Intrahepatic Assembly of Very Low Density Lipoproteins

VARIED SYNTHETIC RESPONSE OF INDIVIDUAL APOLIPOPROTEINS TO FASTING*

(Received for publication, November 9, 1984)

Hepatocytes obtained from rats fed for 3 days chow (control) or drinking water only (fasted) were used to examine how metabolic state affects lipogenesis, apolipoprotein synthesis, and the capacity to secrete de novo synthesized triacylglycerol. The secretion of triacylglycerol (mass and 3H-labeled via 3H2O incorporation) by both groups of cells was constant for 30 h. Moreover, cells from fasted rats secreted triacylglycerol at rates which were markedly reduced (mass -84%; 3H-labeled -91%). To assess the relative capacities of the two groups of hepatocytes to augment triacylglycerol secretion in response to stimulated lipogenesis, cells were incubated with increasing concentrations of glucose. Control cells responded to glucose by increasing equally the synthesis and secretion of [3H] triacylglycerol. When cells from fasted rats were challenged with glucose, triacylglycerol secretion was not increased. Rather, it accumulated intracellularly. Double-reciprocal plot analysis of the capacity to augment triacylglycerol secretion in response to glucose showed that cells from fasted rats had a >10-fold decrease in Vmax. Moreover, fasting changed the synthesis and secretion of apolipoproteins selectively: secretion of low molecular weight apo-B was decreased 50%, large molecular weight apo-B unchanged, and apo-E was increased 2-4-fold. Analysis of the lipoproteins from both groups of cells on Bio-Gel A-50m showed that the very low density lipoprotein secreted by cells from fasted rats was smaller. In addition, all of the increased de novo synthesized apo-E secreted by cells from fasted rats eluted after the triacylglycerol-rich lipoproteins. The combined data show that: 1) the synthesis of individual very low density lipoprotein apolipoproteins is independently regulated, and 2) the synthesis (availability) of apo-B determines the capacity of the hepatocyte to assemble/secrete triacylglycerol-rich very low density lipoprotein.

VLDL1 is the major lipid secretory product of the liver. Since much of low and high density lipoproteins is derived from the metabolism of VLDL, the rate of VLDL assembly and secretion will ultimately influence the plasma concentration of most lipoprotein classes (1, 2).

 Newly secreted VLDL isolated from the culture medium of rat hepatocytes contains three major apolipoproteins: large molecular weight apo-B2 (B2), small molecular weight apo-B (B1), and apo-E (3-5). The individual roles that the different apolipoproteins play in regard to VLDL assembly and secretion have not been delineated. Genetic deletion of both molecular weight forms of apo-B results in an inability to secrete triacylglycerol-rich lipoproteins by both the liver and intestine (6). These data suggest that 1) one or both forms of apo-B are essential for VLDL assembly/secetration and 2) (assuming that abetalipoproteinemia is due to a single genetic defect) both forms of apo-B require a common genetic element for their synthesis and/or secretion. Studies by Malloy et al. (7) of a patient with normotriglyceridemic abetalipoproteinemia (specific deletion of the large form of apo-B) show that the presence of the small form of apo-B is sufficient for assembling/secreting triacylglycerol-rich lipoproteins.

In terms of mass, apo-E is the major apolipoprotein of VLDL secreted by cultured rat hepatocytes (4, 5, 8, 9). Although apo-E may be necessary for VLDL assembly/secetration, in the absence of apo-B it is not sufficient as evidenced in patients with abetalipoproteinemia (6).

It is likely that the individual forms of apo-B function differently. In rat, the small form of apo-B is secreted by both the liver and intestine, whereas the large form is made only by the liver (3, 4, 10-12). The small molecular weight apo-B is more prevalent in triacylglycerol-rich lipoproteins, whereas the large form predominates in cholesterol-rich low density lipoproteins (3, 10-14). Recent studies by our group show that in the rat the small molecular weight form is secreted as a phosphoserine-containing protein, whereas little, if any, phosphate is associated with large molecular weight apo-B (15).

The additional finding that lipoproteins enriched with the small molecular weight form of apo-B are metabolized more rapidly than those enriched with large molecular weight form (11-14) suggests that the processes determining which apo-B form is synthesized may have profound consequences in regard to lipoprotein metabolism. Apoprotein content of lipoproteins is important in directing the tissue sites of uptake and degradation (1, 16, 17). Monoclonal antibody studies show that there is reasonable doubt as to the exact molecular weight of the different forms of apo-B, we choose to designate the migration characteristics on SDS-PAGE as either large or small. The large form corresponds to B-100, while the small form corresponds to B-48, nomenclature of Kane et al. (27). We are not convinced that the migration characteristics of the different apo-B forms are strictly due to the amount of amino acid residues (i.e. chain length).
Intrahepatic Assembly of Very Low Density Lipoproteins

TABLE I

Hepatocyte lipid content

<table>
<thead>
<tr>
<th></th>
<th>Triglycerol</th>
<th>Free cholesterol</th>
<th>Cholesterol ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>360 ± 46</td>
<td>92 ± 3</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Fasted</td>
<td>114 ± 5*</td>
<td>58 ± 4*</td>
<td>22 ± 5</td>
</tr>
</tbody>
</table>

* Significant difference.

Fig. 1. Triacylglycerol secretion by hepatocytes from control (fed) and fasted rats. Hepatocytes were obtained and cultured as described under "Materials and Methods." Four h after plating cells in medium + 20% calf serum, the culture medium was changed to serum-free DMEM. At each time point, cells and medium were harvested and the amount of triacylglycerol secreted into the medium was quantitated by glycerol-phosphate dehydrogenase. Each point is the mean ± S.D. of three individual plates of cells.

that in humans the recognition unit for the high affinity "low density lipoprotein" receptor specifically reacts with an epitope unique for the large molecular weight form of apo-B (18, 19). Thus, it is likely that the processes that determine the structure and content of apo-B in plasma lipoproteins are likely to have important influences in regard to the tissue deposition of cholesterol.

Toward our goal of gaining an understanding of the regulation of the synthesis of individual apolipoproteins, we examined how fasting affects the synthesis of VLDL apolipoproteins. The results support the concept that apo-B availability determines the capacity of the hepatocyte to assemble and secrete VLDL triacylglycerol. Moreover, the results show for the first time that the synthesis of individual VLDL apolipoproteins is differently affected by physiologic state (i.e., fasting).

Fig. 2. Secretion of [3H]triacylglycerol by hepatocytes from control and fasted rats. This experiment was performed as described in the legend to Fig. 1, except cells were incubated with [3H]O (1 mCi/ml). At the time indicated, the medium and cells were harvested, the lipids were extracted with CHCl3/MeOH (2:1, v/v) and separated by TLC, and the radioactivity was quantitated. Each point represents the mean ± S.D. of three individual plates of cells.

MATERIALS AND METHODS

All culture reagents and chemicals were obtained from sources previously described (4, 5, 15). [3H]-Amino-acid mixture (154 mCi/mg), [3H]-O (1 Ci/ml), and [35S]methionine (1066 Ci/mmol) were obtained from ICN Biochemicals (Irvine, CA) Bio-Gel A-50m agarose was obtained from Bio-Rad. Male Sprague-Dawley rats weighing 190-250 g were fed a diet consisting of rat chow and drinking water ad libitum or drinking water only for 3 days. Three days was chosen because we found that by this time all rats in the fasted group had lost 10-25% body weight.

Determination of Serum Triacylglycerol and Apolipoprotein Concentrations—Rats were bled via the saphenous. Serum was obtained and used to quantitate triacylglycerol concentrations using glycerol-phosphate dehydrogenase as described (4, 8). The concentrations of apo-B and apo-E were determined by electronimmunoassay as described (20).

Preparation of Hepatocytes and Labeling Studies—Hepatocytes obtained from rats fed for 3 days chow (control) or drinking water only (fasted) were prepared and plated after collagenase digestion using methods which are described in detail (4, 5, 8). After incubating for 4 h with Dulbecco's modified Eagle's medium (DMEM) containing 20% calf serum, the medium was changed to serum-free DMEM. In most experiments, the glucose concentration was 10 mM. Experiments in which the glucose concentration was not 10 mM are designated in the legends. In some cases (see legends), the serum-free medium was methionine-free ([35S]methionine labeling).

Lipid Biosynthesis Studies—Cells from each group were incubated with [3H]-O (1 mCi/ml) for the time listed in the legends. Cells and medium were harvested, and the labeled lipids were extracted as described in detail (5). Lipids were quantitated as follows. Triacylglycerol content was determined by the glycerol-phosphate dehydrogenase assay (5), free and esterified cholesterol contents were assayed...
Intrahepatic Assembly of Very Low Density Lipoproteins

by a previously described gas-liquid chromatography assay (5), and protein content was determined by the method of Lowry (21) using bovine serum albumin as the standard.

The incorporation of \(^{3}H\)O into each lipid class was determined by separating the lipid extracts by TLC using methods described in detail (5).

**Apolipoprotein Synthesis**—Studies in which we determined the synthesis of immunoprecipitable apo-B (Fig. 5) were performed using 60-mm dishes containing 2 ml of DMEM and \(3.9 \times 10^6\) cells. Four h after plating, the medium was changed to serum-free DMEM containing 100 µCi of \(^{3}H\)-amino-acid mixture. The cells and medium were harvested by adding boiling SDS containing buffer B as described originally by Faust et al. (22) (See legend for exact times of incubation.) A rabbit antiserum prepared and demonstrated to be monospecific for both molecular weight forms of apo-B (15) was used to quantitatively precipitate apo-B. This method involved adding 20 µl of antisera to an aliquot representing 25% of the cell and medium extracts. Following an 18-h incubation, protein A-Sepharose was added (for 2 h) at a concentration previously determined to bind all of the immunocomplexes. The protein A-Sepharose beads were washed six times with buffer D (22). The washed beads were boiled in SDS/urea-containing sample buffer and subjected to SDS-PAGE using a 1-20% acrylamide gradient. The individual bands of apo-B were cut from the gels and counted.

In other experiments, cells from both groups were incubated with \(^{35}S\)methionine (200 µCi/2 ml/60-mm culture dish) for the time described in the figure legends. Cells and medium were harvested and immunoprecipitated with rabbit antisera specific for either apo-B or apo-E as described in detail (15). The immunoprecipitates were subjected to SDS-PAGE as described above. The incorporation of \(^{35}S\)methionine into apo-B and apo-E was quantitated by scintillation assay of portions of the SDS-PAGE gel that contained apo-B or apo-E.

**Elution of Secreted Lipoprotein on Bio-Gel A-50m Agarose**—Cells from both groups were incubated in serum-free, methionine-free medium for 2 h, after which \(^{35}S\)methionine (300 µCi/ml) was added. Following a 4-h incubation, the medium was drawn off via suction and concentrated using Aquacide. The concentrate was applied to a 1.5 m x 0.9-cm (inner diameter) column packed with Bio-Gel A-50m agarose. The eluting buffer contained 0.9% NaCl, 0.01% sodium azide, and EDTA. The flow was maintained at 3.6 ml/h using a peristaltic pump. An ultraviolet monitor recorded the absorbance at 280 nm. Fractions containing 1.3 ml (25 drops) were obtained using an Isco fraction collector. The individual fractions were assayed for triacylglycerol and also were immunoprecipitated with antisera specific for apo-B and apo-E using methods reported earlier. The amount of \(^{35}S\) methionine-labeled apolipoproteins was quantitated by scintillation counting.
both groups of rats were isolated, plated, and cultured using identical methods, chemicals, and medium. Thus, differences between groups are due to intrinsic metabolic capacities retained by cells in culture in the absence of the in vivo dietary stimulus. There was no difference between the two groups in gross morphology as judged by phase-contrast microscopy (data not shown). Although cell counting showed that there was equal plating efficiency after 24 h in culture, cell protein contents of fasted cells were only 71% of the values obtained using control cells (in μg/6.5 × 10⁶ cells); 7.67 ± 0.66, control; 5.44 ± 0.52, fasted. In contrast, the amount of DNA and RNA/cell was unchanged (data not shown). These results are consistent with the findings of others showing that livers from fasted rats contain less protein/liver mass (23). Results are, therefore, expressed relative to number of cells.

Hepatocytes from fasted rats contained 58% less triacylglycerol and 37% less free cholesterol (Table I). Although the cholesterol ester concentration was increased by 47%, this increase was not statistically significant (0.05 > p < 0.1).

**Triacylglycerol Secretion**—Four h after plating the cells, the medium was changed to serum-free medium and the rate of triacylglycerol secretion was determined. Both groups of cells secreted triacylglycerol at constant (linear) rates throughout the 30-h experiments (Fig. 1). However, the slope of the accumulation in medium triacylglycerol of cells from fasted rats was only 16% of that obtained from control cells (Fig. 1, in μg/6.5 × 10⁶ cells/h: 13.1, control; 2.2, cells from fasted rats).

Incorporation of ³H₂O into de Novo Synthesized Lipids—Secretion of [³H]triacylglycerol by both groups of cells was constant throughout the 30-h experiment (Fig. 2). Rates of [³H]triacylglycerol secretion were calculated from the slopes of the linear functions (Fig. 2). Cells from fasted rats secreted de novo synthesized [³H]triacylglycerol at a rate which was only 11% of the rate exhibited by cells from control rats. Thus, there was a concordant decrease in both mass (Fig. 1) and de novo synthesized (Fig. 2) [³H]triacylglycerol secretion.

**Effect of Glucose on Lipogenesis and Lipid Secretion**—We examined the capacity of the two different groups of hepatocytes to respond to increased carbon unit availability (i.e. glucose). Control hepatocytes responded to glucose by increasing the synthesis of all lipids examined (Fig. 3). The response to glucose by cells from fasted rats was markedly different from that obtained with control cells. Whereas, phospholipid synthesis was stimulated similarly by both groups, cells from fasted rats displayed almost no stimulation of cholesterol and cholesterol ester synthesis.

In control cells, triacylglycerol synthesis was the most responsive to glucose stimulation when compared to other lipid classes. The proportion of the total [³H]triacylglycerol synthesized that was secreted was constant (50%). Although triacylglycerol synthesis by cells from fasted rats was stimulated 2.4-fold by >20 mM glucose, triacylglycerol secretion was not significantly affected. In cells from fasted rats at glucose concentrations >20 mM, where the synthesis of [³H] triacylglycerol was greatest, secretion apparently could not keep up with synthesis so that the amount of intracellular [³H]triacylglycerol increased more than the amount of [³H] triacylglycerol that was secreted into the medium.

Differences in the ability of the two groups to augment [³H] triacylglycerol secretion in response to glucose were analyzed by Lineweaver-Burk double-reciprocal analysis (Fig. 4). Hepatocytes from fasted rats displayed a >10-fold decrease in the apparent Vₘₐₓ (in [³H]triacylglycerol cpm secreted per 6.5 × 10⁶ cells/h): 22,200, control; 1600, fasted. A protein and Apolipoprotein Synthesis—The data show that cells from fasted rats displayed a remarkable inability to secrete the lipid components associated with VLDL (Fig. 3). We examined the possibility that there was also a coordinate decrease in apolipoprotein synthesis.

Both groups of cells exhibited the same rate of cellular protein synthesis (i.e. the incorporation of ³H-amino-acid mixture into trichloroacetic acid precipitates was similar in both groups of cells (data not shown)). These data agree with those of others showing that fasting does not effect liver cell...
Intrahepatic Assembly of Very Low Density Lipoproteins

Fig. 5. Rate of incorporation of \(^3\)H-amino-acid mixture into immunoprecipitated apo-B. Cells were prepared and cultured as described in the legend to Fig. 1 except the serum-free culture medium contained 100 \(\mu\)Ci/2 ml \(^3\)H-amino-acid mixture. At the time indicated, the cells and medium were harvested by boiling in SDS-containing buffers as described under "Materials and Methods." Rabbit antiserum shown to be specific for apo-B (15) was added in an amount that was found to quantitatively complex apo-B in each sample. After an overnight incubation, the immunocomplexes were bound to protein A-Sepharose and were then subjected to SDS-PAGE. The portions of the gel containing either large or small molecular weight apo-B were cut out, and the amount of radioactivity was quantitated. Each point represents the mean \pm S.D. of three individual immunoprecipitates. The solid lines represent the control cells, while the dashed lines represent the cells from fasted rats. Asterisks show significant differences.

A CELLULAR LARGE MOLECULAR WEIGHT APO B
B. CELLULAR SMALL MOLECULAR WEIGHT APO B
C. SECRETED LARGE MOLECULAR WEIGHT APO B
D. SECRETED SMALL MOLECULAR WEIGHT APO B

Apooprotein synthesis was determined by immunoprecipitating extracts of cells and medium (labeled with \(^3\)H-amino-acid mixture). A time course showed that there was a rapid increase in immunoprecipitable \(^3\)H-apo-B in the cell, which reached a steady state after 4 h (Fig. 5). Secretion of both forms of apo-B first showed a lag followed by a linear increase which persisted throughout the 8-h experiment. Except for the last time point (for which cells from fasted rats contained less small molecular weight apo-B), there was almost no difference between the two groups in the cellular content of either large or small molecular weight apo-B. Furthermore, both groups of cells secreted similar amounts of de novo synthesized large molecular weight apo-B. In contrast, cells from fasted rats secreted significantly less (-50%) small molecular weight apo-B throughout the 8-h experiment. In addition, we performed similar experiments using \(^[35S]\)methionine and also obtained similar results (i.e. using three different hepatocyte preparations, the secretion of labeled small molecular weight apo-B was decreased 42-67%, whereas secretion of labeled large molecular weight apo-B was unchanged).

Immunoprecipitation of the medium using an antiserum specific for apo-E showed that cells from fasted rats displayed a 2-fold increase (Fig. 6). There was no difference in the amount of immunoprecipitated apo-E in the cells (data not shown). This experiment was repeated three times, and similar results were obtained. The stimulation of de novo apo-E secretion displayed by cells from fasted rats varied between 2 and 4-fold.

In addition, although glucose stimulated triacylglycerol synthesis by both groups of cells (albeit cells from fasted rats were less responsive than were control cells (Fig. 3)), glucose did not affect apolipoprotein synthesis (data not shown). These results are similar to those reported showing that free fatty acids (4) and glucose (5) stimulated triacylglycerol secretion, but did not affect apolipoprotein synthesis. Thus, the
Intrahepatic Assembly of Very Low Density Lipoproteins

Increased secretion of triacylglycerol that accompanies glucose-stimulated lipogenesis does not involve increased apolipoprotein synthesis.

Determination of Re-uptake of \[^{35}S\]Methionine-labeled Apolipoproteins—To examine the possibility that the results reflect possible changes in the re-uptake of de novo synthesized apolipoproteins, cells were incubated with \[^{35}S\]methionine (to label the newly secreted apolipoproteins). The culture medium containing the labeled apolipoproteins was then added to fresh hepatocytes or plastic dishes (no cell controls). After an 18-h incubation, the amount of \[^{35}S\]methionine-labeled apolipoproteins remaining was determined. As shown in Table II, incubation with hepatocytes caused no decrease in either apo-B or apo-E compared to incubation on plastic dishes alone. In addition, we found that there was less than a 10% loss of apolipoproteins following either incubation. These data show that cultured rat hepatocytes do not take up newly secreted VLDL (4, 5).

The results show that as a result of fasting, the liver cell adapts by attenuating several (but not all) processes involved in triacylglycerol synthesis and VLDL assembly/secretion. These processes include lipogenesis, synthesis of small molecular weight apo-B, and the rate of VLDL assembly/secretion. In marked contrast, fasting increases the synthesis of apo-E, and almost all of the increased apo-E is secreted unassociated with triacylglycerol.

When cultured in identical medium in the absence of the in vivo dietary stimulus, hepatocytes from fasted rats maintained a decreased rate of triacylglycerol secretion for at least 30 h. These results show that cells from fasted rats have a decreased rate of triacylglycerol mass secretion (Fig. 1) that is matched by decreased rates of triacylglycerol synthesis (Fig. 2). Fasting is known to inhibit the activity of lipogenic enzymes (24, 25). It is likely that as a result of this inhibition the triacylglycerol pool designated for export via the VLDL secretion pathway is diminished.

The decreased capacity of cells from fasted rats to assemble and secrete triacylglycerol-rich VLDL corresponds to a specific decrease in the synthesis and secretion of small molecular weight apo-B. Since the rate of secretion of large molecular...
Intrahepatic Assembly of Very Low Density Lipoproteins

Fig. 7. Elution of newly secreted lipoproteins on Bio-Gel A-50m agarose. Cells prepared as described in the legend to Fig. 1 were incubated with methionine-free culture medium for 2 h, after which [35S]methionine (200 μCi/2 ml) was added. After 4 h, the medium was harvested as described under "Materials and Methods." The medium (centrated by Aquacide) was eluted through a glass column that contained Bio-Gel A-50m agarose. The void volume is shown (V0). Each fraction contained 25 drops (1.3 ml). A is the elution of lipoproteins secreted by hepatocytes from control rats, while B is from fasted rats. The recording of absorbance at 280 nm is shown by the solid lines. Each other fraction (even-numbered) was precipitated with antisera specific for apo-E. The immunoprecipitates were subjected to SDS-PAGE as described in the legend to Fig. 5. The amount of 35S-labeled apo-E in these fractions is shown by the dashed lines.

In five experiments (separate hepatocyte preparations), we have found the synthesis and secretion of apo-A-I is the same by cells from control and fasted rats. apo-B, apo-A-I, and apo-E was not decreased, the decrease in VLDL triacylglycerol secretion exhibited by cells from fasted rats was not due to a general impairment in secretion per se. In addition, the rate of albumin secretion was also unchanged (data not shown). The finding in this study of a specific reduction in low molecular weight apo-B synthesis and the associated reduction in the capacity to secrete triacylglycerol strengthens the concept that in the rat this form of apo-B determines, at least in part, the capacity of the hepatocyte to assemble and secrete VLDL triacylglycerol. It is also likely that the large molecular weight form of apo-B contributes to triacylglycerol secretion. In fact, in some species (i.e. rabbit (26) and human (27)), the large molecular weight form predominates in lipoproteins produced by the liver.

Our findings that the synthesis of small molecular weight apo-B is decreased, the synthesis of large molecular weight apo-B is unchanged, and the synthesis of apo-E is increased in cells from fasted rats show that the synthesis of these individual apolipoproteins by the liver is under separate metabolic regulation (at least in regard to fasting). Moreover, our results show for the first time that the two major forms of apo-B are regulated independently. It is likely that the two different forms of apo-B require a common genetic element for the synthesis and/or secretion (see Introduction). The specific decrease in small molecular weight apo-B synthesis suggests that fasting inhibits the pathway(s) whereby small molecular weight apo-B diverges from the common genetic element.

Elution of lipoproteins using Bio-Gel A-50m (Fig. 7) showed that fasting also alters the size and apoprotein content of lipoproteins. In cells from fasted rats, the 80–90% decrease in triacylglycerol secretion which was accompanied by only a 50% decrease in the secretion of small molecular weight apo-B resulted in the secretion of smaller lipoproteins (i.e. they eluted at greater volumes). In addition to these size altera-
tions, all of the increased apo-E secreted by cells from fasted rats was secreted unassociated with triacylglycerol (i.e., it is not contained in VLDL). These results are consistent with these findings. To our knowledge this is the first report describing the increased secretion of apo-E displayed by cells from fasted rats corresponds to increased apo-E mRNA synthesis. In addition, using perfused rat livers, Marsh and Sparks (1981) found a decreased rate of secretion of apo-E by hepatocytes from fasted rats. The secretion of apo-E in nascent high density lipoprotein discoidal particles has been reported to be not the result of an artifact of the cultured rat hepatocyte system.

Bell-Quint et al. (3) reported that hepatocytes from fasted rats secrete less VLDL and that they contained more large molecular weight apo-B relative to the small molecular weight form. In addition, using perfused rat livers, Marsh and Sparks (29) found a decreased rate of secretion of the small molecular weight form of apo-B. Our results regarding apo-B secretion by hepatocytes from fasted rats are consistent with these findings. To our knowledge this is the first report describing that fasting increases the synthesis and secretion of apo-E.

The finding that the changes in apolipoprotein synthesis exhibited by hepatocytes from fasted rats correlate with similar changes in the apolipoprotein content of serum shows that changes in hepatic synthesis are an important factor in determining plasma lipoprotein levels.

REFERENCES


* R. A. Davis, R. A. Borchardt, C. A. Reardon, and G. S. Getz, unpublished observations.