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Defect in an Intrahepatic Degradation of Apolipoprotein B in Suncus: An Animal Model of Hypobetalipoproteinemia[1]

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We have previously shown that fatty liver is easily induced in suncus by starvation and that the plasma level of apolipoprotein B (apo B) is very low. We also found that hepatic acyl coenzyme A cholesterol acyltransferase (ACAT) activity is almost absent in the animals, resulting in decreased cholesteryl ester contents in the liver. A deficiency of cholesteryl ester in suncus liver may be one of the reasons for the defect in the assembly process of apo B-containing lipoproteins, leading to a low level of plasma apo B. Another possible explanation for the induction of fatty liver in suncus is a defect in apo B-processing in the liver. In this study, we investigated the hepatic synthetic rate and intrahepatic degradation of apo B using primary cultured hepatocytes derived from suncus and rats. In order to estimate intrahepatic degradation of apo B, we added N-acetylleucyl-leucynorleucinal to the culture medium as an inhibitor of apo B degradation. The basal synthesis of apo B in suncus hepatocytes was 50% of that in rat. Intracellular degradation of apo B was not observed in suncus hepatocytes, while it was obvious in rat hepatocytes. This evidence suggests that the lower secretion rate of apo B-lipoprotein is not due to the intrahepatic degradation of apo B, but may be due to the low synthetic rate of apo B.

Key words: apo B, fatty liver, suncus, VLDL.

Triglyceride esterified in the liver from plasma-derived fatty acid is stored as droplets or secreted in the form of very-low-density lipoprotein (VLDL), whose secretion depends on the synthesis of apolipoprotein (apo) B as well as lipid moieties. Triglyceride is synthesized in smooth endoplasmic reticulum (s-ER), and apo B, which is a vehicle of triglyceride, is synthesized in rough ER (r-ER); then triglyceride and apo B are assembled into a premature VLDL in the pre-Golgi area, and the premature VLDL is modified in Golgi apparatus and secreted in the form of mature VLDL (*1*). In the pre-Golgi area a part of apo B is degraded when the amount of apo B exceeds the amount to be assembled in the premature VLDL, as reported by Davis *et al.* (*2*) and Sato *et al.* (*3*).

Recently we have found that fatty liver is easily induced in the musk shrew, *Suncus murinus* (suncus), by starvation for 24 h and that the change is easily reversed by refeeding (*4*). In this animal, the plasma level of apo B, an essential component of VLDL and LDL, is very low (*5*).

Cianflone *et al.* (*6*) and Tanaka *et al.* (*7*) suggested that the synthesis of cholesterol ester is one of the regulators of apo B secretion. We have shown that the hepatic activity of acyl coenzyme A cholesterol acyl transferase (ACAT) [EC 2.3.1.26], the intracellular cholesterol esterifying enzyme, is almost absent in suncus liver (*8*) and that hepatic cholesterol ester content is significantly low compared with that in the rat (*9*).

Thus, a deficiency of hepatic ACAT may be one of the reasons for the defect of VLDL-assembly (*9*). Another possibility to explain the defect in the assembly process of VLDL is a defect of apo B processing in suncus hepatocytes.

In the present study we focused on the synthesis of apo B and intracellular degradation of apo B in suncus, in comparison with those in rats.

MATERIALS AND METHOD

Animals and Materials—Male suncus (55-75 g body weight) (Central Institute for Experimental Animals, Tokyo) were housed in temperature- and humidity-controlled rooms and fed with laboratory chow (special food for suncus, Central Institute for Experimental Animals). Feeding was stopped 2 h before use. Male Wistar rats, 160-180 g body weight, were housed at natural temperature and humidity. They were fed with laboratory chow, and feeding was stopped 24 h before use. Monospecific anti-rat apo B, anti-suncus apo B (*10*), and anti-suncus albumin antibodies were raised in rabbits in our laboratory; the specificity to the proteins was confirmed by Western blotting

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analysis. Anti-rat albumin IgG was purchased from Organon Teknika (Durham, NC, USA). ALLN (*N*-acetyl leucyl leucyl norleucinal) and bovine serum albumin (BSA) (essentially fatty acid-free) were purchased from Sigma (USA). L-[^{35}S]Methionine *in vitro* cell labeling mix was obtained from Amersham Life Science (Buckinghamshire, England).

Protein A-Sepharose CL-4B was obtained from Pharmacia Biotech (Uppsala, Sweden). Williams' medium E, methionine-free medium generated from a minimum essential medium (selectamine Kit) and penicillin-streptomycin were obtained from GIBCO BRL (NY., USA). Fetal bovine serum (FCS) was purchased from General Scientific Laboratories (Los Angeles, CA). All other chemicals were of the highest purity available.

Isolation and Culture of Hepatocytes—Hepatocytes were isolated from the livers of suncus and rats according to the method of Seglen (*11*). The cells obtained were seeded onto six-well tissue culture plates (9.62 cm^2/well) at the concentration of 0.5×10^6 cells/ml. Cells were incubated in the medium containing Williams' medium E with 2.62 mM sodium hydrogen carbonate ($NaHCO_3$), penicillin 100 units/ml/streptomycin 100 μg/ml, and 10% fetal bovine serum in a humidified atmosphere of 95% O_2 and 5% CO_2 and allowed to attach for 3 days.

Apolipoprotein and Albumin Labeling Studies—To biosynthetically label apo B and albumin, cultured hepatocytes were washed twice with 1 ml of phosphate-buffered saline, and reincubated in 1 ml of serum-free and methionine-free minimum essential medium containing 1.5% bovine serum albumin (BSA). [^{35}S]Methionine (100 μCi) was added and incubated for 2, 4, and 6 h. In some experiments ALLN dissolved in ethanol was added at the concentration of 40 μg/ml (*12*).

After completion of the labeling, the medium was collected and the cells were washed twice with 1 ml of phosphate-buffered saline (PBS) then harvested with 1 ml of lysis buffer (0.15 M NaCl/5 mM EDTA/1% Triton X-100/0.05 M Tris-HCl, pH 8.0).

In pulse-chase experiments, cells were preincubated for 10 min in methionine-free medium containing L-[^{35}S]-methionine (300 μCi/ml), then the medium was replaced with non-radioactive medium containing methionine, and chased for 10, 20, 40, 60, and 90 min. After completion of chase, the cells were harvested and subjected to immunoprecipitation.

Immunoprecipitation of Labeled apo B and Albumin—After labeling, the medium was removed and the cells were extracted by addition of 1 ml of lysis buffer, followed by after twice washing twice with 1 ml of PBS. Newly synthesized Apo B and albumin were precipitated by specific antisera as described previously (*13*). After addition of 10 μl of non-immune rabbit serum to the medium or cell extracts, protein A-Sepharose CL-4B beads were added to remove nonimmune immunoglobulins. Following addition of rabbit antibodies to the medium or the cell extracts, protein A-Sepharose CL-4B beads were added and the samples were incubated for a further 2 h at 4°C. Precipitates were then pelleted and washed twice with a buffer (0.15 M NaCl/5 mM EDTA/1% Triton X-100/0.05 M Tris-HCl, pH 8.0), then twice with another buffer (0.4 M NaCl/0.05 M Tris-HCl, pH 8.0) and once with distilled water. The washed pellets were solubilized in 50 μl of SDS-PAGE electrophoresis sample buffer (2.3% SDS/10% glycerol/0.625 M Tris-HCl, pH 8.2) as described previously (*14*). Samples were boiled for 5 min, any particulates were pelleted, and the supernatants were subjected to SDS-PAGE. The resolving gel contained a 5 to 22.5% gradient of acrylamide. The gels were exposed to an imaging plate of a BAS2000. The radioactivities of the proteins were measured by the BAS 2000, expressed in the arbitrary unit of photostimulated luminescence (PSL).

Protein concentration of hepatocytes was measured by the method of Lowry (*15*).

TCA Analysis—Cell samples of 100 μl and medium samples of 150 μl were precipitated by the addition of 1 ml

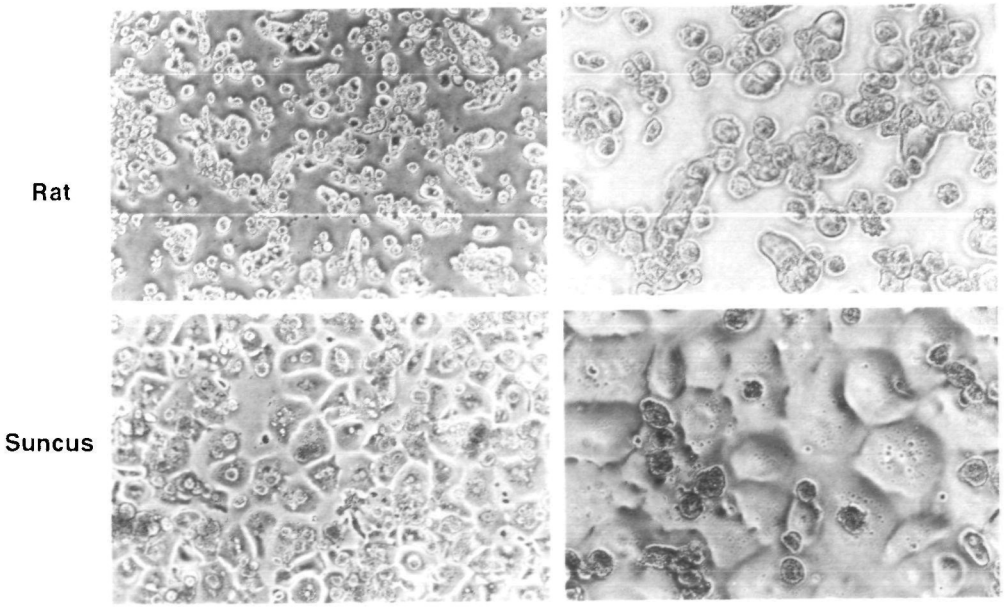

Fig. 1. **Appearance of primary cultured hepatocytes from suncus (lower panels) and rat (upper panels).** Hepatocytes were prepared by the method of Seglen and cultured for 48 h.

of ice-cold 10% trichloroacetic acid (TCA) containing unlabeled methionine as described previously (*14*). The precipitates were washed twice with cold 10% TCA then heated at 95°C for 10 min and cooled in ice. The precipitates were washed sequentially with the following ice-cold solutions: 5% TCA, ethanol-diethyl ether (2:1) and diethyl ether. The precipitates were dried and dissolved in 200 μl of NCS-II tissue solubilizer (Amersham), then 5 ml of Clear-sol I (Nacalai Tesque, Tokyo) was added and the radioactivity was counted in a Liquid Scintillation System LSC-700 (Aloka).

Statistical Methods—All data were analyzed using Student's *t* test for unpaired samples. All values are expressed as means ± SD; differences between means were considered to be significant at the probability level of 0.05.

RESULTS

Figure 1 shows the microscopic appearance of primary cultured hepatocytes from rat and suncus. The hepatocytes from suncus were apparently larger than those from rat, and contain multiple vacuoles, which were not observed in rat hepatocytes.

The time course of protein synthesis in rat and suncus primary cultured hepatocytes showed a linear increase up to 4 h and then a plateau, as shown in the left panel of Fig. 2. Secretion of newly synthesized proteins increased linearly up to 6 h as shown in the right panel of Fig. 2. Since synthesis and secretion of hepatic proteins were in equilibrium at 4 h, we evaluated the apolipoprotein synthesis at 2 and 4 h.

Figure 3 shows the synthesis and accumulation of albumin and apo B in suncus and rat hepatocytes. Since apo B100 was little labeled in these hepatocytes, we measured only apo B 48 in the present study. Albumin synthesis and accumulation were significantly higher in suncus than in rat hepatocytes, by 4-fold at 2 h and 4.5-fold at 4 h. They reached plateau levels between 2 and 4 h in both animals. This may be because albumin is rapidly secreted from hepatocytes in both animals. On the other hand, the synthetic rate of hepatic apo B in suncus was almost the same as that of rat, and showed a linear increase up to 4 h (Fig. 3).

ALLN is well known as an inhibitor of intracellular degradation of apo B. Figure 4 shows that ALLN had no effect on albumin synthesis and accumulation in hepatocytes of both animals, confirming that albumin is not degraded under the hepatocytes in the present experimental conditions.

We investigated the effect of ALLN on the synthesis and cellular accumulation of apo B in a pulse-chase experiment. The accumulation of newly synthesized apo B after a 10 min pulse was significantly higher in rat than in suncus he-

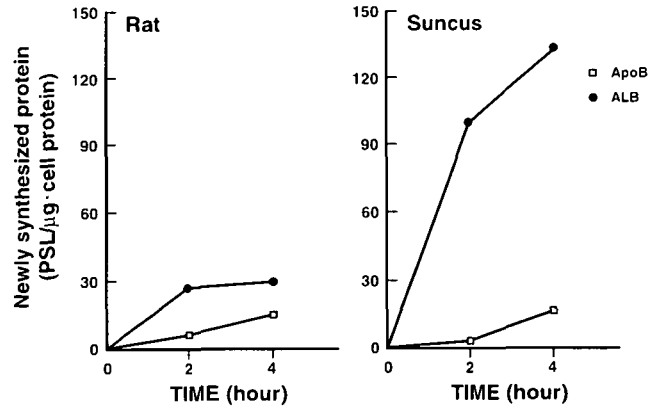

Fig. 3. **Newly synthesized albumin and apo B in rat and suncus primary cultured hepatocytes.** Apo B and albumin were immunoprecipitated from the cell lysates and the medium after protein labeling as described in "MATERIALS AND METHODS."

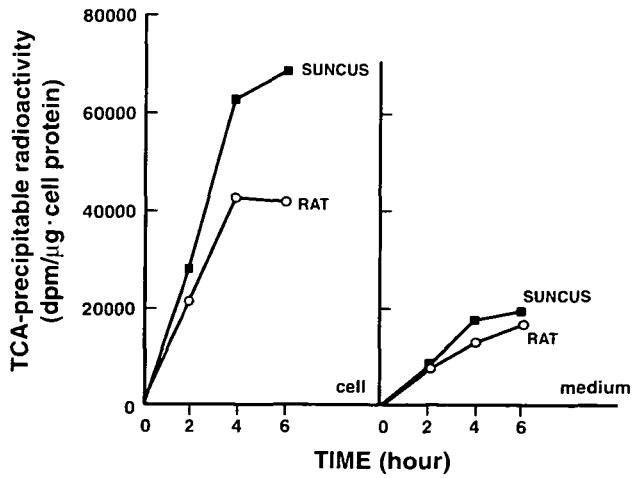

Fig. 2. **Newly synthesized protein in rat and suncus hepatocytes.** After 3 days of growth, the medium was removed and cells were washed twice with PBS, and pulsed for 2, 4 or 6 h with labeling medium containing [^{35}S]methionine. After the labeling, the medium was removed and the cells were washed twice with PBS, and harvested in lysis buffer by scraping. Newly synthesized protein was measured by a TCA precipitation method as described in "MATERIALS AND METHODS."

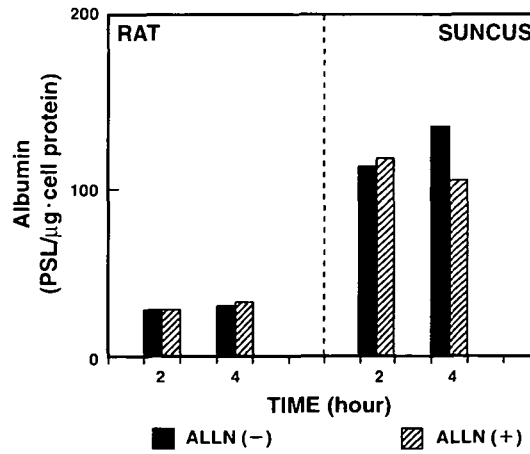

Fig. 4. **Effect of ALLN on albumin synthesis.** Primary cultured hepatocytes were prepared as described in "MATERIALS AND METHODS" and pulsed for 2 or 4 h with labeling medium containing [^{35}S]methionine with or without 40 μg/ml ALLN. Newly synthesized albumin was immunoprecipitated as described in "MATERIALS AND METHODS." The radioactivity of the newly synthesized albumin was measured on a BAS 2000, and expressed in the arbitrary unit of photostimulated luminescence (PSL). The vertical axis shows PSL of apo B per TCA-precipitable radioactivity (dpm).

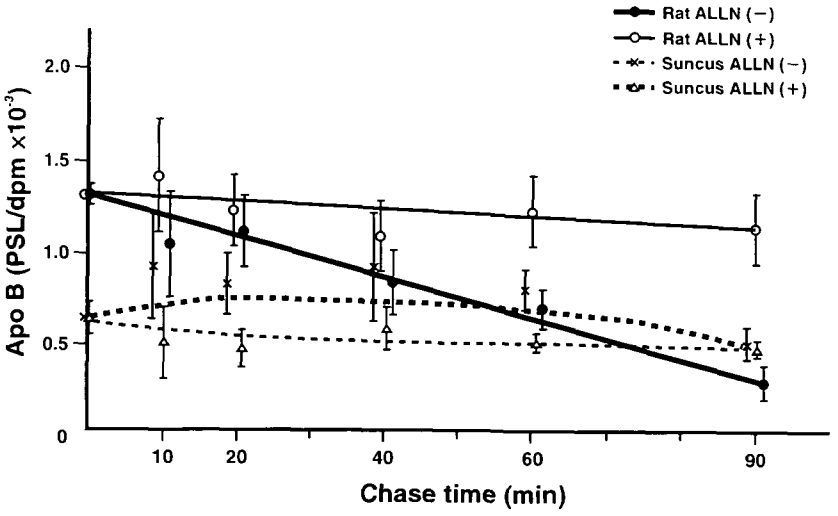

Fig. 5. **Effect of ALLN on apo B synthesis.** The primary cultured hepatocytes were pulsed for 10 min with [^{35}S]methionine and then chased for 10, 20, 40, 60, and 90 min in the presence or absence of ALLN as described in "MATERIALS AND METHODS." Newly synthesized apo B was immunoprecipitated by the specific antibody and subjected to SDS-PAGE. The gel were then exposed to an imaging plate of the BAS 2000. The radioactivity of the newly synthesized apo B thus measured was expressed in the arbitrary unit of photostimulated luminescence (PSL). The vertical axis shows PSL of apo B per TCA-precipitable radioactivity (dpm).

patocytes, as shown in Fig. 5. The synthetic rate of apo B in suncus hepatocytes was estimated as 50% of that in rats. As shown in Fig. 5, newly synthesized apo B decreased linearly up to 90 min during chase in rat hepatocytes, whereas it did not change in suncus, suggesting that intrahepatic degradation of apo B does not occur in suncus. Addition of ALLN significantly inhibited the decrease in newly synthesized apo B in rat, confirming that intracellular degradation of apo B occurs in rat hepatocytes, and is sensitive to ALLN.

DISCUSSION

Since the balance of hepatic lipid is controlled by synthesis of lipid moieties and secretion of VLDL, which is the precursor of atherogenic lipoprotein, low density lipoprotein (LDL), it is important to understand the regulatory mechanism of VLDL assembly and secretion. The major core lipids of VLDL are triglyceride and cholesterol ester, which are assembled with apo B to form premature VLDL. The mechanism by which synthesis of apo B, which occurs in r-ER, is coordinated with synthesis of triglyceride, which takes place on s-ER, is not well known.

We have previously shown that fatty liver is easily induced in suncus by starvation (*4*). The animals may be a good model for studies of the intrahepatic lipid transport pathway. We have previously reported a series of studies on this animal, showing that: (i) plasma apo B is extremely low (*10*); (ii) plasma post heparin lipolytic activity (lipoprotein lipase) is not different from that of rat (data not shown), suggesting that low plasma apo B is not due to an enhanced catabolic rate of VLDL; (iii) VLDL particles in Golgi apparatus are very few (*16*), suggesting that low apo B may be due to a defect of VLDL assembly; and (iv) hepatic ACAT activity, which plays a key role in the formation of cholesterol ester in hepatocytes, was absent in suncus liver (*9*). Cianflone *et al.* (*6*) and Tanaka *et al.* (*7*) suggested that the synthesis of cholesterol ester is one of the regulators of VLDL assembly. We have also shown that hepatic cholesterol-ester content is significantly low compared with that in the rat (*9*). These studies suggest that fatty liver observed in suncus may be induced by a defect in the assembly process of VLDL in suncus.

Two possibilities can be considered. One is a defect of apo B processing in the liver and the other is a defect of microsomal triglyceride transfer protein, which was recently reported to play a key role in VLDL assembly (*17*).

In the present study, we focused on apo B processing in suncus and compared it with that in rat hepatocytes. Apo B is required for hepatic assembly and secretion of VLDL. Increased rates of secretion of apo B-containing lipoproteins from liver appear to be a common abnormality in individuals with hyperlipidemia (*18*). It is therefore of interest to define the regulatory mechanisms involved in the hepatic secretion of apo B-containing lipoproteins. Although changes in the level of apo B mRNA have been observed in cultured hepatocytes and in animal studies under some conditions (*19, 20*), most previous studies have suggested that rapid changes in apo B secretion are not associated with significant changes in the level of apo B mRNA (*21, 22*). Thus, it is believed that regulation of apo B secretion is primarily post-translational. A significant proportion of newly synthesized apo B is rapidly degraded in rat hepatocytes (*2*) and HepG2 cells (*12*). Apo B degradation occurs early in the ER or a pre-Golgi compartment (*2, 23*). Studies with permeabilized HepG2 cells have shown that apo B degradation occurs in the ER by a pH- and temperature-dependent process and is catalyzed by an ALLN-sensitive, calcium-insensitive proteinase (*24*). The regulation of intrahepatic degradation is the most likely post-translational regulation of apo B.

In order to investigate the intrahepatic processing of apo B, we used primary cultured hepatocytes from suncus and rats. The appearance of the hepatocytes from suncus was clearly different from those of rats with respect to size and intracellular vacuoles. The hepatocytes from suncus were larger than those from rats and contained multiple vacuoles, as shown in Fig. 1. These vacuoles may be lipid droplets, because the triglyceride contents were higher in suncus than in rat (data not shown). Although the appearance of the hepatocytes was different from those of rat, protein synthesis and albumin synthesis increased linearly up to 4 h during incubation, as shown in Figs. 2 and 3, suggesting that the cells have the functions of hepatocytes. The synthesis and accumulation of proteins and albumin in hepatocytes from suncus were significantly higher than in those from rats, suggesting that suncus hepatocytes synthe-

size proteins more actively than rat hepatocytes.

Addition of ALLN, a thiol protease inhibitor, to the medium resulted in no decrease in accumulation of newly synthesized apo B in rats, whereas a linear decrease up to 90 min was seen during chase without ALLN, as shown in Fig. 5, suggesting that intracellular degradation of apo B occurs in rat hepatocytes as reported, and is sensitive to ALLN (*2, 25*). On the other hand, no decrease in newly synthesized apo B was observed in suncus hepatocytes with or without ALLN, suggesting that intrahepatic degradation of apo B does not occur in suncus hepatocytes. The synthesis of apo B in suncus hepatocytes was about 50% of that in the rat.

In conclusion, the defect of VLDL assembly is not due to enhanced intracellular degradation of apo B, but may be due to a low synthetic rate of apo B.

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