the suggestion (8,25) that, in the liver, overt DGAT is involved primarily in the utilization of DAG for the synthesis of hepatocyte cytosolic droplet TAG, whereas the role of latent DGAT is to synthesize TAG within the secretory droplets.

FIG. 1. Effect of fenofibrate feeding in rats on the overt and latent DGAT activities of liver microsomes. Two groups of 5 rats each were fed either a powdered diet (control, □) or the same diet supplemented with 0.5% wt/wt fenofibrate (■) for 10 days. Liver microsomes were prepared, and overt (A) and latent (B) DGAT activities were measured as described in RESEARCH DESIGN AND METHODS. Values (means ± SE) are expressed as nanomoles of palmitoyl-CoA incorporated into TAG per minute per milligram of microsomal protein, at 37°C. Asterisks indicate values that are significantly different (P < 0.05) from activities observed in microsomes isolated from control animals.

FIG. 2. Simvastatin feeding downregulates overt DGAT activity without affecting that of latent DGAT. Overt (A) and latent (B) activities of DGAT were measured in liver microsomes isolated from rats fed a control powdered diet (□) or the same diet supplemented with 0.05% simvastatin (■) for 4 days. Activities are expressed as nanomoles of palmitoyl-CoA incorporated into TAG per minute per milligram of microsomal protein, at 37°C. Values are means (± SE) for five rats in each group. The asterisk denotes a statistically significant effect (P < 0.001) of simvastatin on overt DGAT activity, compared with that of microsomes isolated from control rats.

TABLE 1
Triacylglycerol content of livers from rats maintained under different treatment regimens

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>TAG (μmol/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>Fenofibrate</td>
<td>5</td>
<td>8.4 ± 1.2*</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>5</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>Fish oil</td>
<td>5</td>
<td>7.0 ± 0.5</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05.
Microsomes were prepared from their livers, and overt (A) at 37°C CoA converted into TAG/minute per milligram of microsomal protein, five rats in each group and are expressed as nanomoles of palmitoyl-CoA converted into TAG/minute per milligram of microsomal protein, at 37°C. *P < 0.06.

**FIG. 3.** A diet enriched in n-3 PUFA does not mimic the effects of fenofibrate on rat liver microsomal overt or latent DGAT activities. Rats were fed a fish-oil diet (30% menhaden oil by weight, □) or powdered control diet (see RESEARCH DESIGN AND METHODS ) for 10 days. Microsomes were prepared from their livers, and overt (A) and latent (B) activities of DGAT were measured. Values are means (± SE) for five rats in each group and are expressed as nanomoles of palmitoyl-CoA converted into TAG/minute per milligram of microsomal protein.

compartment of the ER, destined for VLDL lipiddation and secretion. In this respect, it is noteworthy that there was a 50% increase in hepatic content of TAG after fenofibrate feeding in spite of the expected PPARα-mediated induction of mitochondrial and peroxisomal fatty acid oxidation in these livers (26,27). This suggests that the fourfold increase in the overt/latent DGAT activity ratio induced by fenofibrate is sufficient to ensure efficient retention of acyl groups within the liver. The concomitantly decreased rate of utilization of DAG and acyl-CoA by latent DGAT for secreted TAG synthesis would contribute toward the inhibition of VLDL-TAG secretion induced by the fibrate (Fig. 4).

Therefore, latent DGAT responds to PPARα activation in a manner similar to that of apoA-I and apoC-III (28) and as would be expected from the hypotriglyceridemic effect of fibrates. However, our data indicate that when the upregulation of fatty acid oxidizing enzymes occurs in parallel with the downregulation of enzymes involved in de novo synthesis of fatty acid—i.e., as occurs after n-3 PUFA feeding (4,29)—the activity of overt DGAT too is downregulated, whereas that of latent DGAT was unaffected (Fig. 4). This indicates that the hypolipidemic effect of n-3 PUFA occurs primarily through their downregulation of the enzymes of de novo fatty acid synthesis and upregulation of those involved in fatty acid oxidation. Under these conditions, the lowered availability of DAG is presumably sufficient to lower the rate of VLDL-TAG secretion, without the requirement for concomitant downregulation of latent DGAT (Fig. 4). Although fatty acids synthesized de novo make a relatively small quantitative contribution toward the overall amount of secreted TAG in both rat and human, there is a strict correlation between the two parameters (30–32), indicating that participation of a distinct pool of fatty acids synthesized de novo within the hepatocyte is essential for TAG synthesis and VLDL-TAG secretion. Indeed, the disruption of the stearoyl-CoA desaturase 1 gene in mice results in the inability of the livers of these animals to either synthesize or secrete TAG, a defect that cannot be rescued by the provision of dietary oleate (33).

**Mechanisms of action of cholesterol depletion and PPARα activation.** A possible mechanism of action of PPARα in the induction of a lipogenic enzyme such as overt DGAT has emerged from the observations of Roglans et al. (34), who showed that gemfibrozil treatment results in the induction of nuclear sterol regulatory element-binding protein (SREBP)-2, owing to the depletion of hepatocyte cholesterol that accompanies the fibrate-induced increase in bile flow. Those authors also reported the increased expression, after gemfibrozil treatment, of phosphatidate phosphatase, which catalyzes the synthesis of DAG, indicating that DAG (34) and TAG (this study) synthesis on the cytosolic (but not luminal) aspect of the ER membrane is coordinately induced by PPARα activation in the liver.

The possibility has been raised that PPARα activation could act indirectly through its upregulation of Δ5- and Δ6-desaturases and the ability of their PUFA products to downregulate lipogenic enzymes (4). In our experiments, however, n-3 PUFA feeding did not mimic the effects of PPARα activation by fenofibrate on either of the two DGAT activities, suggesting that no such indirect action of PPARα activation was involved. That PUFA can act independently of PPARα on hepatic gene transcription is well established (35,36). The depression of overt DGAT activity by dietary n-3 PUFA, in contrast to the action of fenofibrate, provides another example of this phenomenon. A similar effect of n-3 PUFA on (overt) DGAT activity of rat liver microsomes has been observed previously (37), although latent DGAT activity was not measured in those experiments.

Statins inhibit hepatic apolipoprotein B (apoB) secretion (2), presumably through their inhibition of HMG-CoA reductase activity and their lowering of the availability of de novo synthesized cholesterol and cholesteryl esters. Both these lipid components are essential for the assembly and secretion of VLDL particles (38,39). In the present experiments, simvastatin treatment markedly inhibited the expression of overt DGAT activity, suggesting that the expression of the protein responsible for this activity is under the control of cholesterol/oxysterol molecular sensors (SCAP [40], liver X receptor α [LXRα] [41,42]) within hepatocytes.

Several lines of evidence suggest that the changes in overt DGAT activity observed in the present study may be mediated through SREBP-1. Thus, the depletion of hepatocyte cholesterol in vivo, which lowers overt DGAT (Fig.
2), is known to decrease the nuclear content of SREBP-1c concomitantly with the marked increase in mature SREBP-2 expression (43) that accompanies the lowering of LXRα activity (42). Similarly, the feeding of a n-3 PUFA–rich diet, which lowers the activity of overt DGAT (Fig. 3), also lowers nuclear SREBP-1c content through transcriptional and posttranscriptional effects (44,45), the former being mediated by inhibition of LXR binding to LXR response elements (46). It is of interest, therefore, that LXRα-null mice have a lower hepatic cytosolic TAG content than wild-type animals (41), suggesting that cytosolic TAG synthesis is impaired in these animals, as would be predicted from our data.

The cDNA of two proteins that express DGAT activities have been cloned and sequenced from mouse and other mammalian species; they have been designated as DGATs 1 and 2, in the order in which they were described (19,20). To avoid confusion of terminology, we have refrained from using the DGAT I and II nomenclature we used previously (8,9) when describing the overt and latent DGAT activities, respectively, in rat liver ER membranes. It has not been possible, as yet, to ascertain whether either of the cloned cDNAs codes for the activities expressed as overt or latent DGATs, but the existence of separate DGAT genes that code for proteins that are totally unrelated (19,20) agrees with the existence of two DGATs that are targeted to different aspects of the same membrane and respond differently to pharmacological and dietary treatments.

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REFERENCES
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