Glycosylation of apolipoproteins by cultured rat hepatocytes

Effect of tunicamycin on lipoprotein secretion

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Cultured rat hepatocytes were used to measure hepatic synthesis of rat plasma glycoproteins. [^3H]Glucosamine was progressively incorporated into the protein of hepatocyte culture media very-low-density lipoprotein, low-density lipoprotein, high-density lipoprotein and the $\rho > 1.21\text{ g/ml}$ fraction after 3.5 and 6.5 h incubation. Apolipoproteins B, E and C, as well as transferrin, were identified as glycoproteins. The association of radioactivity with apolipoprotein C of hepatocyte very-low-density and high-density lipoproteins suggests that apolipoprotein C-III-3, the only C apolipoprotein in the rat, is synthesized *de novo* by the hepatocytes. Treatment of hepatocytes with tunicamycin, a specific inhibitor of protein glycosylation, resulted in a substantial decrease in [^3H]glucosamine incorporation into hepatocyte very-low-density, low-density and high-density lipoproteins and $\rho > 1.21\text{ g/ml}$ protein, but had little or no effect on secretion. In the rat, hepatic secretion of lipoproteins and transferrin does not appear to be dependent on prior protein glycosylation.

The isolation of very small amounts of labelled lipoproteins from the medium of incubated liver slices or from liver subcellular fractions was previously used to assess hepatic glycosylation of rat plasma apolipoproteins. Mookerjee and his collaborators (Mookerjee *et al.*, 1967; Mookerjee, 1969) demonstrated the incorporation of glucosamine into low-density lipoproteins. Lo & Marsh (1970) studied the labelling of rat low-density and high-density lipoproteins in liver microsomal subfractions and concluded that the Golgi apparatus plays a major role in the attachment of carbohydrate to the lipoprotein after it leaves the endoplasmic reticulum. Interpretation of the data obtained from these experiments is complicated or limited by the use of carrier serum or lipoproteins in the isolation of the hepatic lipoproteins and by the fact that rats were pre-treated with colchicine, an inhibitor of lipoprotein secretion.

Techniques for culturing rat hepatocytes have permitted more direct measurement of hepatic lipoprotein synthesis and secretion (Jeejeebhoy *et al.*, 1975; Davis *et al.*, 1979; Kempen, 1980; Bell-Quint & Forte, 1981). In the present study, hepatic synthesis of rat plasma glycoproteins was

assessed by measuring [^3H]glucosamine incorporation into the protein of VLD, LD and HD lipoproteins and $\rho > 1.21\text{ g/ml}$ fraction secreted by cultured hepatocytes. In addition, tunicamycin, an antibiotic that inhibits glycosylation of newly synthesized protein (Kuo & Lampen, 1974; Schwarz *et al.*, 1976; Struck & Lennarz, 1977; Hickman *et al.*, 1977; Duksin & Bornstein, 1977), was used to determine whether lipoprotein secretion is dependent on apolipoprotein glycosylation.

Experimental

Biochemicals and reagents

Collagenase (type IV; 125–200 units/mg), gentamicin sulphate (600 $\mu\text{g/ml}$) and cross-linked albumin and haemocyanin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Purified rat transferrin and albumin were obtained from Cappel Laboratories, Thud Ridge Farm, Cochranville, PA, U.S.A. [^3H]Glucosamine (30–60 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. Tunicamycin was a gift from Dr. Robert Hamill, Lilly Research Laboratories, Indianapolis, IN, U.S.A. Heat-inactivated foetal calf serum, penicillin/streptomycin and Leibovitz's L-15 medium were obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. Cholesterol oxidase (40 units/ml) and cholesterol esterase

(250 units/g) were purchased from Miles Laboratories Inc., Elkhart, IN, U.S.A. Other purified enzymes were obtained from Boehringer Mannheim, Indianapolis, IN, U.S.A.

Preparation of monolayer cultures of primary rat hepatocytes

Monolayer cultures of hepatocytes were prepared from 200 g male WAG/rij rats as described previously (Bell-Quint & Forte, 1981). Parenchymal cells were isolated by three successive centrifugations at 70 g for 2 min each. After each centrifugation the sedimented cells were resuspended in L-15 culture medium supplemented with 8.3 mM-glucose, 32 m-units of insulin/ml, penicillin (100 units/ml)/streptomycin (0.1 mg/ml) and 10 µg of gentamicin sulphate/ml, pH 7.6 (standard medium), containing heat-inactivated foetal-calf serum (20%, w/v). The washed cell pellet was weighed and suspended in this medium to yield a 0.12 g/ml cell suspension. Of the cell suspension 0.5 ml was placed in collagen-coated 60 mm plastic Petri dishes in 2.0 ml of standard medium containing heat-inactivated foetal-calf serum (20%, w/v) to yield 10^6 cells. From each rat 60–70 monolayers were obtained, and hepatocytes isolated from two to three rats were routinely used for each experiment. The cells were incubated at 37°C in humidified air.

After 24 h, the monolayers were divided into two groups; 25 µl of 25 mM-NaOH was added to each plate of one group and tunicamycin (1 µg/ml) contained in 25 µl of 25 mM-NaOH was added to each plate of the other group. After 30 min incubation, the media were removed and 2.5 ml of fresh serum-free standard medium was added to each of the rinsed monolayers. In experiments measuring [^3H]glucosamine incorporation, the serum-free standard medium contained 45 µCi of [^3H]glucosamine. Lipoproteins were isolated from the culture media after 3.5, 6.5 or 18 h. The hepatocytes were 85–90% viable as determined by Trypan Blue exclusion during the course of the study.

Isolation of hepatocyte culture media lipoproteins

The pooled culture media from each group (approx. 100 monolayers) were first centrifuged at

700 g at 5°C to remove cell debris and then concentrated to 30–40 ml by ultrafiltration under N_2 in an Amicon stirred cell with PM-30 membranes. The concentrated media were dialysed extensively at 5°C against a 154 mM-NaCl solution containing 0.24 mM-EDTA and 3 mM- NaN_3 . Lipoproteins were isolated sequentially from rat plasma and hepatocyte culture media by preparative ultracentrifugation by previously described methods (Bell-Quint & Forte, 1981).

Analyses

Protein was determined by the method of Lowry *et al.* (1951). Enzymic methods were used to determine cholesterol (Allain *et al.*, 1974) and triacylglycerol (Bucolo & David, 1973) content. Phospholipid phosphorus was determined by the method of Bartlett (1959).

Apolipoproteins were analysed by disc-gel electrophoresis in 10 and 3.5% polyacrylamide containing 0.1% sodium dodecyl sulphate by the method of Weber & Osborn (1969). All gels were stained with 0.2% Coomassie Blue and destained in 10% acetic acid. The relative intensities of various bands were estimated by densitometry.

Protein radioactivity in lipoprotein and $\rho > 1.21$ g/ml fractions was measured using the procedure described by Mans & Novelli (1961). Apolipoprotein bands were sliced from stained gels, digested and counted in liquid-scintillation solution by the method of Mahin & Lofberg (1966). Total recovery of applied radioactivity was 85–95%.

Results

Incorporation of [^3H]glucosamine into hepatocyte culture media lipoprotein and $\rho > 1.21$ g/ml protein

The synthesis of glycosylated apolipoproteins and $\rho > 1.21$ g/ml proteins by cultured rat hepatocytes was measured by incubating cells with [^3H]glucosamine and determining the incorporation of radioactivity into trichloroacetic acid-precipitable protein. The results of these experiments are shown in Table 1. The increased incorporation of [^3H]glucosamine into all of the fractions at 6.5 h, compared with at 3.5 h, parallels the time-related increases in the concentrations of the lipoproteins

Table 1. Time-related incorporation of [^3H]glucosamine into hepatocyte culture media lipoprotein and $\rho > 1.21$ g/ml protein

Monolayers of rat hepatocytes were incubated in serum-free standard medium containing [^3H]glucosamine for 3.5 or 6.5 h.

Time (h)	$10^3 \times \text{Radioactivity (c.p.m./g of cell protein)}$			
	VLD lipoprotein	LD lipoprotein	HD lipoprotein	$\rho > 1.21$ g/ml fraction
3.5	45.8	4.0	15.4	28950
6.5	97.8	12.4	57.6	75495

and $\rho > 1.21$ g/ml fraction isolated from the culture medium, and suggests progressive glycosylation of newly synthesized proteins.

The protein fraction of $\rho > 1.21$ g/ml contains the highest amount of radioactivity at both incubation periods. Of the lipoprotein fractions, approx. 64% of the radioactivity is associated with VLD-lipoprotein protein, 6% LD-lipoprotein protein and 29% with HD-lipoprotein protein. The specific radioactivity (c.p.m./ μ g of lipoprotein protein) of hepatocyte VLD lipoprotein increases from 263 at 3.5 h to 379 at 6.5 h, LD lipoprotein from 39 (3.5 h) to 118 (6.5 h) and HD lipoprotein from 69 (3.5 h) to 183 (6.5 h).

Identification of glycoproteins synthesized by cultured rat hepatocytes

To identify which proteins are glycoproteins, hepatocytes were incubated in serum-free medium containing [3 H]glucosamine for 6.5 h and the secreted apolipoproteins and $\rho > 1.21$ g/ml protein were separated by 10 and 3.5% sodium dodecyl sulphate/polyacrylamide-gel electrophoresis respectively. Radioactivity associated with the various protein bands was determined after relative quantities of the proteins were estimated by scanning densitometry.

Fig. 1 shows the apolipoprotein content of rat

Fig. 1. Rat plasma and hepatocyte culture media (6.5 h) apolipoproteins determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Samples (a)–(f) were electrophoresed in 10% gels, whereas (g) was electrophoresed in 3.5% polyacrylamide. Gels (a), (b) and (c) correspond to plasma VLD lipoprotein, LD lipoprotein and HD lipoprotein respectively, and (d), (e) and (f) to hepatocyte culture medium VLD lipoprotein, LD lipoprotein and HD lipoprotein respectively. Gel (g) is the $\rho > 1.21$ g/ml fraction; 55–60 μ g of protein were applied to each gel. The nomenclature of the apolipoprotein bands follows that recommended by Swaney *et al.* (1974) as follows: B, apolipoprotein B; A-IV, apolipoprotein A-IV; E, apolipoprotein E; A-I, apolipoprotein A-I; C, C apolipoproteins.

hepatocyte culture medium VLD lipoprotein, LD lipoprotein and HD lipoprotein after 6.5 h of incubation. Apolipoproteins of rat plasma lipoprotein fractions are shown for comparison. The major apolipoproteins were identified on the basis of protein standards electrophoresed in the same system and of previously published values (Swaney *et al.*, 1974). Hepatocyte culture medium and plasma VLD lipoprotein contain apolipoproteins B, E and C as the major constituents. Hepatocyte LD lipoprotein contains primarily apolipoprotein B. Although plasma LD lipoprotein contains a prominent apolipoprotein B band, other components including apolipoproteins E and A-I are also present. The presence of the latter two apolipoproteins is probably attributable to HD lipoprotein, a large, less-dense, apolipoprotein E-enriched particle that is isolated from rat plasma at ρ 1.006–1.063 g/ml (Mahley, 1978). Hepatocyte HD lipoprotein, like plasma HD lipoprotein, contains apolipoproteins A-I, E and C. In contrast with plasma HD lipoprotein, however, the hepatocyte particles also contain protein material of mol.wt. $> 100\,000$; we have recently identified apolipoprotein B as a constituent of hepatocyte HD lipoprotein (Bell-Quint *et al.*, 1981). Fig. 1 also shows that albumin and transferrin are the major proteins recovered in the hepatocyte culture medium $\rho > 1.21$ g/ml fraction.

The incorporation of [3 H]glucosamine into the apolipoproteins of hepatocyte VLD lipoprotein, LD lipoprotein and HD lipoprotein is shown in Fig. 2. Radioactivity is associated with apolipoproteins B, E and C of the lipoproteins, which suggests that these proteins are glycosylated within the hepatocyte before secretion. LD lipoprotein also incorporates [3 H]glucosamine into an unidentified component. In contrast, apolipoprotein A-I, the major protein of hepatocyte HD lipoprotein is apparently not glycosylated since negligible amounts of 3 H are associated with this protein.

Separation of hepatocyte culture media apolipoprotein B proteins by electrophoresis in 3.5% polyacrylamide disc gels as described previously (Bell-Quint *et al.*, 1981) indicates that [3 H]glucosamine is incorporated predominantly into the approx. 243 000-mol.wt. B protein of hepatocyte VLD lipoprotein and HD lipoprotein and into the 345 000-mol.wt. apolipoprotein B component of hepatocyte LD lipoprotein (results not shown).

The specificity of [3 H]glucosamine incorporation is illustrated by the data in Fig. 3. The majority of the radioactivity incorporated into the hepatocyte $\rho > 1.21$ g/ml fraction is associated with transferrin (peak I), a previously identified glycoprotein (Gordon & Louis, 1963; Wise & Oliver, 1966). The small percentage of radioactivity appearing in the albumin region (peak II) probably results from

Fig. 2. Incorporation of $[^3\text{H}]$ glucosamine into hepatocyte apolipoproteins

Labelled lipoproteins isolated from the culture medium after 6.5 h of incubation were separated by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels. Protein was stained with Coomassie Blue G-250 and scanned with a densitometer (—); the major protein peaks are identified as follows: B, apolipoprotein B; E, apolipoprotein E; A, apolipoprotein A-I; C, C apolipoproteins. Radioactivity in the gel slices is indicated by the bar columns.

incomplete separation of the two protein peaks; it has been clearly demonstrated by others (Peters *et al.*, 1971) that albumin is not a glycoprotein.

Effect of tunicamycin on glycosylation and secretion of hepatocyte lipoprotein and $\rho > 1.21$ g/ml protein

Table 2 shows the effect of tunicamycin on the secretion of, and $[^3\text{H}]$ glucosamine incorporation into, hepatocyte lipoprotein and $\rho > 1.21$ g/ml protein after 6.5 h incubation. Exposure of hepatocytes to tunicamycin results in substantial inhibition of protein glycosylation as judged by the decreased incorporation of $[^3\text{H}]$ glucosamine into hepatocyte lipoprotein and $\rho > 1.21$ g/ml protein relative to control. In contrast, tunicamycin has no apparent effect on the secretion of hepatocyte LD lipoprotein, HD lipoprotein and $\rho > 1.21$ g/ml protein. The secretion of hepatocyte VLD-lipoprotein protein is somewhat decreased by the drug (26%); however, the inhibition of VLD lipoprotein glycosylation is considerably more pronounced (82%). Lipoprotein

Fig. 3. Incorporation of $[^3\text{H}]$ glucosamine into hepatocyte $\rho > 1.21$ g/ml fraction after 6.5 h of incubation

Labelled $\rho > 1.21$ g/ml fraction was separated by electrophoresis on 3.5% sodium dodecyl sulphate/polyacrylamide gels. Protein was stained with Coomassie Blue G-250 and scanned with a densitometer (—); peaks I and II represent transferrin and albumin respectively. Radioactivity in the gel slices is indicated by the bar columns.

Table 2. Effect of tunicamycin on glycosylation and secretion of hepatocyte lipoprotein and $d > 1.21$ g/ml protein. Monolayers of rat hepatocytes were incubated in serum-free standard medium for 6.5 h. Other details of the incubation conditions are given in the Experimental section. Protein secretion data are means $\pm$ S.E.M. ($n = 5$).

	$[^3\text{H}]$ Glucosamine incorporated (% of control value)	Protein secreted (% of control value)
VLD lipoprotein	18	74 ± 0.10
LD lipoprotein	29	102 ± 0.05
HD lipoprotein	26	103 ± 0.07
$d > 1.21$ g/ml fraction	12	106 ± 0.05

secretion by treated and untreated cells undergoes the same time-related changes that we reported previously (Bell-Quint & Forte, 1981). VLD lipoprotein synthesis predominates after 6.5 h (approx. 60% of total), whereas equal quantities of LD lipoprotein and HD lipoprotein are secreted. After 18 h, VLD lipoprotein is almost undetectable, whereas the concentration of LD lipoprotein and HD lipoprotein continues to increase. There is also no change in either the distribution of apolipoproteins or in the chemical composition of the hepatocyte lipoproteins after treatment with tunicamycin.

Discussion

In the present paper, glycosylation of rat hepatic VLD-, LD- and HD-lipoprotein apolipoproteins was measured under the same experimental conditions using cultured rat hepatocytes. Based on the incorporation of [^3H]glucosamine into lipoprotein protein, apolipoproteins B, E and C were identified as newly synthesized glycoproteins. Hepatocyte VLD lipoprotein and HD lipoprotein contained all three apoglycoproteins, whereas LD lipoprotein contained apolipoprotein B and an unidentified glycoprotein.

The identification of hepatocyte VLD- and LD-lipoprotein apolipoprotein B as glycoproteins is consistent with previous reports in which radioactive glucosamine was found incorporated into the heparin-precipitable protein of the $p > 1.063 \text{ g/ml}$ fraction secreted by rat liver slices and subcellular particles (Delahunty & Mookerjee, 1974) and into apolipoprotein B of VLD-lipoprotein isolated from rat hepatic Golgi fractions (Dolphin & Rubinstein, 1977). The presence of glycosylated apolipoprotein B in hepatocyte HD lipoprotein is a new finding, but is compatible with previous reports of the isolation of apolipoprotein B-containing HD lipoprotein from rat liver perfusates (Fainaru *et al.*, 1977) and from the medium of cultured rat hepatocytes (Bell-Quint *et al.*, 1981). We have calculated a 10-fold lower estimated specific radioactivity of apolipoprotein B in hepatocyte LD lipoprotein compared with hepatocyte VLD lipoprotein, which suggests that a larger intracellular pool of the 345 000-mol.wt. LD lipoprotein B protein was glycosylated compared with the 242 000-mol.wt. B protein of VLD lipoprotein. Alternatively, the VLD- and LD-lipoprotein apolipoprotein B proteins may be differentially exposed to glycosylating enzymes in the Golgi of the hepatocytes. We previously reported a lower estimated specific radioactivity of hepatocyte LD-lipoprotein apolipoprotein B compared with VLD-lipoprotein B based on [^3H]leucine incorporation studies (Bell-Quint & Forte, 1981).

The incorporation of [^3H]glucosamine into the C apolipoproteins of VLD lipoprotein and HD lipopro-

tein suggests that apolipoprotein C-III-3, the only carbohydrate-containing C protein in the rat (Herbert *et al.*, 1974), is synthesized *de novo* by the hepatocytes. In contrast with our data, other investigators have reported synthesis of VLD lipoprotein deficient in apolipoprotein C by suspensions of rat hepatocytes (Jeejeebhoy *et al.*, 1975) and by rat hepatocyte monolayers (Davis *et al.*, 1979). The reason for this discrepancy is not clear. However, it is possible that the high apolipoprotein C content of hepatocyte VLD lipoprotein in our studies results from the transfer of newly synthesized C apolipoproteins carried by hepatocyte HD lipoprotein to VLD lipoprotein.

Our studies with tunicamycin suggest that secretion of rat hepatic VLD lipoprotein, LD lipoprotein and HD lipoprotein is not dependent on the *N*-glycosidic linkage of oligosaccharide chains to apolipoproteins B, E and C. Dissociation of VLD-lipoprotein synthesis and secretion by cultured chick hepatocytes from apolipoprotein B glycosylation has also been established by Struck *et al.* (1978). Our studies also suggest that carbohydrate attachment to lipoproteins is not required for lipid binding since the lipid composition of hepatocyte lipoproteins secreted by cells treated with tunicamycin was not significantly different from that of control hepatocyte lipoproteins.

Measurements of lipoprotein secretion after 18 h of incubation by control hepatocytes and hepatocytes treated with tunicamycin indicated a substantial decrease in the concentration of VLD lipoprotein isolated from the media relative to the 6.5 h incubation period. As reported previously (Bell-Quint & Forte, 1981), the decreased concentration of media VLD lipoprotein after prolonged incubation appears to be due in part to catabolism or degradation of the particles by the hepatocytes. The disappearance of unglycosylated hepatocyte VLD lipoprotein from the medium in the present study suggests that the carbohydrate moiety of the VLD lipoprotein is not required for cellular uptake or degradation of the particles. High-affinity receptors that bind and degrade rat hepatic VLD lipoprotein, LD lipoprotein and apolipoprotein E-enriched HD lipoprotein have been demonstrated on perfused livers and liver membranes from control and oestrogen-treated rats (Windler *et al.*, 1980). Cultured rat hepatocytes may prove useful in elucidating the role of cellular glycoprotein synthesis and hepatic lipoprotein glycosylation in the receptor-mediated uptake of lipoproteins by the liver.

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