Pathogenesis of Carbohydrate-Induced Hypertriglyceridemia Using HepG2 Cells as a Model System

Katherine Cianflone, Smadar Dahan, Juan Carlos Monge, and Allan D. Sniderman

This study compares the effects of glucose and fatty acid on hepatic lipid synthesis and apolipoprotein (apo) B secretion. To do so, varying concentrations of either glucose or oleic acid were added to the medium in which HepG2 cells were being incubated. Intracellular triacylglycerol and cholesteryl ester synthesis and secretion were measured by addition of radioisotopic tracers and by determination of mass, whereas apo B concentration in the medium was measured by a specific enzyme-linked immunosorbent assay. The data indicate that increasing concentrations of glucose in the medium resulted in increased synthesis of triacylglycerol within the cell and increased secretion of triacylglycerol into the medium. Apo B secretion into the medium, however, did not change, and intracellular synthesis and secretion of cholesteryl ester did not change as well. By contrast, addition of oleic acid to the medium resulted in increased synthesis and secretion of both cholesteryl ester and triacylglycerol, and this was associated with increased secretion of apo B into the medium. Thus, a carbohydrate load resulted in secretion of normal numbers of triacylglycerol-enriched apo B particles by this hepatocyte cell line, whereas a fatty acid load led to the secretion of increased numbers of apo B particles, which were essentially normal in composition. (Arteriosclerosis and Thrombosis 1992;12:271–277)

KEY WORDS • hypertriglyceridemia • apolipoprotein B • HepG2 cells

Hypertriglyceridemia can result from disorders of clearance or from disorders of overproduction.1,2 It is obviously, therefore, not a unitary entity. Two different clearance defects have been recognized: the first involves the rate of triacylglycerol hydrolysis (lipoprotein lipase and apolipoprotein [apo] CH deficiency fall in this category), whereas the second involves impaired removal of chylomicron and very low density lipoprotein (VLDL) remnants (with type III hyperlipoproteinemia being the prototype of this class of disorders).3 Two different genetic forms of hepatic overproduction of VLDL have also been recognized: familial hypertriglyceridemia and familial combined hyperlipidemia. The former is characterized by the secretion of normal numbers of triacylglycerol-enriched VLDL particles, whereas the latter is characterized by secretion of increased numbers of VLDL particles of normal composition.4–6

However, little is yet known of the processes that regulate VLDL secretion. Almost all available in vitro evidence supports the view that apo B production rates are modulated by posttranslational mechanisms,7,8 and we have advanced the hypothesis that cholesteryl ester synthesis is critical in this regard, at least so far as the response to increased delivery of fatty acids to the liver is concerned.9 Nevertheless, it has been well documented that carbohydrate feeding also can lead to increased plasma triacylglycerols,10,11 presumably through increased delivery of glucose to the liver. Accordingly, the present studies were undertaken to compare the response of HepG2 cells on the one hand to an exogenous glucose load and, on the other, to an exogenous fatty acid load. Our expectation was that the latter would result in secretion of increased numbers of apo B particles, whereas the former would result in an increased content of triacylglycerol per apo B particle. Our hypothesis was that these differences would relate to differences in cholesteryl ester synthesis induced by the two different metabolic loads.

Methods

Tissue Culture

HepG2 cells obtained from the American Tissue Culture Collection (Rockville, Md.) were routinely grown in minimum essential medium supplemented with 10% fetal calf serum and 100 IU penicillin/streptomycin in 75-cm² flasks with 15 ml medium in a 37°C incubator with 5% CO₂. Flasks were subcultured every 7 days, with a split ratio of 1:3. The cells were dislodged from the culture flask with 0.25% trypsin in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) for 5 minutes at 37°C. For experiments, cells were plated out at a density of 1.3×10⁴ cells/cm² in 17-mm dishes (24-well plates) or 35-mm dishes. For the density gradient experiments, cells were used in T75 flasks.

Experimental Conditions

[2-3H]glycerol (final specific activity, 200 mCi/mmol); [1-¹⁴C]acetic acid, sodium salt (final specific activity,
Lipid Quantification

The remaining radioactive free oleate. The sample was dissolved in 1 ml 0.1N NaOH and collected. The phospholipids were taken as the nonmigTating lipids (cholesteryl ester, triacylglycerol, and phospholip-ids). The phospholipids were extracted with 2 ml heptane/isopropanol (3:2, vol/vol). After 30 minutes, the extracts were removed and the cells were washed once with an additional 1 ml heptane/isopropanol (3:2, vol/vol) and added to the previous extract. The soluble cell protein was dissolved in 1 ml 0.1N NaOH and collected.

Lipid Quantification

Cell lipid extracts were evaporated under nitrogen and redissolved in a known volume of chloroform/methanol (2:1, vol/vol). An aliquot was applied to a thin-layer chromatography plate prewashed in chloroform/methanol (2:1, vol/vol). The lipids were separated by running in hexane/ether/acetic acid (75:2:5; vol/vol/vol); the lipid spots were visualized by exposure to iodine vapor and identified by comparison to reference lipid standards. The phospholipids were taken as the nonmigrating polar lipids in this solvent system and include phosphatidylycholine and sphingomyelin. The silica gel was scraped into vials containing scintillation cocktail, and the radioactivity was counted in a scintillation counter (Beckman Instruments, Palo Alto, Calif.). For mass assays, the triacylglycerol band was scraped from the plates, eluted with isopropanol, and measured by the method of Neri and Frings.13 Cholesteryl ester mass was quantified by gas–liquid chromatography on a Hewlett-Packard Model 5830A gas chromatograph as described by Huff et al.14

Aliquots of the medium were also extracted with 5 volumes of heptane/isopropanol (1:1, vol/vol), and the organic phase was washed twice with 1 ml isopropanol/heptane (4:1, vol/vol) and 1 ml 0.05% KOH to remove the remaining radioactive free oleate. The sample was then processed in the same way as the cell extracts to measure medium triacylglycerol and cholesteryl ester.

For density gradient experiments, cells in T75 flasks were incubated as described previously, and the media from two to three flasks were pooled. The medium was concentrated to less than 10-fold by dialysis against 50% polyethylene glycol in 10,000 molecular-weight-cutoff dialysis tubing. A 1-ml sample was then adjusted to a density of 1.10 g/ml and layered under a discontinuous gradient of 1 ml of d=1.07 g/ml, 1 ml of d=1.05 g/ml, 1 ml of d=1.02 g/ml, and 1 ml of d=1.0063 g/ml using potassium bromide salt solutions (total volume, 5 ml). The sample was centrifuged for 24 hours at 100,000g in a Beckman L8-80 ultracentrifuge to form a linear gradient. The bottom of the tube was then pierced, and 0.5-ml aliquots were collected. Each fraction, including the starting fractions, was analyzed for apo B by enzyme-linked immunosorbent assay, for [3H]glycerol incorporation into triacylglycerol, and for [14C]acetate incorporation into cholesteryl ester. The density of the solution was measured by refractive index and compared against a set of calibrated potassium bromide standard solutions.

Protein and Apolipoprotein Quantification

Cell protein was measured by the method of Bradford,13 using BSA as a standard. Apo B and apo A-I concentrations in the medium were measured by sandwich enzyme-linked immunosorbassay16 as modified by Ortho Diagnostics (La Jolla, Calif.), using a standard curve of 0.025–0.400 µg/ml apo B. Each point in each experiment is the average of triplicate determinations and is expressed per milligram cell protein±SEM. Significance was measured by paired Student’s t test between the test value and the control value. An individual control was run for each experiment.

Results

The effects of increasing concentration of oleate in the medium on intracellular lipid synthesis and secretion of apo B and apo A-I into the medium are shown in Figure 1. Consistent with our previous results, triacylglycerol and cholesteryl ester synthesis increased in parallel. Apo B secretion into the medium also increased substantially, while apo A-I release was unaltered. These results contrast with those obtained by increasing the medium concentration of glucose (Figure 2). In this instance, intracellular triacylglycerol synthesis increased to the same extent as in the oleate experiments (373±103% glucose versus 344±33% oleate nmol/mg cell protein), but neither cholesteryl ester synthesis nor phospholipid synthesis changed significantly. Once again, apo A-I secretion was unaffected, but this time apo B secretion was unaffected as well. Cells exposed to 50 mM glucose or 1 mM oleate manifested cytoplasmic lipid droplets as determined by light microscopy.

The changes in cellular lipids as determined by radioactive tracer were confirmed by mass analysis of the triacylglycerol and cholesteryl ester as shown in Table 1. There was a three to fourfold increase in cellular triacylglycerol mass on incubation with either oleate or glucose for 24 hours. In contrast, there was no
change in cellular cholesteryl ester mass with glucose, but there was an increase with the oleate incubation.

Not only did the number of secreted apo B particles differ in the two circumstances, but also their composition differed as well. These results are shown in Figure 3. With the oleate challenge, there was little difference in either the cholesteryl ester to apo B or the triacylglycerol to apo B ratio over the range of oleate concentrations examined (Figure 3, left panel). On the other hand, carbohydrate challenge changed the composition of the secreted apo B particle but not the number of particles, in that there was a pronounced increase in the
TABLE 1. Determination of HepG2 Cellular Lipid Mass: Triacylglycerol and Cholesteryl Ester

<table>
<thead>
<tr>
<th>Addition</th>
<th>Triacylglycerol (nmol/mg cell protein)</th>
<th>Cholesteryl ester (nmol/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% BSA</td>
<td>246±42</td>
<td>4.2±0.4</td>
</tr>
<tr>
<td>1% BSA+50 mM glucose</td>
<td>791±191</td>
<td>4.1±0.4</td>
</tr>
<tr>
<td>p</td>
<td>0.05</td>
<td>NS</td>
</tr>
<tr>
<td>1% BSA+0.8 mM oleate</td>
<td>1,170±355</td>
<td>6.1±0.4</td>
</tr>
<tr>
<td>p</td>
<td>0.005</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

Cells were incubated in 35-mm dishes as described in "Methods" in the indicated media for 24 hours. Cellular mass was determined and expressed as nanomoles per milligram cell protein±SD (n=8). p values were calculated by t test for two means against basal (1% bovine serum albumin [BSA]). NS, not significant.

Discussion

The mechanism of carbohydrate-induced hypertriacylglyceridemia has long been of interest, and we believe the present data allow this phenomenon to be viewed from yet another perspective, a perspective that provides insights into the mechanisms that regulate secretion of hepatic apo B particles. In these experiments, triacylglycerol synthesis has been augmented either by increasing delivery of fatty acids to the liver or by increasing de novo fatty acid synthesis within the liver. Although triacylglycerol synthesis increased in both cases, the consequences differed. With a fatty acid challenge, the number of apo B particles secreted increased, although their composition did not change. With a carbohydrate challenge, the number of apo B particles secreted did not change, but their composition did. In the first instance, the extra triacylglycerol synthesized was removed from the cells in more particles, so the triacylglycerol to apo B ratio but no change in the cholesteryl ester to apo B ratio over the range of glucose concentrations tested (Figure 3, right panel). When both glucose and oleate were added to the medium, more apo B particles were secreted and they were triglyceride enriched (results not shown).

The effect of addition of glucose or oleate on the distribution of the secreted apo B particles within a density gradient was also examined. In essence, the increased triacylglycerol to apo B ratio induced by glucose was associated with a shift in the secreted apo B particles toward the more buoyant densities. This can be seen in the density gradient profile of the medium lipoproteins shown in Figure 4. The upper panel shows the distribution of secreted lipoproteins in basal medium (1% BSA) as measured by apo B mass and [3H]glycerol incorporation into triacylglycerol. After oleate incubation, there was no change in size distribution (middle panel). However, in the presence of glucose, the density shifted toward a less dense fraction (lower panel). The amount of apo B associated with the peaks was 74%, 81%, and 79% of the total. Similarly, the amount of triacylglycerol associated with the peaks was 77%, 82%, and 73%.

The importance of the duration of the incubation and the concentration of glucose in the medium is illustrated in Figure 5. At the lowest concentration of glucose (5 mM), there was little increase in triacylglycerol in the medium even after an 8-day incubation, whereas at 25 mM and even more markedly at 50 mM, triacylglycerol secreted into the medium rose progressively and markedly over the 8-day time course (Figure 5, upper panel). This contrasts with the results for cholesteryl ester secreted into the medium, for which there was little change (Figure 5, middle panel). Of importance, there was no difference in apo B concentration in the medium for any glucose concentration at any time point (Figure 5, lower panel).

**Figure 3.** Line plots showing effect of oleate and glucose on secreted triacylglycerol (TG) and cholesteryl ester (CE) to apolipoprotein (apo) B (B) ratio. Cells were incubated for 24 hours as described in the legends to Figures 1 and 2. Triacylglycerol (○) and cholesteryl ester (●) secreted into the medium are expressed per secreted apo B (nanomoles lipid per microgram apo B)±SEM. *p<0.0125, **p<0.025.
Figure 4. Line plots showing effect of oleate and glucose on the size distribution of secreted lipoprotein particles. HepG2 cells in T75 flasks were incubated with basal 1% bovine serum albumin (BSA) medium (upper panel), 1% BSA+0.8 mM oleate (middle panel), or 1% BSA+50 mM glucose (lower panel) for 24 hours. The percentage distributions of apolipoprotein B (○) and triacylglycerol (●) are shown in each panel. The medium was then concentrated and separated on a continuous density gradient as described in "Methods."

Figure 5. Line plots showing long-term effect of glucose on lipoprotein lipid and apolipoprotein (apo) B secretion in HepG2 cells. Cells were incubated as described in "Methods" for 24 hours–8 days in 1% bovine serum albumin medium supplemented with 5.5 (○), 25 (●), or 50 (□) mM glucose. Results are expressed as nanomoles triacylglycerol (TG) per milligram soluble cell protein (upper panel), nanomoles cholesteryl ester (CE) per milligram cell protein (middle panel), or micrograms apo B per milliliter medium per milligram cell protein (lower panel)±SEM. *p<0.01, **p<0.0025.
while in the second, the same end was achieved by packaging more triacylglycerol per particle, resulting in a more buoyant particle.

We have previously proposed that the critical event regulating the apo B response to increased delivery of fatty acids to the liver is the rate of cholesteryl ester synthesis in the rough endoplasmic reticulum. Our model posits that the newly synthesized cholesteryl ester molecule causes a newly formed apo B molecule to enter the phospholipid bilayer of the rough endoplasmic reticulum and then to move along to the junction with the smooth endoplasmic reticulum where triglyceride is added to the apo B–cholesteryl ester complex, following which the lipid–apo B complex is extruded into the lumen of the endoplasmic reticulum. The fact that cholesteryl ester synthesis is much less in toto than triacylglycerol synthesis does not argue against the model, in that only a small number of cholesteryl ester molecules synthesized in the rough endoplasmic reticulum may be necessary to achieve a critical effect.

The present results are in accord with this model. With an exogenous fatty acid load, cholesteryl ester synthesis increases in parallel with triacylglycerol synthesis and apo B secretion increases as well. By contrast, with an endogenous fatty acid push, that is, fatty acids generated by de novo synthesis, cholesteryl ester synthesis does not increase and apo B secretion does not change either. Therefore, in situations where triacylglycerol and cholesteryl ester synthesis can be dissociated, apo B secretion follows cholesteryl ester synthesis, not triacylglycerol synthesis.

All these studies, however, have been conducted in HepG2 cells. Notwithstanding that they retain many of the biologic capacities of normal hepatocytes, HepG2 cells are a transformed cell line and do not primarily secrete VLDL. Nevertheless, there is a close correspondence between the present results and much that has been done before. Ahrens and colleagues were the first to demonstrate that increased dietary intake of carbohydrate resulted in increased plasma triacylglycerol levels and that this phenomenon occurs in normals as well as in hypertriglyceridemic patients. Since then, in both rats and humans, several studies have shown that carbohydrate loads result in the secretion of triacylglycerol-enriched VLDL particles without a change in the number of such particles secreted.

The present results may also provide insight into the molecular defects responsible for familial combined hyperlipidemia and familial hypertriglyceridemia. The former is characterized by the secretion of increased numbers of VLDL particles. Were fatty acid flux to the liver to increase for any reason, our data demonstrate that the consequences would be an increased rate of intracellular triacylglycerol synthesis and an increased secretion rate of VLDL particles of normal composition. The metabolic features of familial combined hyperlipidemia and hyperapobetalipoproteinemia are identical and one defect has now been identified in a subset of patients: reduced cell membrane receptors for acylation stimulating protein, which might reduce the maximal rate of triacylglycerol synthesis in the peripheral tissues and which, as a consequence, would result in increased fatty acid influx to the liver.

On the other hand, the present data also suggest a hypothesis for the nature of the metabolic defect in familial hypertriglyceridemia, namely that intracellular synthesis of fatty acids from glucose is increased and that this results in the secretion of normal numbers of triacylglycerol-enriched apo B particles. Although this hypothesis remains to be tested, the present results at least amplify the evidence that it is intrahepatic cholesteryl ester synthesis and not triacylglycerol synthesis that plays the key role in regulating hepatic apo B secretion.

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References

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