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 acytransferase 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

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Received for publication 23 October 2001 and accepted in revised form 27 February 2002.

AEAT, acyl-CoA:ethanol acyltransferase; apoB, apolipoprotein B; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticular; HMG, 3-hydroxy-3-methylglutaryl; LXR, liver X receptor; PPARα, peroxisome proliferator–activated receptor α; PUFA, polyunsaturated fatty acids; SREBP, sterol regulatory element-binding protein; TAG, triacylglycerol; TLC, thin-layer chromatography.

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 (DAG) 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 lumenal 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 lumenal 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 memebrane (14,15). The ability of such transfer to result in the incorporation of cytosolic acyl moieties into ER lumenal TAG has been demonstrated experimentally in cell-free reconstituted systems in vitro (16,17). A role for latent DGAT in the synthesis of a secretion-dedicated pool of TAG on the lumenal 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 PPARα agonist fenofibrate 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-[^{14}C]palmitoyl-CoA (50 μCi/μmol) was obtained from Amersham Life Sciences. [9,10-^{3}H(N)]triolein (1 μCi) was obtained from Dupont NEN Research Products (Hounslow, U.K.). Mannose-6-phosphate, fenofibrate, alamethacin, phosphatidylglycerol, and the Infinity Reagent triacylglycerol detection kit were purchased from Sigma (Poole, U.K.). Palmitoyl-CoA was from Lipid Products (Surrey, U.K.). Silica-gel 60 thin-layer chromatography (TLC) plates were purchased from Merck. Rosiglitazone and simvastatin were gifts from SmithKline Beecham and Merck, respectively.

Animal studies. Female Wistar rats (180–230 g) were maintained in a controlled light (12 h light/12 h dark) environment and in accordance with local animal welfare regulations. Before the start of feeding experiments, all animals were maintained on a standard pelleted laboratory diet (RMP1; Special Diets Services, Edinburgh, U.K.) (6). Three days before experimental feeding commenced, each batch of 10 animals was divided randomly into two groups and given the same diet that had been ground to a fine powder, for a period of familiarization with powdered diet intake. Preliminary experiments indicated that absolute DGAT activities vary within the year. Therefore, a different set of five control animals was paired with each group of five animals on the experimental diets to eliminate any possible seasonal effects. At the start of the experimental feeding period, each treatment group of animals was switched to powdered diet to which was added either 0.5% (wt/wt) fenofibrate (21) or 0.05% simvastatin (22). The fish-oil and corn-oil diets were based on the isocaloric high-fat diets described in Pegorier et al. (23), containing 30% by weight of menhadden oil or corn oil, respectively. The control animals were fed powdered diet throughout; all diets were fed ad libitum. None of the experimental diets resulted in any change in the daily food or water intake compared with controls. All diets were fed for 10 days, except that containing simvastatin, which was fed for 4 days.

Preparation of liver microsomes and DGAT assays. Animals were killed by cervical dislocation, and freshly excised 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 as described previously (8). The final microsomal membrane suspension was divided 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 acitivities 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 $MgCl_2$, and 1 mg/ml defatted BSA. The mixture was warmed to 65°C and sonicated using a 2.5-mm microprobe sonicator (Kontes, Burkard Scientific, Uxbridge, U.K.) operated at 20 μm and 80% of maximal power, for 20 periods of 15 s, over 40 min. The mixture was then diluted with assay buffer to give final lipid concentrations of 1 and 0.8 mmol/l, respectively, and palmitoyl-CoA containing 22,000 dpm of 1-[^{14}C]palmitoyl-CoA was added to bring the final concentration to 100 μmol/l. The final mixture was then sonicated for 15 s at 37°C before aliquoting into assay tubes. Ethanol, in which alamethacin was delivered, acted as substrate for measurement of AEAT activity. The final concentration of ethanol (15 mmol/l) in the assay mixture was the same for intact and permeabilized microsomal samples. All assays were initiated by the addition of 40 μg microsomal protein (in 50 μl) and terminated, after 1 min, by the addition of 0.75 ml chloroform/methanol (2:1, vol/vol) containing 20 μg/ml trioleoylglycerol as carrier and 2,000 dpm [9,10-^{3}H(N)]triolein as internal standard. Chloroform-extractable material was separated by TLC on silica gel 60 analytical plates using hexane/diethyl ether (4:1, vol/vol) as developing solvent. The separated lipid classes were then visualized using iodine vapor, and the areas associated with TAG and cholesteroyl ester were scraped into separate scintillation vials. After addition of 10 ml Optifuor scintillant (Packard), the associated ^{3}H and ^{14}C radioactivities were quantified. Measurement of AEAT activity in the same assay as that of DGAT enabled us to determine, simultaneously, the degree of intactness of the individual microsomal membrane preparations. AEAT is known to be exclusively latent in distribution (24). Consequently, AEAT activity detected in microsomes not previously exposed to alamethacin treatment was assumed to be due to leakiness of the microsome vesicles to palmitoyl-CoA, owing to membrane damage, inside-out sealing, or both. Therefore, we used the AEAT activity measurements to correct the observed overt and total DGAT activities for lack of vesicle integrity. The formulae used were as follows:

$$\text{overt DGAT} = D_0 - [(D_t - D_0)A_0/A_t]$$

$$\text{latent DGAT} = (D_t - D_0)A_t/(A_t - A_0)$$

where D_0, A_0, D_t, and A_t represent the DGAT and AEAT activities measured before and after alamethacin treatment.

Whereas the proportion of rightside-out and/or intact microsomes of preparations from rats in any individual dietary groups was highly reproducible, it differed for membranes obtained from livers of animals in the different experimental groups, suggesting that different dietary and pharmacological treatments of animals altered membrane lipid composition. The most intact microsomal vesicles (AEAT 94% latent) were obtained from fenofibrate-fed rats, whereas the most fragile (AEAT 85% latent) were those obtained from simvastatin-fed animals, in which membrane cholesterol content would be expected to be diminished. Membrane vesicles from control animals were intermediate (AEAT 90% latent). The absolute activities of AEAT were also affected by the different diets, but as regulation of the expression of this enzyme was not the subject of this work, the effects were not pursued further.

Lipid extraction of liver samples was performed using chloroform/methanol (2:1, vol/vol) as solvent, and TAG content was determined using the Infinity Reagent kit (Sigma) as described in the manufacturer's manual. Protein determination was made using BioRad Reagent (BioRad) BSA as standard.

RESULTS

Effects of fenofibrate feeding on hepatic overt and latent DGAT activities. The effects of fenofibrate feeding of rats on overt and latent activities of DGAT are shown in Fig. 1. Fenofibrate feeding for 10 days raised overt DGAT activity by 90% from 1.30 ± 0.22 to 2.44 ± 0.51 nmol · min^{-1} · mg^{-1} microsomal protein ($P < 0.05$). By contrast, latent DGAT activity was decreased by 55% from 1.9 ± 0.37 to 0.86 ± 0.26 nmol · min^{-1} · mg^{-1} microsomal protein ($P < 0.05$). As a result, the ratio between the two activities (overt/latent) 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).

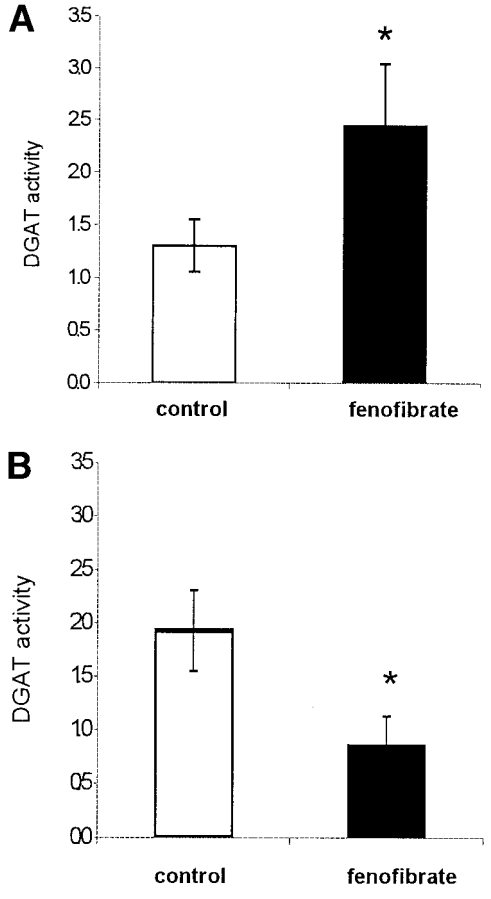

FIG. 1. Effect of fenofibrate feeding in rats on the overt and latent DGAT activities of liver microsomes. Two groups of 5 rats each were fed either a powdered diet (control, □) or the same diet supplemented with 0.5% wt/wt fenofibrate (■) for 10 days. Liver microsomes were prepared, and overt (*A*) 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.

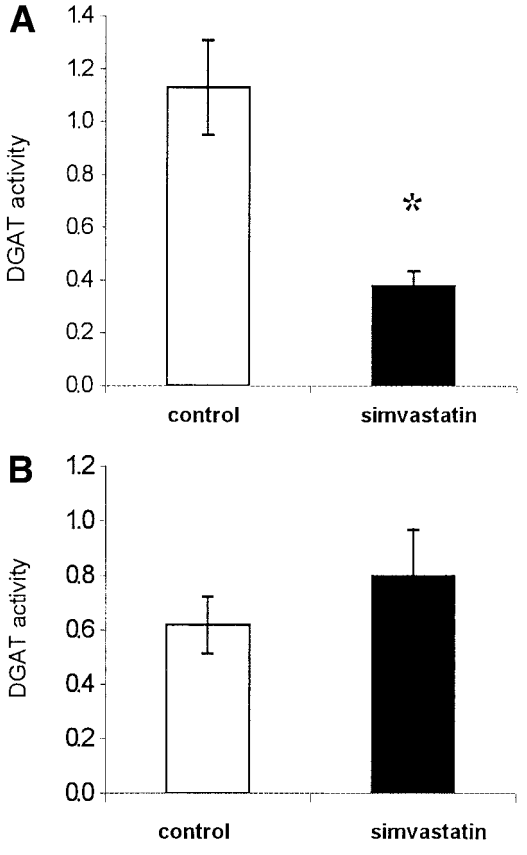

FIG. 2. Simvastatin feeding downregulates overt DGAT activity without affecting that of latent DGAT. Overt (*A*) and latent (*B*) activities of DGAT were measured in liver microsomes isolated from rats fed a control powdered diet (□) or the same diet supplemented with 0.05% simvastatin (■) for 4 days. 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.

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).

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

Treatment	*n*	TAG (μmol/g liver)
Control	20	5.7 ± 0.7
Fenofibrate	5	8.4 ± 1.2*
Simvastatin	5	4.5 ± 0.6
Fish oil	5	7.0 ± 0.5

Data are means ± SE. *$P < 0.05$.

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

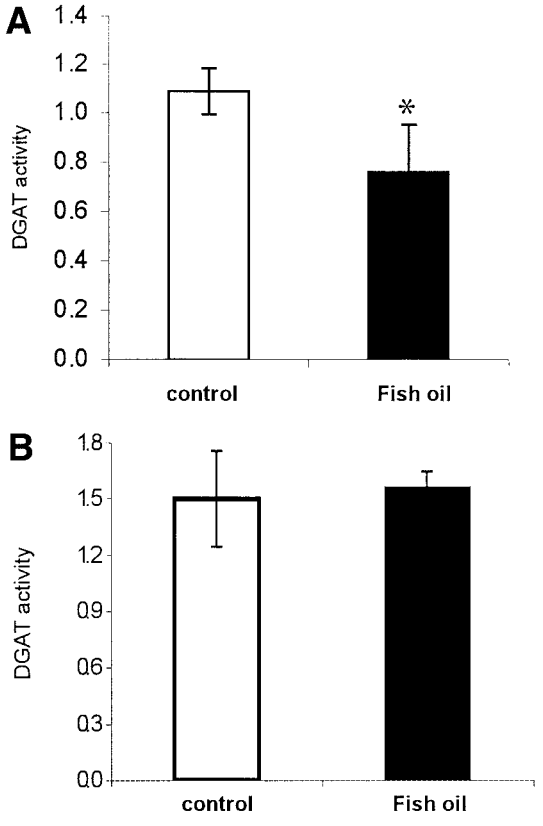

FIG. 3. A diet enriched in *n-3* PUFA does not mimic the effects of fenofibrate on rat liver microsomal overt or latent DGAT activities. Rats were fed a fish-oil diet (30% menhadden oil by weight, ■) or powdered control diet (see RESEARCH DESIGN AND METHODS) for 10 days. Microsomes were prepared from 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, at 37°C. **P* < 0.06.

compartment of the ER, destined for VLDL lipidation 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 is 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 liver 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 lumenal) 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.

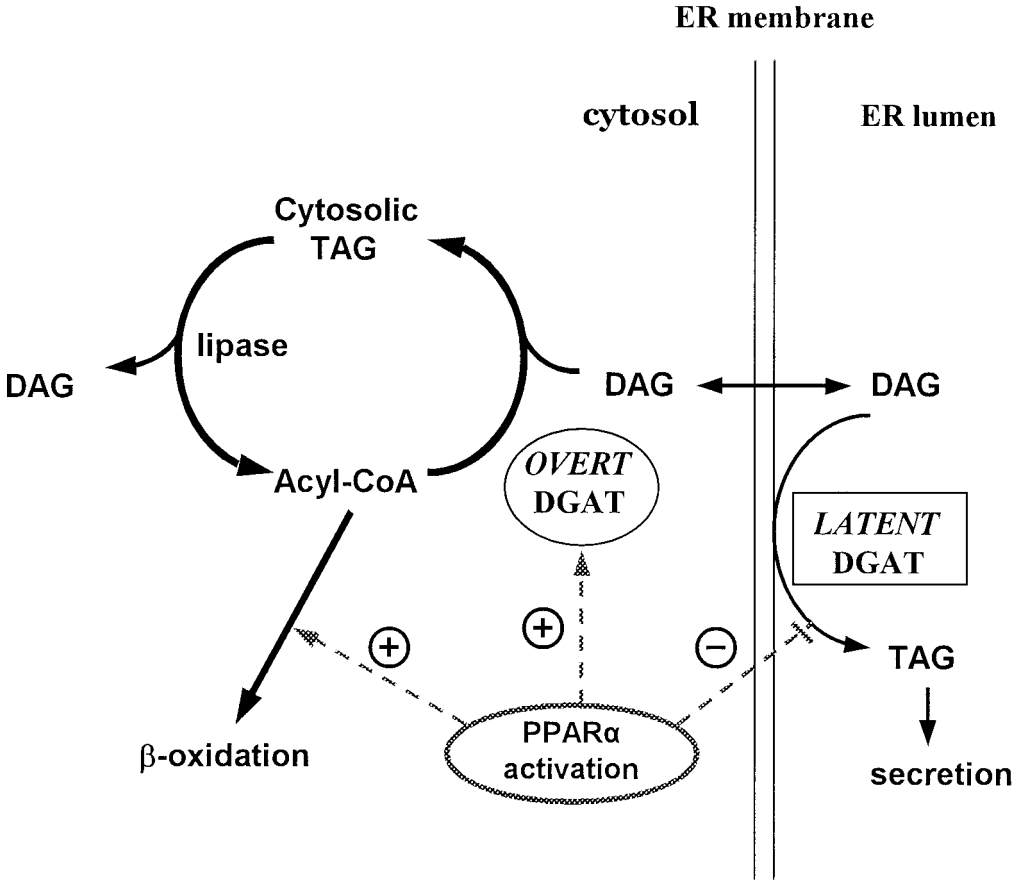

FIG. 4. Coordinate effects on enzymes of fatty acid oxidation and on overt and latent microsomal DGAT activities in fibrate-mediated inhibition of hepatic VLDL-TAG secretion. Activation of PPARα by fibrates activates hepatic enzymes involved in long-chain fatty acid oxidation and microsomal overt DGAT, but downregulates that of latent DGAT (Fig. 1). These coordinated changes are suggested to result in the increased retention of long-chain fatty acids within the liver for intrahepatic oxidation within mitochondria and peroxisomes and to contribute toward the inhibition of hepatic VLDL-TAG secretion mediated by fibrates.

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.

ACKNOWLEDGMENTS

This work was funded by the British Heart Foundation and the Scottish Executive (SEERAD).

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