DISSOCIATION BETWEEN RATE OF HEPATIC LIPOPROTEIN SECRETION AND HEPATOCYTE MICROTUBULE CONTENT

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ABSTRACT

The fact that colchicine inhibits hepatic secretion of very low density lipoprotein (VLDL) particles has been interpreted to mean that microtubules are involved in hepatic VLDL secretion. To further define this relationship, we have attempted to see if changes in hepatic VLDL secretion are associated with changes in hepatocyte microtubule or tubulin content. Accordingly, hepatic secretion of VLDL was increased in rats, and the hepatocyte content of both microtubules (using quantitative morphometric methods) and tubulin (using a time-decay colchicine binding assay) was determined. In acute experiments, VLDL secretion was increased by perfusion of isolated rat livers for 2 h with varying concentrations of free fatty acids (FFA). Results indicate that hepatic VLDL triglyceride (TG) secretion at perfusate FFA levels of 0.7 μEq/ml is threefold greater ($P < 0.01$) than when livers are perfused without added FFA. However, no differences are observed in the content of microtubules in these livers: specifically, microtubules occupy 0.029% of hepatocyte cytoplasm in livers perfused without FFA and 0.030% of cytoplasm in livers perfused with FFA. In chronic experiments, rats were fed for 1 wk with either standard rat chow or a hyperlipidemic (sucrose/lard) diet. With the experimental diet, plasma triglyceride levels increase threefold over controls, and liver VLDL-TG production, as determined by [^{3}H]glycerol turnover studies, is 55% greater ($P < 0.01$) than controls. However, microtubules occupy 0.027% of the cytoplasm of hepatocyte cytoplasm whether rats are on standard or hyperlipidemic diets. Furthermore, the tubulin content of isolated hepatocytes does change, and represents 1% of hepatocyte soluble protein, irrespective of diet. These results suggest that increases in hepatic VLDL secretion can occur without any demonstrable change in hepatocyte assembled microtubule or tubulin content, and raise questions as to the role played by microtubules in hepatic VLDL secretion.

KEY WORDS microtubule · tubulin · hepatocytes · very low density lipoprotein secretion · hypertriglyceridemia

Very low density lipoproteins (VLDL) are important secretory products of the liver (4, 7, 8, 18). Although the pathway of synthesis, intracellular transport and secretion of these particles has been well defined, the factors regulating their intracellular translocation and secretion are still largely unknown. However, circumstantial evidence has led to the suggestion that microtubules are in-

volved in this process. The evidence for this view is based largely on the fact that the administration of colchicine and vinblastine sulfate, agents known to interfere with the assembly of microtubules, markedly inhibit the release of VLDL from liver under in vitro (9, 11) and in vivo (16, 19) circumstances. Furthermore, if these agents are added to the perfusate during liver perfusion, there is a loss of microtubules from hepatocytes associated with the accumulation of VLDL particles in Golgi vacuoles throughout the cells (11, 16). By implication, these observations suggest that VLDL intracellular transport and secretion (but not VLDL production) is affected by the loss of microtubules. Other basic functions of the liver (such as oxygen consumption, maintenance of ATP levels and glucose and urea production) remain normal after liver perfusion with antimicrotubule agents (16), providing additional evidence for the view that the effect of colchicine and vinblastine on hepatic VLDL secretion is a specific one and is related to their effects on microtubules.

In this present study, we have attempted to further define this relationship between microtubules and hepatic VLDL secretion. Specifically, we have attempted to see if there is a relationship between changes in hepatic VLDL secretion and hepatocyte microtubule content. For this purpose, we have used a combined morphometric and biochemical approach to identify and quantitate hepatocyte microtubules and tubulin in animals in which acute and chronic increases in lipoprotein secretion were experimentally induced.

MATERIALS AND METHODS

Experimental Protocol

Male Sprague-Dawley rats were used for all experiments. Before study, the rats were fed standard rat chow and maintained on a 12-h light/dark (6 a.m./6 p.m.) cycle. Hepatic lipoprotein secretion in these animals was altered by two methods: (*a*) in the first procedure, livers from fed 250-g rats were perfused at different free fatty acid (FFA) levels in order to acutely modify rates of VLDL triglyceride (TG) production and secretion. Perfusate samples were removed at regular intervals in order to determine VLDL-TG secretion rate, and liver samples were obtained at the end of the perfusion period for electron microscope morphometric analyses; (*b*) in the second procedure, rats weighing 180–200 g were fed either standard rat chow or a diet (Teklad Test Diets, Madison, Wis.) which produces chronic hyperlipoproteinemia in rats (2). The experimental diet contained 350 calories/100 g chow; diet constituents (as percent calories) were 66% sucrose, 12% lard, and 22% casein. Standard rat chow has a similar number of calories/gram, but contains (as percent calories) 60% vegetable starch, 11% unsaturated oils, and 29% animal proteins. Rats were fed the different diets from 1–4 wk. On the final day of the diet, food was removed from the cages between 7 and 8 a.m., and experimental procedures were begun 5 h later. Analysis of tail blood samples indicated that approx. 80% of the rats on the experimental diet developed hypertriglyceridemia (serum TG levels > 200 mg/100 ml), and only these rats were studied further. These rats were used for two purposes. In some animals, VLDL-TG turnover rates were carried out to confirm the fact that the diet-induced hypertriglyceridemia was due to increased production and secretion of VLDL. Other animals were simply decapitated, blood was taken for TG determination, and liver samples were removed for tubulin and microtubule analyses.

Measurements

PERFUSION STUDIES: Livers from fed rats were perfused in an *in situ* cyclic perfusion system by techniques previously described by Mondon and Burton (10). The basal perfusing medium consisted of a filtered mixture of fresh 90% defibrinated rat blood and Krebs-Ringer's bicarbonate buffer (containing 3 g of bovine serum albumin/100 ml), which was recycled through the liver at flow rates of 1.0 ml/min/g liver. Livers perfused with this basal mixture produced and secreted only minimal amounts of VLDL-TG. In order to increase hepatic production and secretion, the basal perfusing medium was enriched with a physiological concentration of a free fatty acid (oleic acid) prepared in recycled rat serum. The concentration of oleic acid averaged 160 μmol/ml and was infused at a rate of 1.68 ml/h; under these conditions, perfusate FFA levels averaged 0.6–0.8 μEq/ml during the course of the experiment. Perfusate samples of 1.0 ml were removed from the recycling perfusate of all rats at 0, 30, 60, 90, and 120 min and were stored at −20°C for subsequent FFA and TG determinations. Mean FFA levels were determined by averaging the 30–90-min values of each liver. Net VLDL-TG production was calculated from TG changes in perfusate concentration corrected for sampling losses and infusate additions at each time-point between 30 and 90 min; these values are expressed as milligrams of TG secreted/h/g liver weight. At the end of each perfusion, liver samples from the perfused animal were obtained for electron microscopy.

In order to determine the size of the lipoprotein particles secreted during hepatic perfusion, particles of density <1.006 were isolated from the perfusate, negatively stained, and subjected to diameter measurements as described in a previous publication from this laboratory (15).

TG TURNOVER STUDIES: VLDL-TG turnover rates were determined by following the rate of removal

of prelabeled VLDL-TG from the plasma. This approach has been described and validated in a previous publication (6). In brief, rats on the standard or hyperlipidemic diets were injected with plasma containing VLDL-TG which had been prelabeled in vivo with [^{3}H]glycerol. After the administration of the labeled VLDL-TG, the tail was amputated proximal to the site of injection, and 0.4 ml of blood was collected into capillary tubes rinsed with a 5% EDTA solution at approx. 5, 10, 15, and 20 min after the injection. (These samples took 1–2 min to collect and, in all subsequent calculations, the time used for a collection period was the mean of the beginning and ending times of the blood collection.) The plasma was separated by centrifugation and stored frozen until analyzed. A lipid extract was made of each sample, evaporated to dryness, and the radioactivity was measured by liquid scintillation counting (Beckman LS-235, Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) using a standard toluene scintillation mixture. The half-time ($t_{\frac{1}{2}}$) of VLDL-TG removal was directly determined from these measurements by a least squares linear regression analysis, and the VLDL-TG turnover rate was calculated from the following formula:

$$\text{VLDL-TG turnover rate} = (\ln_2 \div t_{\frac{1}{2}}) \times$$

$$\text{(Plasma TG concentration)} \times \text{(plasma volume)}.$$

Since these studies were carried out under steady-state conditions, VLDL-TG turnover rate = VLDL-TG removal rate = VLDL-TG secretion rate. For the purposes of this communication, we shall subsequently refer to this measurement as VLDL-TG secretion rate.

Lipoproteins of density <1.006 were isolated from the serum of rats on standard and hyperlipidemic diets and analyzed for differences in diameter (15).

MORPHOMETRIC PROCEDURES: Samples of tissue from the left lobe of the liver were obtained from rats at the conclusion of perfusion experiments or after the animals had consumed the different diets for 1 wk. The liver was finely minced and submerged in 2% glutaraldehyde (0.1 M cacodylate, pH 7.0, 22°C) overnight. The following day, the tissue was postfixed for 1 h in 1% osmium tetroxide in Palade's veronal buffer (pH 7.0) and stained en bloc for 1 h with 2.0% uranyl acetate in veronal buffer (pH 5.5). The blocks were subsequently dehydrated and embedded in Epon-Araldite plastic. Periportal regions were identified, and thick (about 0.5 μm) and thin (about 0.05 μm) sections were cut from one block of each liver sample.

Thick sections from each block were stained with toluidine blue, and estimates of cell size cell were obtained by length (L) and width (W) measurements on 50 cells from each block. The mean cell diameter was obtained by application of the formula, $\sqrt{L \times W}$ for each cell measured.

For ultrastructural morphometry (estimation of microtubule content), two photographs were taken at × 16,000 of each of six nucleated cells from each block; to avoid bias, photographs were taken at 12 and 6 o'clock of each cell without regard to the organelle composition of the region of the cell being photographed. One additional photograph was obtained from a Golgi region of each cell. Quantitative estimates of the fraction of the cytoplasmic volume occupied by microtubules in randomly selected areas (as well as in the Golgi area of hepatocyte) were obtained by point counting stereological techniques (22), as has been previously applied to microtubules in this laboratory (12, 13, 14, 20).

TUBULIN ASSAY: To determine the tubulin content of cells from control rats and rats on the hyperlipidemic diet, isolated hepatocytes were obtained by *in situ* perfusion of livers with collagenase (50 mg/100 ml) in modified Swim's S77 medium (pH 7.4, 37°C, 95% O_2–5% CO_2) as described in previous communications (5, 12). Hepatocytes from one control and one experimental rat were obtained simultaneously (through the use of a multichannel perfusion box) and subjected to analysis on the same day. Hepatocytes prepared in this fashion showed greater than 90% viability by the trypan blue exclusion test, and ultrastructural morphometric analyses indicated that the separated cells survived the isolation procedure without significant morphological damage, i.e., values for the mean volume densities of mitochondria, lysosomes, peroxisomes, rough and smooth endoplasmic reticulum, and microtubules were identical to values obtained for cells from glutaraldehyde-perfused livers (12).

For tubulin quantification, washed hepatocytes were suspended in glutamate-phosphate buffer at 4°C and homogenized until no intact cells were visible with the light microscope. Undiluted extracts were centrifuged at 100,000 *g* for 45 min, and the soluble fraction were utilized for determination of colchicine-binding activity. Aliquots of the cell supernates were incubated with [*methyl*-^{3}H]colchicine (sp act = 0.5 Ci/mol), and time decay binding reactions were carried out in phosphate-glutamate buffer as previously described by Wilson et al. (23) and as recently carried out on isolated liver cells from control rats in our laboratory (12). In brief, aliquots of the soluble supernate from cells were allowed to undergo degradation with time: a series of four samples were taken 2 h apart while the extracts were decaying and each sample was then incubated with colchicine (2×10^{-6} M) for 2 h at 37°C. Binding mixtures contained 1×10^{-5} M vinblastine sulfate to help stabilize colchicine-binding activity. Subsequently, aliquots of the incubation mixture was passed through 1 × 13 cm columns of BioGel P10 to separate bound colchicine complex from free colchicine, and the radioactivity of the bound fraction was determined by liquid scintillation counting. The initial colchicine-binding capacity (IBC) of the fractions was determined from extrapolation of the decay points to zero time of incubation. A previous study on hepatocytes had indicated that, under the conditions of these experiments, the

majority of the colchicine-binding activity found in the soluble cell extract was, in fact, due to tubulin (12). As such, the quantity of tubulin present in the hepatocyte soluble extract could be estimated by comparing the IBC of extracts of hepatocytes with the IBC of the soluble extracts of embryonic brain determined under identical conditions (23); thus,

$$\%\ \text{Tubulin in hepatocyte supernatant} = \frac{\text{IBC hepatocyte supernatant}}{\text{IBC chick brain supernatant}} \times 42\%.$$

TG and FFA levels: Serum TG levels were determined by an enzymatic method based on Wahlfeld's technique (3) and FFA levels by the colorimetric method of Akio et al. (1).

RESULTS

Acute Stimulation of Hepatic TG Secretion

As indicated in Table I, the level of FFA with which livers are perfused has a dramatic effect on hepatic VLDL-TG secretion rate. When livers from normal rats are perfused at a low FFA concentration, the VLDL-TG secretion rate is 0.64 mg/h/g liver. When physiological levels of oleic acid (approximately that found in serum from 24-h fasting rats) are added to the perfusing medium, the VLDL-TG secretion rate is increased to a level of 1.93 mg/TG/h/g liver.

The observed threefold increase in TG secretion is primarily the result of an increase in VLDL particle number, since no substantial increase in particle size occurs. The mean VLDL diameter of 100 randomly obtained particles from the perfusate of each of two animals infused with low levels of FFA is 0.047 μm; similar measurements on particles from two rats infused with high levels of FFA show the mean diameter to be 0.048 μm. This difference in VLDL diameter results in a 6% increase in the average volume of the particles secreted by livers of rats infused with high levels of FFA.

Despite the substantial stimulation of hepatic VLDL-TG secretion observed in these experiments, Table I indicates that there is no change in the microtubule content of hepatocytes obtained from the perfused livers. Thus, microtubules occupy approx. 0.03% of the cytoplasm of hepatocytes under basal conditions, and this is not changed when the VLDL-TG secretion rate is tripled. To determine whether hepatocyte microtubules have a specific relationship to the Golgi region of the cell (where nascent VLDL particles are repackaged in preparation for their movement toward the cell periphery), separate analyses of the microtubule content of Golgi areas were carried out. These measurements show that slightly more microtubules are present in the Golgi area than in other areas of the cell, but that the content of microtubules in Golgi regions does not appear to change when the VLDL-TG secretion is increased (mean [±SE] volume density [$\times 10^2$] of microtubules in Golgi areas is 0.033 ± 0.002 as compared to 0.029 ± 0.002 in non-Golgi areas of lives perfused without added FFA; in Golgi and non-Golgi areas of livers perfused with FFA, these values are 0.033 ± 0.003 and 0.030 ± 0.003, respectively). These observations demonstrate that increases in microtubule content of hepatocytes do not accompany acute increases in VLDL secretion in perfused livers.

TABLE I

Effect of Variation in Perfusate FFA Levels on Hepatic VLDL-TG Secretion Rate and on Hepatocyte Microtubule Content (Mean ± SE)

No. of perfusions	FFA levels*	VLDL-TG secretion rate*	Microtubules
	μEq/ml	*mg/h/g liver*	*vol density × 10²*
8	0.12 ± 0.01	0.64 ± 0.17	0.029 ± 0.003
8	0.70 ± 0.04	1.93 ± 0.14†	0.030 ± 0.003

* Based on the mean values determined between 30 and 90 min of perfusion.

† $P < 0.01$ as compared to livers perfused without added FFA.

Chronic Stimulation of Hepatic VLDL-Triglyceride Secretion

Fig. 1 indicates that serum triglyceride levels of animals on the hyperlipidemic diet are increased two-threefold; the onset of the hypertriglyceridemia occurs rapidly (within 3 days of consuming the diet) and remains constant for 4 wk. For convenience, a period of 7 days on the diet was the interval chosen for further experiments.

During this 7-day interval, total body weight gain and food consumption were similar in all animals regardless of diet. No significant differences were observed in liver weight (as related to total body weight) or in hepatocyte size as determined by measurements at the light microscope level.

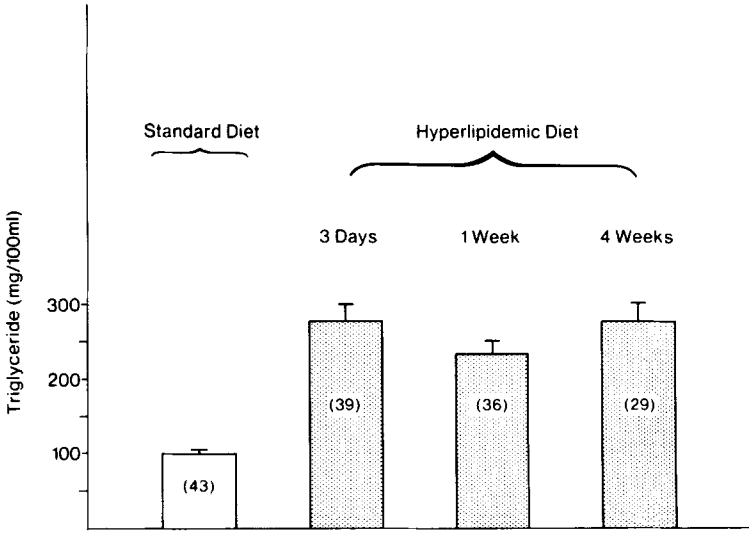

FIGURE 1 Effect of standard and hyperlipidemic diets on mean (±SE) serum TG levels. Food was removed from fed rats at 8 a.m., and blood was obtained for determination of TG levels between 2 and 4 p.m. The numbers in the parentheses refer to the number of rats studied in each group.

In Fig. 2, we have plotted the VLDL-TG secretion rate against the TG concentration that obtained during the determination of VLDL-TG turnover. These results suggest that the rats in this study comprise a single population, in which higher VLDL-TG secretion rates are associated with increases in serum TG levels. Furthermore, since the rats consuming the hyperlipidemic diet had higher levels of both VLDL-TG secretion and TG concentration, it is clear that the cause of their hypertriglyceridemia was a diet-induced increase in VLDL-TG secretion (see also Table II). This increase in VLDL-TG secretion is assumed to be entirely of hepatic origin, since postabsorptive intestinal TG secretion was found to be unaffected by the change in diet (our unpublished observation). In addition, this increase in TG secretion appears to be the result of a primary change in number (rather than size) of VLDL particles. The mean diameter of lipoprotein particles obtained from the serum of two animals on standard diet is 0.047 μm as compared to 0.050 μm for the diameter of particles obtained from two animals on the hyperlipidemic diet. The increase in diameter of VLDL obtained from rats fed the hyperlipidemic diet results in a 21% change in particle volume.

Despite the increases observed in hepatic VLDL-triglyceride secretion in animals on the hyperlipidemic diet, no changes were observed in the microtubule content of hepatocytes from these animals (Table II); hepatocyte microtubule volume density was found to be approx. 0.03% of the cytoplasm regardless of the diet that the animals had consumed. Again, microtubules were 15% more prevalent in Golgi areas than in non-Golgi areas, but these differences were unrelated to diet.

Because of the chronic nature of this portion of the study, it seemed reasonable to question whether the pool size of hepatocyte tubulin had undergone changes in any way related to the observed diet-induced secretory changes. Accordingly, paired time-decay colchicine-binding assays were carried out on the soluble extract of isolated hepatocytes from animals on control and hyperlipidemic diets. The final column in Table II indicates that the initial colchicine-binding capacity (IBC) of the soluble protein of the isolated hepatocytes was not altered as a result of the animals) having consumed the hyperlipiemic diet. When compared with the IBC and tubulin content of embryonic chick brain prepared under identical conditions (12), it appears that tubulin comprises 1% of the soluble protein of hepatocytes whether obtained from animals fed a standard or hyperlipidemic diet.

DISCUSSION

The present study was undertaken in an effort to

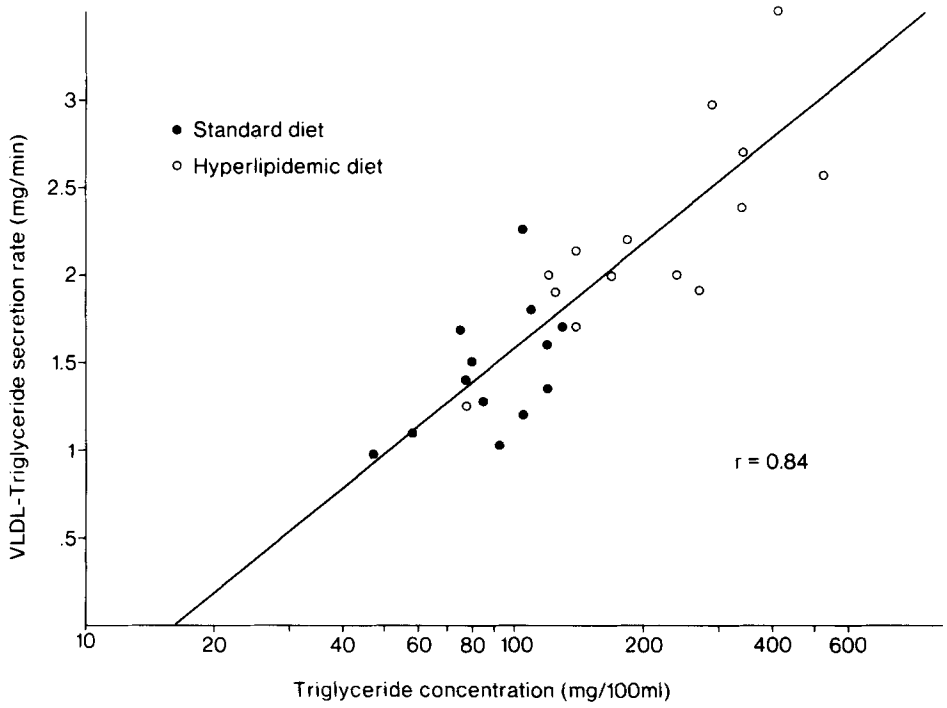

FIGURE 2 The relationship between the logarithm of serum TG concentrations and VLDL-TG secretion rates in rats fed standard (closed circles) or hyperlipidemic diets (open circles). The best fit line was obtained by the least squares techniques, and the correlation coefficient (r = 0.84) between the two variables was highly statistically significant ($P < 0.001$).

TABLE II

Effect of Diet on VLDL-TG Secretion, Hepatic Microtubule, and Tubulin Content (Mean ± SE)*

Diet	VLDL-TG secretion	Microtubules	IBC
	mg/min	*vol density × 10²*	*mol colchicine/μg protein*
Standard diet	1.45 ± 0.10 (n = 13)	0.026 ± 0.003 (n = 13)	$4.79 \times 10^{-14} \pm 0.20 \times 10^{-14}$ (n = 4)
Hyperlipidemic diet	2.24 ± 0.14† (n = 14)	0.027 ± 0.002 (n = 11)	$5.20 \times 10^{-14} \pm 0.32 \times 10^{-14}$ (n = 4)

* Rats were fed different diets for 7 days, after which triglyceride turnover studies were performed or tissue taken for microtubule and tubulin analysis.

† $P < 0.01$ as compared to control.

further define the role that microtubules play in the intracellular transport and secretion of VLDL-TG by liver parenchymal cells. It was our intent to modify hepatic VLDL-TG secretion in individual rats, and to observe changes in the content of microtubules and tubulin of hepatocytes from these animals. In this regard, we took advantage of two well documented facts: (*a*) Liver cells are capable of rapidly responding to the availability of extracellular FFA by increasing FFA uptake and esterification into TG and by increasing the release of this TG in the form of VLDL particles (24); and (*b*) rats fed diets high in carbohydrates and/or in specific sugars such as sucrose or fructose will become hypertriglyceridemic (21). In this manner, acute and chronic animal models for increased VLDL-TG secretion were developed, and hepatocytes from these rats were examined for evidence of changes in cellular microtubule and/or tubulin content. The results of the combined experiments appear to indicate that substantial increases in hepatic acute and chronic secre-

tion of triglyceride can occur without a demonstrable change in hepatocyte content of either the assembled or unassembled form of microtubule protein.

Before commenting upon the significance of these findings, it is essential that we examine the actual experimental observations more closely. The experimental design was based upon the assumption that the increase in triglyceride reaction resulted from an increase in the number of VLDL particles packaged and exported by the liver. However, that need not have been the case, and the observed increase in VLDL secretion could have resulted from an increase in the size of the VLDL-particles, without any change in the number of particles being secreted. In this instance, there would be no reason to expect to see an associated increase in microtubule content, and the relevance of these results to the role played by microtubules in hepatic lipoprotein secretion would be quite different. However, this was not the case. Therefore, formulation as to the functional role fulfilled by microtubules in hepatic lipoprotein secretion must take into account the fact that more VLDL particles were secreted in both experimental situations studied.

A more formidable problem in the interpretation of our results relates to the quantification of hepatocyte microtubule or tubulin content, and caution must be applied to the observation that neither form of microtubule protein increased in content in parallel with the increase in hepatic VLDL-TG secretion. Indeed, there are at least three ways in which changes in microtubule content could have occurred without being detected. In the first place, increases in microtubule turnover (rate of microtubule assembly or disassembly) could have occurred without measurable changes in either microtubule or tubulin content. Secondly, it is possible that our means of preserving and measuring microtubules in tissues is selective for a particularly stable population of microtubules (which may be unrelated to secretory events) or is insensitive to shifts in intermediary polymeric forms of microtubule protein (which might be important to the secretory process). Thirdly, the methods used to increase hepatic VLDL-TG secretion may have led to parallel decreases in other hepatic secretory products. For example, an increase in content of hepatic microtubule protein associated with VLDL-secretion could have been offset by a parallel decrease in hepatic microtubule protein involved in the secretion of albumin. Any one or any combination of these alternatives could have led to a situation in which increased hepatic VLDL-TG secretion was, in fact, associated with commensurate increases in hepatocyte microtubule or tubulin content which we would not have detected. For the most part, these possibilities cannot be directly tested by methods available to us. It should be emphasized, however, that despite these theoretical questions the most likely interpretation of the results is still that the content of hepatocyte microtubule or tubulin does not change significantly in association with significant increases in the number of VLDL particles secreted by the liver.

Given the qualifications discussed above, what can we now say as to the role played by microtubules in hepatic lipoprotein secretion? The simplest relationship would be for microtubule content and VLDL secretion to change in parallel. Since this does not seem to be the case, we believe it most unlikely that microtubules subserve a regulatory function in hepatocyte VLDL secretion. On the other hand, microtubules could play a permissive role in VLDL secretion wherein a basal number of microtubules (or a basal amount of microtubule protein) would be required for secretory events to proceed at all. If one accepts this formulation, VLDL secretion could be increased without a change in microtubule content; for example, the role of microtubules may be only to insure the appropriate intracellular location of other organelles (see reference 14), which in turn may be important for the intracellular transport and/or secretion of VLDL. Finally, it is theoretically possible that microtubules are not involved in any fashion with hepatocyte VLDL secretion, and that the effect of colchicine and other antimicrotubule agents on VLDL secretion is coincidental and/or associated with some tubulin-like colchicine-binding component of membranes (17). Unfortunately, our results do not permit us to make a definitive choice between these various alternatives. However, they serve to emphasize the need for additional studies aimed at defining precisely what role microtubules play in the hepatic secretion of VLDL.

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