Microsomal membrane-associated apoB is the direct precursor of secreted VLDL in primary cultures of rat hepatocytes

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Abstract **Brefeldin A (BFA) added to primary cultures of rat hepatocytes, at a concentration of 0.2 μg/ml, prevented the assembly of newly synthesized apolipoprotein B (apoB) into mature, secretory VLDL but did not prevent the secretion of apoB as denser particles (HDL apoB), or of albumin. The unassembled apoB remained associated with the membranes of the cellular microsomal fraction. There was no effect of BFA on the removal of apoB from the lumen of these vesicles. VLDL apoB formed only a minor component of the total apoB in the microsomal lumen. Higher (5 μg/ml) concentrations of BFA were required to prevent the secretion of HDL apoB and albumin. Under these conditions apoB accumulated in the microsomal lumen, as well as in the membranes of these vesicles. Again, apoB VLDL formed only a minor proportion of the total lumenal apoB. ApoB-48 VLDL and apoB-100 VLDL assembly could be restored by removing BFA from the medium. This reactivation of VLDL assembly was accompanied by an increased removal of apoB from the microsomal membranes, but there was no detectable increase in the small quantity of VLDL apoB that was recovered from the microsomal lumen. In the absence of BFA, during pulse-chase experiments the pattern of change in the specific radioactivity of microsomal membrane apoB was similar to that of the secreted VLDL apoB whereas that of the lumenal apoB resembled that of the secreted HDL apoB. The results suggest that membrane-associated apoB is the main direct precursor of secreted VLDL apoB in primary cultures of rat hepatocytes and that VLDL assembly does not involve primarily microsomal lumenal apoB as an intermediate.**—Hebbachi, A-M., and G. F. Gibbons. **Microsomal membrane-associated apoB is the direct precursor of secreted VLDL in primary cultures of rat hepatocytes.** ***J. Lipid Res.*** **2001.** 42: **1609–1617.**

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The assembly of hepatic VLDL occurs in at least two distinct stages [1–3; reviewed in Ginsberg (4) and Olofsson, Asp, and Bóren (5)]. The first stage requires an interaction between newly translated apolipoprotein B (apoB) and lipids in the membrane of the endoplasmic reticulum (ER). In McArdle RH-7777 cells this form of apoB remains associated with the microsomal membrane as particles with the density of HDL (6). The other VLDL precursor consists of an apoB-free particle of neutral lipid (1, 7, 8) that, in McArdle RH-7777 cells, requires the synthesis of neutral lipid from exogenous fatty acids (9). The formation of both precursors requires microsomal triglyceride transfer protein (8, 9). The final stage of VLDL assembly involves the fusion of these two precursors. It is generally agreed that the synthesis of the apoB-containing VLDL precursor occurs either co- or posttranslationally in the ER membrane (6, 10). The intracellular site of the fusion process that produces mature VLDL is more controversial and evidence has been presented that implicates both the ER (1, 11, 12) and the Golgi (13, 14). Neither has it yet been unequivocally established whether the fusion process is a lumenal or membrane-associated event. Elucidation of the mechanism involved in the fusion step has been facilitated by the use of brefeldin A (BFA), a fungal metabolite (15). At relatively high concentrations, BFA disturbs the functional integrity of the secretory pathway by interfering with GTP exchange on ADP-ribosylation factor 1 (ARF-1) (16), a small G protein that forms part of the COP-I complex, recruitment of which to intracellular membranes is essential for vesicle budding (17, 18). This causes disassembly of the Golgi complex and redistribution of the Golgi resident proteins into the ER (19–21). In McArdle RH-7777 cells lower concentrations of BFA specifically suppress the bulk addition of neutral lipid to the apoB-containing VLDL precursor, thus preventing the maturation phase of VLDL assembly. The formation of the VLDL precursor is unaffected and the secretory apparatus remains intact (2, 6). Inhibition of GTP exchange on ARF-1 explains the inhibitory effect of BFA on the bulk

Abbreviations: apoB, apolipoprotein B; BFA, brefeldin A; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

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lipid addition step of VLDL production (22). We have utilized BFA to study the intracellular kinetics of apoB metabolism when the final stage of VLDL assembly is specifically inhibited in primary cultures of rat hepatocytes, a model in which preformed intracellular neutral lipid is mobilized to form the apoB-free VLDL precursor (23). These results, together with those obtained from measurements of changes in the specific radioactivity of newly synthesized apoB, have provided evidence that microsomal membrane-associated apoB is the precursor for VLDL maturation in primary liver cell culture.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade. Waymouth's medium (methionine free) and Dulbecco's PBS were obtained from GIBCO (Paisley, Scotland). [^{35}S]methionine (1,175 Ci/mol) was from NEN Life Science Products (Zaventem, Belgium). BSA (fatty acid free), BFA, dexamethasone, glutamine, alanine, serine, lactate, pyruvate, methionine, and protein A Sepharose CL4B were obtained from Sigma (Poole, Dorset, UK). Anti-rat apoB antiserum was raised in rabbits as described previously (24). Anti-rat albumin antiserum was obtained from ICN Pharmaceutical (Basingstoke, UK). Acrylamide-bisacrylamide (40%, w/v) was obtained from Bio-Rad (Hemel Hempstead, UK). Anti-human apoB antibody and the secondary antibody (donkey anti-sheep IgG) for use in the ELISA were obtained from Boehringer and Scottish Antibodies (Carluke, Scotland), respectively. Male Wistar rats were purchased from Harlan UK (Bicester, UK) and fed a low fat diet [rat and mouse no. 3 breeding diet purchased from Special Diet Services (Witham, Essex, UK)]. Tissue culture plates and plastic disposables were obtained from Becton Dickinson (Cowley, Oxford, UK).

Preparation and culture of rat hepatocytes

Hepatocytes were prepared from male Wistar rats under sterile conditions by collagenase perfusion under conditions described previously (25). The isolated cells were suspended in Waymouth's medium MB 752/1 containing fetal bovine serum (10%, v/v), penicillin (90,000 U/l), streptomycin (90,000 μg/l), glutamine (3.6 mM), alanine (0.36 mM), and serine (0.45 mM). The viability of the hepatocytes was measured by trypan blue exclusion and was usually between 90% and 95%. The cell suspension (0.65×10^6 cells/ml) was plated out onto dishes previously coated with rat tail collagen (26), until the cells formed a monolayer (4 h). After this period, the medium was removed, the monolayer was washed twice with PBS, and the cells were cultured for 0.5 h in serum-free, methionine-free medium containing the supplements described in Hebbachi, Brown, and Gibbons (27). This is subsequently referred to as supplemented medium. Cells were then cultured for a further 20 min in the same medium either in the absence or presence of BFA in ethanol (see below) at a concentration of 0.2 or 5.0 μg/ml. At the end of this period a trace amount of [^{35}S]methionine (150 μCi) was added to each dish for a further period of 1 h (pulse period) with or without BFA as appropriate. The medium was removed and the monolayer was washed twice with PBS. At this stage, some dishes were removed to prepare microsomes (total ER). To the remaining cells was added supplemented medium containing unlabeled methionine (10 mM) (chase medium). The cells were cultured for further periods of 0.5 and 2 h in the presence or absence of BFA (0.2 or 5 μg/ml in 20 μl of ethanol), after which the medium was removed and the cells were harvested. Control dishes contained 20 μl of ethanol only. At the end of each culture period, the medium was removed to isolate VLDL and HDL. Cells were harvested and used for preparing a total microsomal fraction as described previously (28). In some experiments, cells were pulsed in the presence of BFA (5 μg/ml) and chased for 2 h in the presence (5 μg/ml) or absence of BFA. In separate experiments apoB was pulse labeled for 1 h in the absence of BFA and the cell cultures were chased for 1, 2, 4, and 16 h. The specific radioactivity of total apoB (apoB-100 and apoB-48) in the microsomal membrane, microsomal lumen, and secreted lipoproteins (VLDL and HDL) was determined.

Preparation of subcellular fractions from cultured rat hepatocytes

Total ER (microsomes) was prepared from cultured rat hepatocytes by methods previously described (28, 29). The microsomal fraction was separated into membranes and lumenal contents. Total microsomes were suspended in 100 mM sodium carbonate (pH 11.5) at a concentration of up to 1 mg of protein per ml and incubated on ice for at least 30 min (30). This procedure served to open the vesicles and released the lumenal contents and peripherally associated membrane proteins from the intact membranes. Membrane and lumenal contents were separated by ultracentrifugation (30).

Separation of VLDL and HDL particles from the cell medium and microsomal lumen

At the end of each culture period, the cell medium was centrifuged in a Beckman (Fullerton, CA) 50.4 fixed angle rotor for 16 h at 40,000 rpm. The floating VLDL was separated from the denser particles ($d > 1.006$) in the medium infranate by tube slicing. HDL was separated from the infranates as described previously (27). The contents of the microsomal lumen were fractionated into the corresponding density ranges in an identical manner.

Immunoprecipitation and electrophoresis of labeled apoB and albumin

Labeled apoB was immunoprecipitated from samples of cells, microsomal membranes, and lumenal contents and from VLDL and HDL fractions of the medium and microsomal lumen, using a polyclonal anti-rat apoB antibody raised in rabbits (24). Similarly, radiolabeled albumin was immunoprecipitated from these fractions by using anti-rat albumin antiserum. All immunoprecipitates were isolated with protein A Sepharose as described previously (27). The resulting protein A Sepharose bead suspension was heated with sample buffer at 75°C for 20 min followed by centrifugation. After centrifugation, the supernatants containing either the immunoprecipitated labeled apoB-48 plus apoB-100 or labeled albumin were subject to SDS-PAGE in a 3–20% gradient polyacrylamide gels (31). Bands on gels containing either apoB-48, apoB-100, or albumin were visualized with Coomassie Brilliant Blue R and the dried gel was exposed to X-ray film for 48 h. Bands containing labeled apoB-48, apoB-100, or albumin were excised from the appropriate gel and solubilized with 30% H_2O_2 and NCS tissue solubilizer (NEN Life Science Products). After neutralizing the solubilizer with glacial acetic acid, scintillation fluid (Optiphase) was added and radioactivity was determined in a Beckman LS-6500 scintillation counter.

Other analytical methods

Cellular protein was determined colorimetrically by the method of Lowry et al. (32). Total apoB mass (apoB-100 and apoB-48) in isolated lipoproteins (VLDL and HDL), microsomal membrane, and lumen was determined by an ELISA method described in Wilkinson et al. (33).

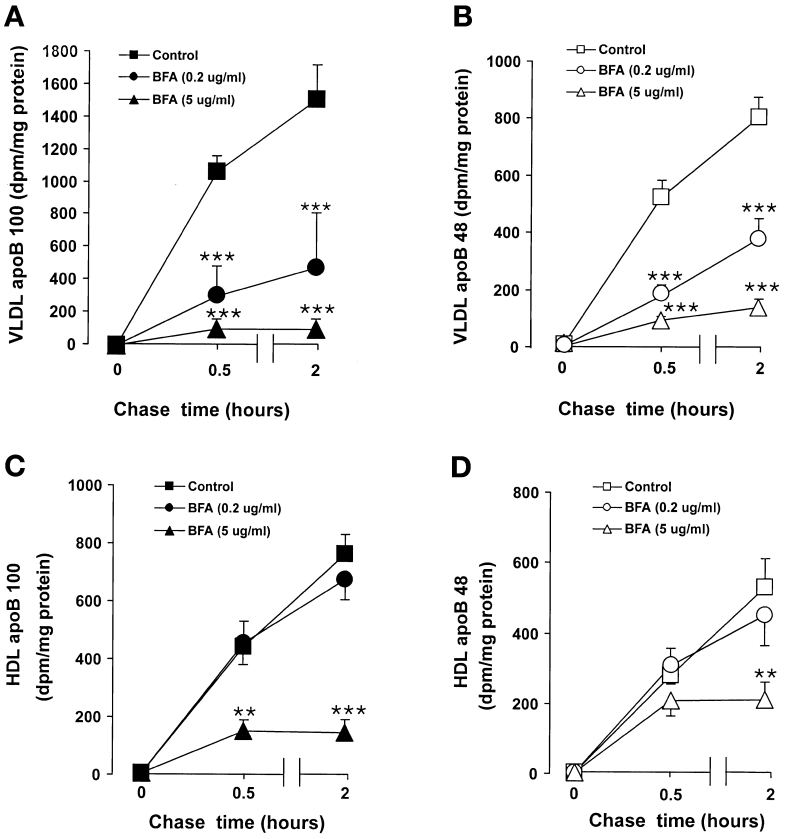

Fig. 1. Differential effects of BFA on the secretion of newly synthesized apoB associated with VLDL (A and B) and HDL (C and D). Cultured rat hepatocytes were incubated with or without BFA (0.2 or 5.0 μg/ml) and then pulse labeled with [^{35}S]-methionine for 1 h with or without BFA as appropriate. The medium was changed and the cells were chased in a medium containing 10 mM methionine with or without BFA (0.2 or 5.0 μg/ml) as appropriate. After removal of the medium at either 30 or 120 min, fractions containing VLDL and HDL were separated by ultracentrifugation. The radioactivity associated with apoB-100 and apoB-48 in each fraction was determined. Each value represents the mean ± SEM of five independent experiments. Values marked with asterisks were significantly different from the respective control value (** $P < 0.01$ and *** $P < 0.001$, respectively).

RESULTS

Selective manipulation of VLDL assembly by BFA delays the removal of apoB from the microsomal membrane, but not from the microsomal lumen

Rat liver preparations in vitro secrete apoB-100 and apoB-48 in both the VLDL and HDL density ranges (15, 34, 35). Similar results were obtained in the present work during the secretion of newly synthesized apoB labeled with [^{35}S]methionine after a 1-h pulse (**Fig. 1**). When a low concentration of BFA (0.2 μg/ml) was added to the medium of the cells 20 min before the start of the pulse label, there was a significant, 2- to 3-fold decline in the secretion of apoB-100 and apoB-48 associated with VLDL during the subsequent 2-h chase period (Fig. 1A and B). There was no significant effect of this low BFA concentration on the secretion of either apoB-100 or apoB-48 as HDL (Fig. 1C and D). Neither was there any effect on the secretion of newly synthesized albumin (**Fig. 2**), which suggested that the bulk secretory apparatus remained intact and functional at this low concentration of BFA. A similar experiment carried out at a higher concentration (5 μg/ml) of BFA, as expected, blocked the secretion not only of VLDL apoB (Fig. 1A and B), but of HDL apoB (Fig. 1C and D) and albumin (Fig. 2), confirming that the integrity of the secretory apparatus had broken down under these conditions.

The selective decrease in the secretion of apoB-48 and apoB-100 VLDL at the low concentration of BFA was accompanied by an almost total block in the release of apoB-100 and apoB-48 from the microsomal membranes during the 2-h chase period (**Fig. 3A** and **B**). In cells that had been pulsed and chased in the absence of BFA there were decreases in the amounts of membrane-associated

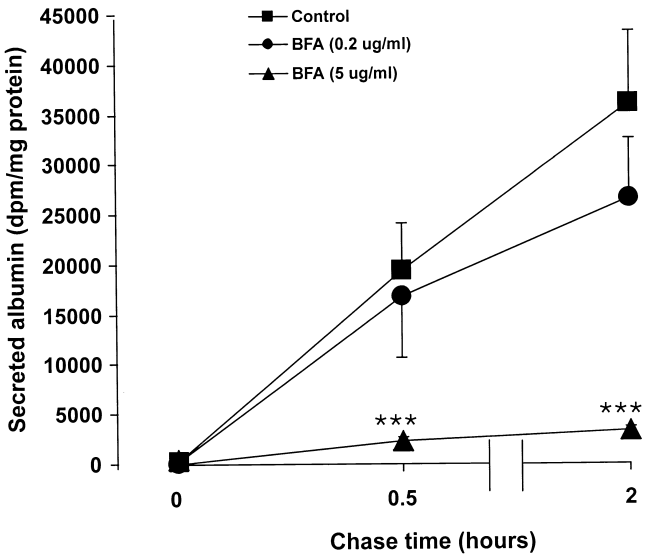

Fig. 2. Effects of BFA at different concentrations on the secretion of newly synthesized albumin. Cultured rat hepatocytes were treated as described in the legend to Fig. 1. Radiolabeled albumin secreted into the medium was determined. Each value represents the mean ± SEM of three independent experiments. *** Values significantly different from the corresponding control values ($P < 0.001$).

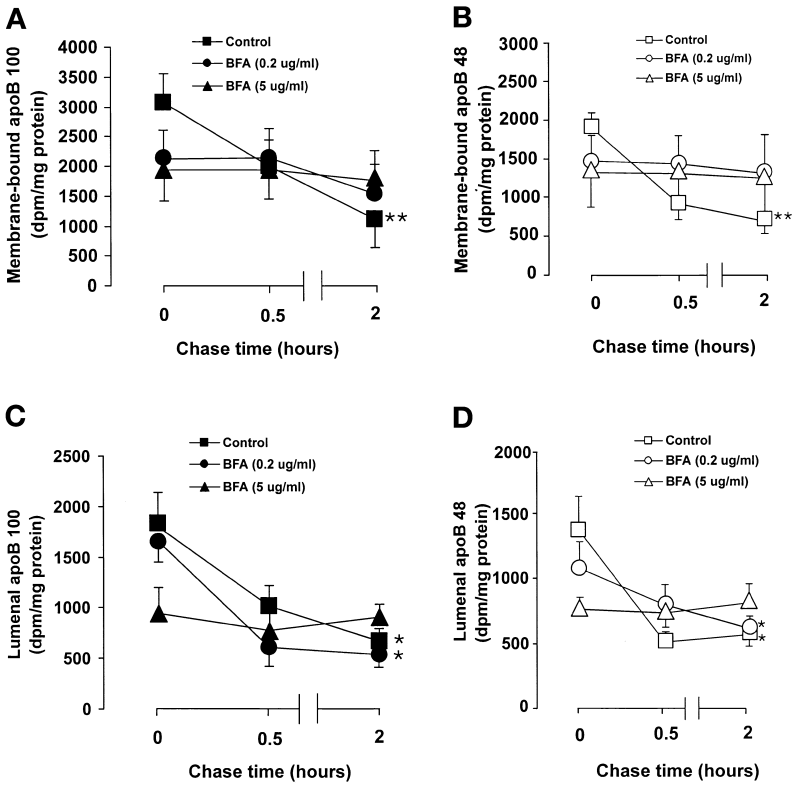

Fig. 3. A low concentration (0.2 μg/ml) of BFA prevents the removal of newly synthesized apoB from the microsomal membrane (A and B) but not from the microsomal lumen (C and D). Cultured rat hepatocytes were treated with or without BFA (0.2 or 0.5 μg/ml) as described in the legend to Fig. 1. After chase periods of 30 and 120 min the cells were harvested and the microsomal fractions were isolated. Membrane and lumenal fractions were separated after treatment with sodium carbonate. Radioactivity associated with newly synthesized apoB-100 and apoB-48 was determined after immunoprecipitation and SDS-PAGE. Each value represents the mean ± SEM of five independent experiments. Values marked with asterisks were significantly different from the corresponding values at zero time (* $P < 0.05$ and ** $P < 0.01$).

apoB-48 and apoB-100 of 63 ± 3.6% and 64 ± 6.2%, respectively ($P < 0.01$ in each case), after 2 h compared with the amounts present after the pulse label. In the presence of BFA (0.2 μg/ml) the corresponding values were 11 ± 6% and 27 ± 7.8% (NS). Increasing the concentration of BFA in the medium to 5 μg/ml blocked the secretion of apoB as the small, HDL-like particles, as well as VLDL apoB, and had no further effect on the removal of membrane-bound apoB. Both concentrations of BFA (0.2 and 5 μg /ml) also decreased the amount of label associated with the membranes at the end of the pulse. Compared with the membrane, smaller amounts of apoB appeared in the lumen at the end of the pulse (i.e., the start of the chase period). Also, the effects of BFA on the pattern of apoB release from the microsomal lumen differed from that in the membrane (Fig. 3C and D). For instance, with BFA at 0.2 μg/ml, which almost completely blocked apoB loss from the microsomal membrane, there remained a substantial removal from the lumen. Thus, in the lumen of the control cells, the amounts of apoB-100 and apoB-48 removed during the 2-h chase were 67 ± 3.7% and 59 ± 3.7%, respectively. With BFA at 0.2 μg/ml, these values were 63 ± 5.6% and 43 ± 7%, which were not significantly different from the control values. Substantial inhibition of apoB removal from the lumen was achieved only at a BFA concentration of 5 μg/ml, a concentration identical to that required to inhibit the secretion of HDL apoB. Although under these conditions there was a decreased amount of labeled apoB in the lumen at the end of the pulse, there were virtually no losses of either apoB-48 or apoB-100 during the 2-h chase period. Because measurements were not made between 0 and 30 min of chase, the pulse maximum level of incorporation of label may have occurred during this period as a result of apoB chain elongation. The percentage decreases calculated above, therefore, may represent minimal values.

The distribution of labeled albumin between the membranes and lumen of the microsomes was different from that of apoB. In this case, most of the label was associated with the lumen at the end of the 1-h pulse (**Fig. 4A** and **B**). Most of this material (82 ± 3% of initial) was transported out of the lumen during the 2-h chase in the absence of BFA. The low (0.2 μg/ml) concentration of BFA had little effect on the removal of lumenal albumin (65 ± 6% of initial removed after 2-h chase). In the presence of the higher (5 μg/ml) concentration of BFA, transfer of labeled albumin out of the lumen was almost completely blocked. The effects of BFA on the pattern of removal of the smaller amounts of labeled albumin from the membrane were similar to those in the lumen. In this case, again, there was little effect of BFA at 0.2 μg/ml, but removal was almost completely abolished at the higher BFA concentration (Fig. 4).

Restoration of VLDL assembly from apoB occurs in parallel with an increased loss of membrane-associated apoB

It has been reported previously that in McArdle RH-7777 cells, the BFA-mediated inhibition of VLDL assembly can be reversed by removal of BFA from the medium of the cells, provided that fatty acid is present (2, 6, 15). To investigate whether primary hepatocyte cultures, which assemble VLDL from endogenous lipid stores, behaved in a similar way, cells that were preincubated and pulse labeled

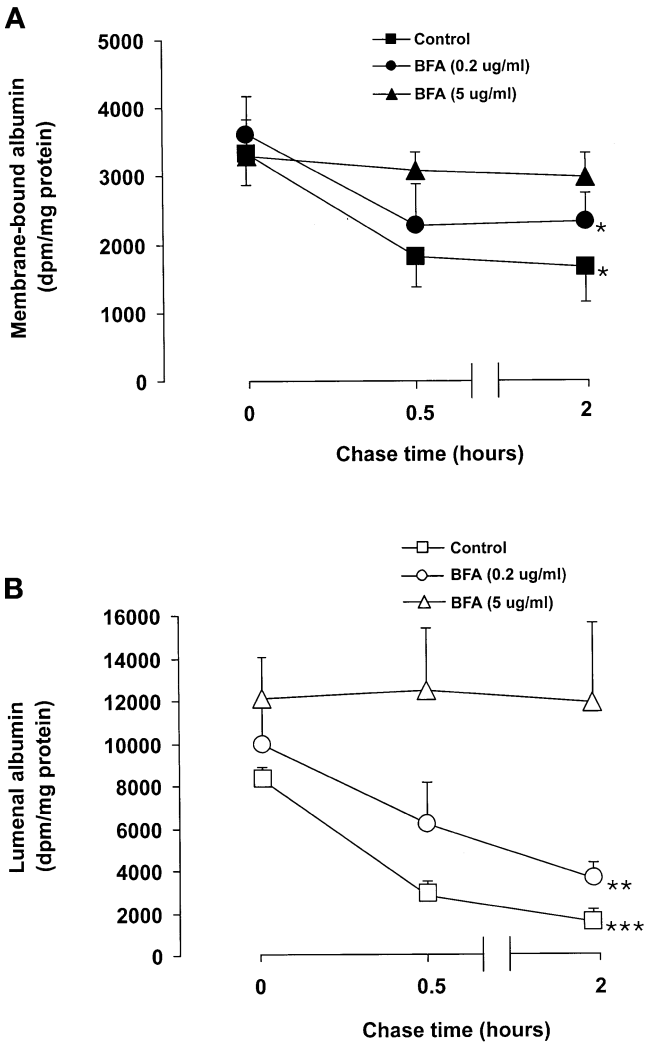

Fig. 4. Effects of BFA (0.2 and 5.0 μg/ml) on the removal of newly synthesized albumin from the microsomal membranes and lumen. Cells were cultured and treated with or without BFA (0.2 or 5.0 μg/ml) according to the legend to Fig. 1. At 30 and 120 min of chase cells were harvested and the microsomes were isolated. The lumenal and membrane fractions were obtained as described in the legend to Fig. 3. Labeled albumin was obtained from each fraction by immunoprecipitation. Each point represents the mean ± SEM of four independent determinations. Values marked with asterisks were significantly different from the corresponding values at zero time (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$), respectively.

for 1 h with BFA (5 μg/ml) were transferred to a BFA-free medium and chased for 2 h. As in all the previous experiments, extracellular fatty acid was not present. The secretion rates of labeled VLDL apoB and HDL apoB from these cells were compared with those from cells in which BFA had been present during both the pulse label and the chase. **Figure 5** shows that, when BFA was removed at the start of the 2-h chase, the secretion of VLDL apoB-48 and VLDL apoB-100 increased 3- to 5-fold compared with that observed when BFA was present throughout (Fig. 5C). Changes in the subcellular distribution of apoB-48 and apoB-100 were also examined under these conditions. Thus, when BFA was present during the chase, there was a negligible loss of microsomal membrane apoB-100 and apoB-48. These losses increased to 44% and 33% of that present at the end of the pulse when VLDL assembly and secretion were restored after removal of BFA (Fig. 5A). Subfractionation of the lumenal contents of the microsomes showed that, in cells pulse labeled in the presence of BFA, only minute fractions of the total apoB-48 and apoB-100 were present in particles with the density of VLDL. Most of the apoB appeared in particles in the HDL density range (Fig. 5B). When BFA (5 μg/ml) was present during the chase, disappearance of both VLDL apoB and HDL apoB from the lumen was prevented. If, however, the cells were transferred to a BFA-free medium after a pulse label in the presence of BFA, apoB HDL disappeared from the lumen during the subsequent 2-h chase (Fig. 5B). As mentioned above, this pattern was accompanied by a disappearance of membrane-associated apoB. However, the disappearance of relatively large quantities of labeled apoB from both the membrane and lumenal HDL was not accompanied by a significant increase in the amounts of labeled VLDL apoB in the lumen, as might have been expected from a precursor-product relationship. Even when cells were pulsed in the absence of BFA, lumenal VLDL constituted only a fraction of the total lumenal apoB (20.9 ± 5.9% for apoB-100 and 25.2 ± 8% for apoB-48) (**Fig. 6**). This proportion did not change significantly during a 0.5-h chase (17.7 ± 3.3% for apoB-100 and 32 ± 9% for apoB-48) or after a 2-h chase (15.6 ± 6.9% and 17 ± 10%, respectively) even though there was a substantial loss of labeled apoB-100 and apoB-48 from the microsomal membrane during this period (Fig. 3A and B).

Changes in the specific radioactivities of apoB associated with the microsomal membrane and the microsomal lumen suggest that these two pools do not equilibrate

In a further series of experiments we sought to establish the relationship between apoB associated with the microsomal lumen, with the microsomal membrane, and with secreted HDL and VLDL apoB by comparing changes in their specific radioactivities at various times during a 16-h chase after a 1-h pulse label. Microsomes were prepared at the end of the 1-h pulse label (zero time) and then at 1, 2, 4, and 16 h during a chase in the presence of unlabeled methionine. BFA was not present at any point during these experiments. At each time point the microsomal membrane was separated from the lumenal contents by treatment with sodium carbonate. At corresponding time points the medium was retained and separated into fractions of VLDL density and HDL density. In each case total mass of apoB (apoB-100 plus apoB-48) was determined by ELISA and total apoB radioactivity was determined by summation of the label associated with apoB-100 and apoB-48 after SDS-PAGE. **Figure 7** shows that the pattern of the time-dependent changes in the specific radioactivity of VLDL apoB differed from that of HDL apoB. For instance, whereas the specific activity of the VLDL apoB decreased sharply between 2 and 16 h (after a small increase between 1 and 2 h), reaching 27% of the initial (1-h) value after 16 h, that of the HDL apoB did not decrease

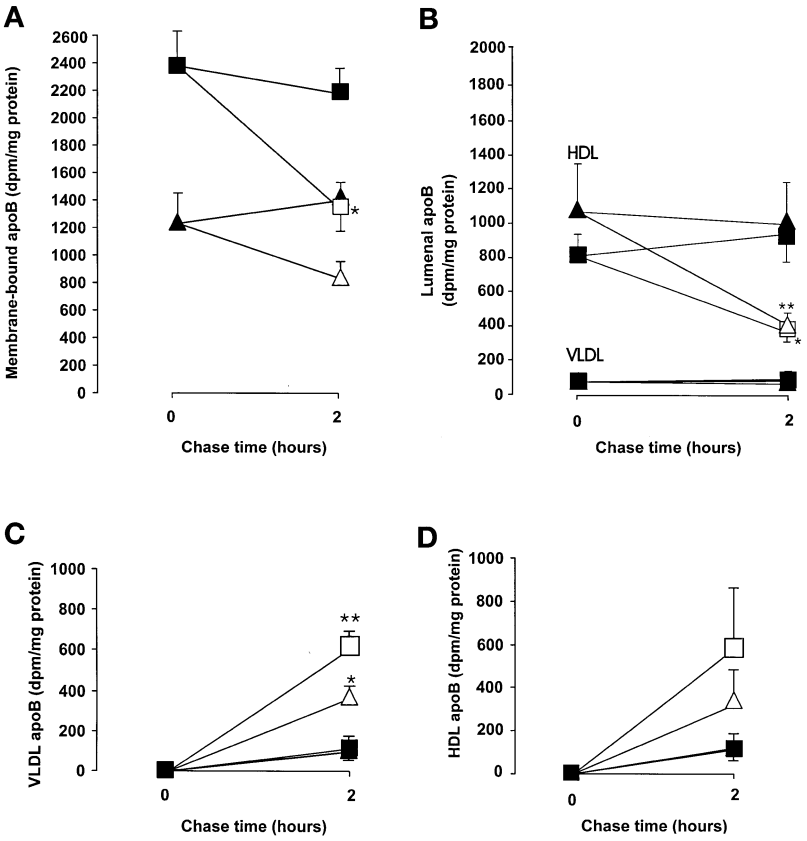

Fig. 5. Removal of BFA from the culture medium restores VLDL apoB secretion but does not increase VLDL apoB in the microsomal lumen. Cells were cultured for 20 min in the presence of BFA (5 μg/ml) and then pulse labeled for 1 h with [^{35}S]methionine and BFA. The medium was removed and the cells were chased for 2 h either with (closed symbols) or without (open symbols) BFA (5.0 μg/ml). Cells were harvested and the medium was retained for separation of the VLDL (C) and HDL (D) fractions. The microsomal fraction of the cells was isolated and the membrane (A) and lumenal (B) fractions were obtained as described in the legend to Fig. 3. Labeled apoB-100 and apoB-48 associated with the membrane were determined. The microsomal lumenal contents were separated into VLDL- and HDL-containing fractions and the radiolabel associated with apoB-100 and apoB-48 in each fraction was determined. Each value represents the mean ± SEM of three independent experiments. Values marked with asterisks are significantly different from those in which BFA was present during the chase (* $P < 0.05$ and ** $P < 0.01$, respectively). ApoB-100 (solid and open squares); apoB-48 (solid and open triangles).

from the initial value during the 16-h chase. The pattern of change in the specific activity of the microsomal membrane apoB resembled that of the VLDL apoB although at all time points the latter value was considerably lower. Thus the final specific activity of the membrane apoB was only 23% of the initial value at the end of the pulse. By contrast, there was relatively little change in the specific activity of the lumenal apoB and the final value was 80% of the initial value at the end of the pulse. This pattern of change broadly resembled that of the secreted HDL apoB rather than that of the secreted VLDL apoB.

DISCUSSION

The biosynthesis of VLDL from newly synthesized apoB consists of a number of distinct stages (1–8, 15, 22). In the first of these, apoB cotranslationally or posttranslationally becomes associated with a small amount of lipid, resulting in a small, secretion-competent VLDL precursor. This step probably requires, at some point, an interaction between newly translated apoB and lipids in the membrane of the ER (6, 15). The other precursor of VLDL consists of an apoB-free lipid droplet (1, 7–9) that, in primary hepatocytes, is recruited into the secretory pathway by lipolytic mobilization of intracellular triacylglycerol (36). The final stage of VLDL production requires the fusion of these precursors to give large, triacylglycerol-rich particles of mature VLDL. Within this broad framework, the detailed aspects of each of the component stages of VLDL assembly remain to be elucidated, although the overall process is suppressed by insulin (37). One of the most important of these unresolved issues concerns the location of the intracellular site that accommodates the fusion of the VLDL precursors. Evidence has been presented that supports the involvement of the rough ER (RER) (11); smooth ER (SER) (1, 7); a specialized post-RER, pre-Golgi compartment (38); and Golgi (13, 14, 39) for the site of VLDL maturation. Important advances have been made in this area by Olofsson and colleagues (6, 15, 40), who have developed a methodology that permits the study of each of the steps of VLDL assembly independently in the McArdle RH-7777 cell line. These authors showed that a low concentration of the fungal metabolite BFA was able to specifically block the bulk addition of neutral lipid to the apoB-containing VLDL precursor. Under these conditions the assembly and secretion of VLDL apoB were inhibited but the secretion of dense, HDL-like particles containing apoB remained constant. At this low concentration of BFA, the functional integrity of the secretory apparatus remained intact. Under these conditions, apoB remained associated with the microsomal membrane (15) and could be removed as small, HDL-like particles by treatment with deoxycholate (6, 40). Removal of BFA from the medium was accompanied by disappearance of membrane-associated apoB and VLDL apoB secretion was restored. These authors concluded that membrane-associated apoB-containing particles were the precursors for the bulk lipid

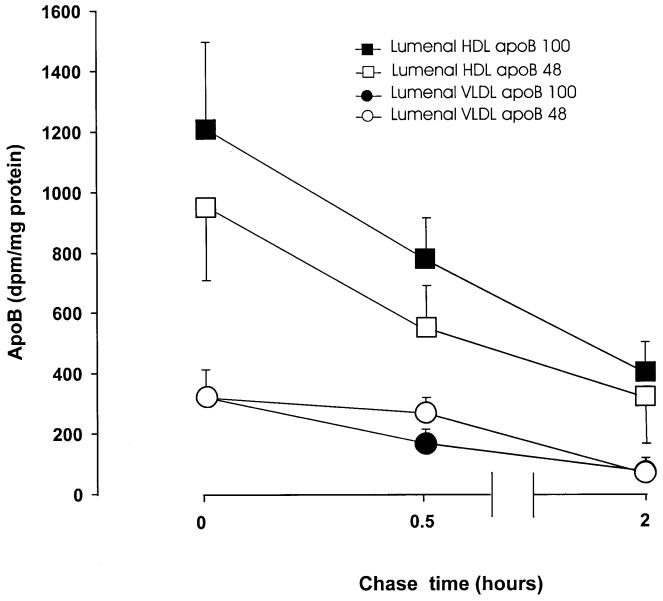

Fig. 6. Disappearance of microsomal membrane-associated apoB is not accompanied by an increase in the lumenal content of VLDL apoB. Rat hepatocytes were cultured, pulse labeled, and chased in the absence of BFA as described in the legend to Fig. 1. At 30 and 120 min of chase, the microsomal lumenal contents were obtained as described in the legend to Fig. 3 and were separated into fractions containing VLDL and HDL by ultracentrifugation. The amounts of labeled apoB-48 and apoB-100 in each fraction were then determined. Each point represents the mean ± SEM of four independent experiments.

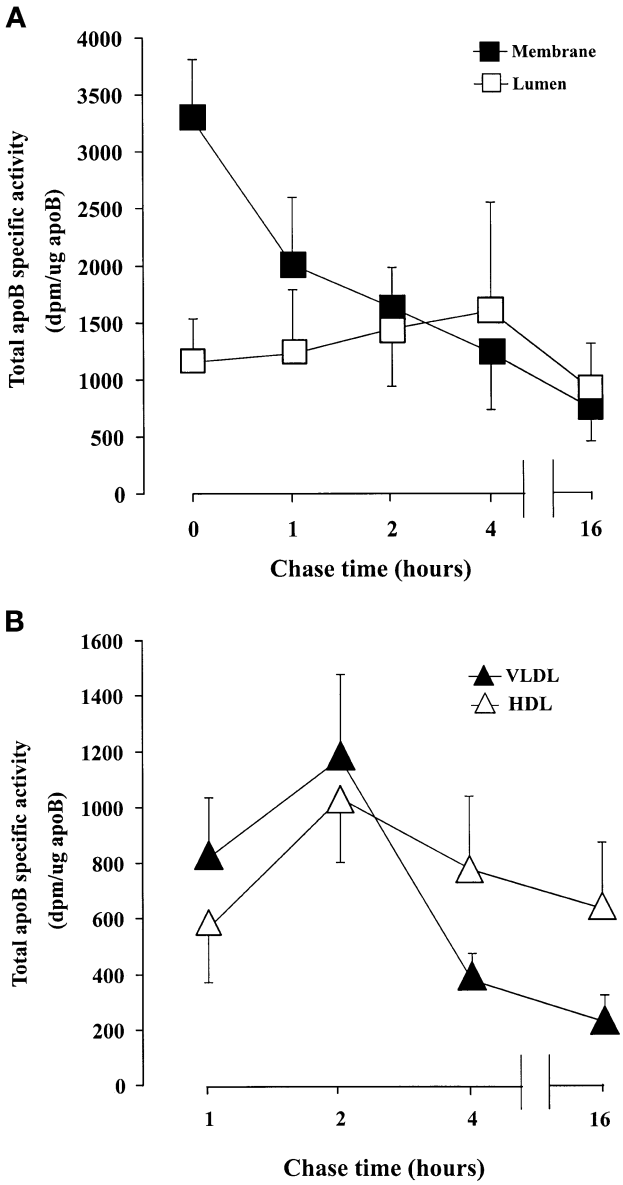

Fig. 7. Differences in specific radioactivities of newly synthesized apoB in the microsomal membranes and lumen (A) and secreted HDL and VLDL (B) during a pulse chase. Rat hepatocytes were cultured and pulse labeled in the absence of BFA as described in the legend to Fig. 1. Cells from some dishes were harvested at the end of the pulse label. Others were chased for periods ranging from 1 to 16 h. At the end of each chase period cells were harvested and the medium was retained. The medium was separated into fractions containing VLDL and HDL. Microsomes were obtained from the cells and separated into membrane and lumenal contents. The total mass of apoB in the microsomal membranes, lumen, and secreted VLDL and HDL fractions was determined by ELISA. The total radioactivity associated with the apoB-48 and apoB-100 in these fractions was obtained by summation of the amounts of label in the individual apoB-48 and apoB-100 bands after electrophoresis. Each point represents the mean ± SEM of four independent experiments.

addition step that culminated in the production of mature VLDL.

In the present work, we have adapted this methodology for the study of VLDL assembly in primary cultures of hepatocytes that, unlike McArdle RH-7777 cells, are able to utilize endogenously stored triacylglycerol for the maturation step of VLDL assembly (41). These results show that a low (0.2 μg/ml) concentration of BFA remained capable of specifically blocking utilization of endogenous lipid stores for the maturation phase of VLDL assembly in primary hepatocyte cultures (Fig. 1A and B). The secretion of apoB-100 HDL and apoB-48 HDL was resistant to inhibition at this low concentration of BFA (Fig. 1C and D), as was the secretion of newly synthesized albumin (Fig. 2). The specific inhibition of VLDL apoB secretion under these circumstances was accompanied by an almost complete blockage of labeled apoB-100 and apoB-48 transport out of the microsomal membranes, which were obtained from a mixture of RER- and SER-derived vesicles (Fig. 3A and B). There was no inhibition of apoB-100 and apoB-48 transfer out of the microsomal lumen (Fig. 3C and D). Most of this newly synthesized lumenal apoB was present as HDL at the end of the pulse (Fig. 5B). A BFA concentration of 5 μg/ml was required to suppress both the secretion of apoB HDL (Fig. 1C and D) and the removal of labeled apoB HDL from the microsomal lumen (Fig. 5B). The almost complete blockage of albumin secretion (Fig. 4) suggested that, under these conditions, the functional integrity of the secretory apparatus had been compromised. In the present work, the efficiency of the sodium carbonate extraction procedure in releasing lumenal protein was shown by the recovery of approximately 80% of

the total microsomal albumin in the lumen after sodium carbonate treatment (Fig. 4).

In McArdle RH-7777 cells, the BFA-mediated suppression of VLDL maturation was reversed when BFA was removed from the medium after the apoB-containing VLDL precursor had been synthesized (15). This finding was exploited in the present work to establish the kinetics of intracellular apoB metabolism after restoration of the VLDL maturation stage in primary cultures of rat hepatocytes. The results obtained show that when apoB was pulse labeled for 1 h in the presence of BFA (5 μg/ml), removal of BFA during a subsequent 2-h chase increased the secretion of both VLDL apoB-48 and VLDL apoB-100 (Fig. 5C). Under these conditions, transfer of labeled apoB-48 and apoB-100 out of the microsomal membrane was also increased. The apparent precursor-product relationship between microsomal membrane apoB and secreted VLDL apoB was not accompanied by an increased amount of VLDL apoB in the microsomal lumen (Fig. 5B). In this compartment, the major consequence of BFA removal was a decrease in the absolute amount of labeled apoB HDL. Thus, when conditions favorable for VLDL maturation from its apoB-containing precursor were restored, labeled apoB lost from either the microsomal membrane or from lumenal HDL apoB did not appear in lumenal VLDL. If, under these conditions, microsomal lumenal VLDL was indeed a precursor of secreted VLDL, then the turnover of the latter must have been high. This would appear unlikely in view of a kinetic study of rat hepatocytes, which suggested that transfer of apoB out of the ER is the rate-determining step in the overall secretion of VLDL (12). This latter observation may explain the low level of VLDL apoB in the microsomal fraction. Furthermore, in another experiment in the absence of BFA, when apoB was pulse labeled for 1 h, the small proportion of apoB present in the microsomal lumen as VLDL did not increase during a short (0.5-h) chase (Fig. 6).

The absence of a precursor-product relationship between the bulk membrane-associated pool of apoB and lumenal apoB in the microsomal fraction was supported by the results shown in Fig. 7, which shows that these two pools become metabolically distinct soon after the synthesis of apoB. The large decline in the specific radioactivity of secreted VLDL apoB mirrored that of the membrane-associated pool (Fig. 7A) whereas that of lumenal apoB remained relatively constant (Fig. 7B). The small amounts of labeled apoB in the microsomal lumen probably represent apoB that has become prematurely uncoupled from the major pathway of VLDL assembly at some early stage in the microsomal membrane and that is released into the lumen by sodium carbonate treatment. The relative constancy of the specific radioactivity of the lumenal apoB pool reflects the relatively small changes observed in the specific activity of the secreted apoB-HDL fraction.

Because we made no distinction between the specific activities of apoB-100 and apoB-48 in this experiment, the above-described interpretation assumes that both species behave similarly during the assembly of VLDL. On the basis of evidence from McArdle cells (15) this might not be entirely correct. Nevertheless, in the present case, there did not appear to be a selective removal of either isoform of apoB from the microsomal membrane during a pulse chase, as evidenced by the constancy of the labeled apoB-100:apoB-48 ratio in the membrane, and that of the secreted VLDL (**Table 1**).

TABLE 1. ApoB-100:apoB-48 ratios of microsomal membrane lumenal VLDL and secreted VLDL

Time after Pulse	Microsomal Membrane	Secreted VLDL
(*min*)		
0	2.01 ± 0.38	
30	1.84 ± 0.73	2.25 ± 0.38
120	1.90 ± 0.37	2.33 ± 0.46

Cells were cultured, pulse labeled, and chased in the presence or absence of BFA as described in the legend to Fig. 1. The labeled apoB-48 and apoB-100 contents of the microsomal membrane, microsomal lumen VLDL, and secreted VLDL were determined as described in the legends to Figs. 3 and 5. Each value is the mean ± SEM of four independent experiments.

In summary, the present results suggest that the maturation phase of VLDL assembly in primary cultures of rat hepatocytes can be driven by an apoB-free pool of neutral lipid derived from endogenous stores rather than from exogenous fatty acids. If the mechanism involved in the assembly of VLDL in primary hepatocytes is similar to that which occurs in McArdle RH-7777 cells, then membrane-associated HDL apoB is the precursor for the bulk lipid addition step (40). At which point in the secretory apparatus this event takes place is not clear. Previous pulse-chase studies in rat hepatocytes have suggested that apoB is normally transferred between ER and Golgi membranes and that this process is interrupted by orotic acid consumption (28). The present results also suggest that if the membrane-associated apoB HDL becomes derailed from the normal assembly process and enters the microsomal lumen, then it becomes inaccessible to the VLDL lipid precursor pool and maturation cannot take place. Instead, these lumenal particles are the precursors of secreted apoB HDL. In general, the present study provides further support for the view that the small, apoB-containing precursor of VLDL is associated with the microsomal membrane of the cell (2, 6, 15, 22, 40).

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