Postnatal development of hepatocellular apolipoprotein B assembly and secretion in the rat

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Abstract In this study, we explored the paradox that in suckling rats the serum concentration of LDL is high although the liver secretes only minimal quantities of VLDL, the presumed precursor of LDL. Freshly isolated hepatocytes and hepatocytes in primary culture obtained from adult (90 days old) and suckling (17 days old) rats were used to investigate the synthesis and secretion of apolipoprotein B (apoB) and lipids as well as the density profile of secreted apoB-containing lipoproteins. Furthermore, the effects of dexamethasone and oleate on apoB biogenesis were investigated in primary cultures of hepatocytes from adult and suckling rats. Hepatocytes from suckling rats were unable to assemble mature VLDL but secreted apoB as primordial lipoprotein particles in the LDL-HDL density range. Intracellular degradation of apoB was also reduced in hepatocytes from suckling rats compared with that in hepatocytes from adults. The immaturity in VLDL assembly and apoB degradation of hepatocytes from suckling rats could be overcome by treating the cultures with dexamethasone plus oleate or dexamethasone alone. The lower microsomal triacylglycerol transfer protein (MTP) mRNA concentrations in hepatocytes from suckling rats in comparison with hepatocytes from adult rats were not reflected in lower MTP activity levels. Furthermore, dexamethasone plus oleate treatment had no effect on MTP activity although VLDL assembly and secretion were clearly stimulated. We conclude that, during the suckling period of the rat, serum LDL is directly produced by the liver. This is a result of impaired hepatic VLDL assembly, which is a consequence of low triglyceride synthesis and an inefficient mobilization of bulk lipids in the second step of VLDL assembly.—Plonné, D., H-P. Schulze, U. Kahlert, K. Meltke, H. Seidolt, A. J. Bennett, I. J. Cartwright, J. A. Higgins, U. Till, and R. Dargel. **Postnatal development of hepatocellular apolipoprotein B assembly and secretion in the rat.** ***J. Lipid Res.*** **2001.** 42: **1865–1878.**

Supplementary key words atherosclerosis • direct LDL secretion • primary hepatocytes • rat development

Throughout postnatal development rats undergo dramatic changes in their nutritional and hormonal state. During the suckling period the high fat, low carbohydrate diet is accompanied by high glucagon and low insulin and glucocorticoid plasma levels (1). At the time of weaning the nutrition becomes poor in fat and rich in carbohydrates, which is associated with an increase in the insulin/glucagon ratio and the glucocorticoid concentration (2–8). In parallel, remarkable changes occur in lipid and lipoprotein metabolism. During suckling most of the fatty acids taken up as triglycerides with the milk are directed into β-oxidation and ketogenesis for energy production whereas hepatic lipogenesis is generally low (8–11) due to low gene expression of lipogenic enzymes (12). In spite of substantially reduced hepatic triglyceride synthesis, livers of sucklings contain large amounts of triglycerides (13) but secrete only minimal quantities of VLDL, so that nearly all triglycerides in plasma are of intestinal origin (14–16). On the other hand, apart from the decreased hepatic apolipoprotein B-48 (apoB-48) production, because of the lower apoB mRNA editing in the liver of sucklings (17–20) the synthesis and secretion of apoB-100, the main structural component of VLDL, is the same in hepatocytes from suckling and adult animals (16). The different metabolic situations in suckling and adult rats are also reflected by different serum profiles of apoB-containing lipoproteins. During the suckling period, serum VLDL apoB concentrations are about four times lower, whereas the LDL apoB concentrations are 4- to 5-fold higher than in adult animals (20–22). The high concentration of LDL in suckling rat serum raises the question of the origin of this lipoprotein at that period of development. Because of the low hepatic VLDL secretion and the predominant production of apoB-48 by the intestine it seems unlikely that the LDL fraction arises from triglyceride-rich lipoproteins, but rather implies their direct hepatic secretion.

Abbreviations: MTP, microsomal triacylglycerol transfer protein; SER, smooth endoplasmic reticulum.

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This hypothesis is supported by the fact that the visceral yolk sac of the rat (23–26) and Hep-G2 cells (27, 28), both of which have low triglyceride synthesis or availability, secrete apoB as lipoprotein particles in the LDL but not in the VLDL density range. However, the pattern of apoB-containing lipoproteins secreted by livers of suckling rats is unknown and neither is its hormonal and metabolic regulation.

In the present study we have grown hepatocytes from suckling rats (17 days old) and adult rats (90 days old) in suspension culture and in primary culture, and used them to investigate the production and secretion of apoB-containing lipoproteins at these two typical stages of development. Even though hepatocytes in suspension culture might reflect the in vivo metabolic conditions with sufficient accuracy they do have limitations. Freshly isolated cells can only be used for up to 2 h with good viability. They can neither generate cell-cell contacts nor attach to an extracellular matrix, both of which are important for normal hepatocellular function. We have therefore carried out most experiments on primary cultures and confirmed the results as far as possible, using freshly isolated hepatocytes in suspension. Our experiments demonstrated that without additives hepatocytes from suckling rats do not produce VLDL particles but secrete apoB in the LDL-HDL density range because of immature apoB assembly. Only after addition of both oleic acid and dexamethasone to the culture medium do hepatocytes from suckling animals secrete apoB in the VLDL density range, similar to hepatocytes prepared from adult rats.

MATERIALS AND METHODS

Materials

L-[^{35}S]methionine (370 MBq/ml; 37 TBq/mmol), [α-^{32}P]UTP (3,000 Ci/mmol), Hyperfilm-MP, and Amplify were from Amersham Buchler (Braunschweig, Germany). [^{3}H]glycerol (7.4 GBq/mmol) and [^{3}H]mevalonolactone (740 GBq–1.48 TBq/mmol) were from NEN Life Science Products (Boston, MA). Williams' medium E as a powder medium, leupeptin, and fatty acid-free BSA were from Serva (Heidelberg, Germany). Aprotinin was from Fluka (Berlin, Germany). DNase and tRNA were from Boehringer Mannheim (Mannheim, Germany). Collagenase (Worthington type CLS) was from Seromed Biochrom KG (Berlin, Germany). Soluene 350 was obtained from Packard (Groningen, The Netherlands). All other chemicals were of analytical grade. Anti-rat apoB antiserum was prepared as described (29), using purified serum LDL from fetal rats (23).

Animals

Suckling (17-day-old) and adult (90-day-old) Wistar rats of the strain Han-Wist were used for all experiments. Adult rats were housed under standard conditions and fed a standard laboratory chow diet (Altromin, Lage, Germany) with drinking water ad libitum. Suckling rats were allowed free access to maternal milk until they were used for hepatocyte preparation.

Isolation and culture of rat hepatocytes

Hepatocytes from adult and suckling rats were isolated by a collagenase digestion method according to Seglen (30) as modified by Zimmermann et al. (31). Generally, after isolation the viability of the hepatocytes was higher than 85% as measured by the trypan blue exclusion test. After isolation the hepatocytes were used either in suspension culture or in primary culture.

For suspension culture, 25 $\times$ 10^6 freshly isolated hepatocytes were resuspended in 5 ml of Williams' medium E supplemented with 1.2% BSA, 25 mM $NaHCO_3$, and gentamicin (50 μg/ml) and incubated with gentle shaking at 37°C under a 95% O_2/5% CO_2 atmosphere for a maximum of 2 h.

For primary culture, hepatocytes were resuspended at a density of 0.75 $\times$ 10^6 cells/ml (suckling rats) or 0.45 $\times$ 10^6 cells/ml (adult rats) in Williams' medium E supplemented with 10% fetal calf serum (FCS), 25 mM $NaHCO_3$, and gentamicin (50 μg/ml). Two milliliters of the cell suspension was plated in collagen-coated 35-mm plastic culture dishes and maintained at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. After 2 h, the plating medium and nonadherent cells were aspirated from each plate and replaced with 1.5 ml of feeding medium [Williams' medium E supplemented with 10% FCS, 0.6% BSA (fatty acid free), 25 mM $NaHCO_3$, and gentamicin (50 μg/ml) and, if indicated, with 0.4 mM oleate and/or 100 nM dexamethasone]. The cultures were used for experiments 24 h after plating.

Experiments with hepatocytes in suspension culture

To radiolabel secreted apoB-100 and apoB-48, 5 ml of the hepatocyte suspension was incubated with 25 μl of [^{35}S]-methionine (10 MBq). After 2 h of incubation, the medium was obtained by centrifugation; EDTA (2.5 mM), leupeptin (0.1 mM), and aprotinin (0.2 mM) were added and the density of the medium was adjusted to d = 1.21 g/ml by addition of solid KBr. The incorporation of [^{35}S]methionine into secreted apoB and the density profile of the secreted lipoproteins were determined as described previously (26).

To determine synthesis of triglycerides or cholesterol and cholesteryl esters 1 mM [^{3}H]glycerol or 0.1 mM [^{3}H]mevalonolactone, respectively, was added to the medium. After a 2-h incubation, lipids in cells and media were extracted by the method of Folch, Lees, and Stanley (32). Triglycerides, cholesteryl esters, and free cholesterol were separated by TLC, using the solvent system hexane–diethyl ether–glacial acetic acid 81:17:2 (v/v/v). The spots were visualized with iodine vapor and scraped off, and the radioactivity was measured by β counting. As was proven in prior experiments the incorporation of [^{35}S]methionine, [^{3}H]glycerol, and [^{3}H]mevalonolactone into apoB, or lipid, was linear over the 2-h incubation period (data not shown).

Experiments with hepatocytes in primary culture

For incorporation studies, the medium was changed to Williams' medium E without methionine (for apoB) or with methionine (for lipids) containing 1.2% BSA, 25 mM $NaHCO_3$, and gentamicin (50 μg/ml) and 0.4 mM oleate and/or 100 nM dexamethasone was added as indicated. To radiolabel apoB, cells were incubated with 5 μl of [^{35}S]methionine per ml of medium for 4 h and the cells and media were separated by centrifugation. Radioactivity incorporated into apoB-100 and apoB-48 was determined by immunoprecipitation, as described by Sparks and Sparks (33) followed by SDS-PAGE. After autoradiography the apoB-100 and apoB-48 bands were excised from gels and the radioactivity was measured as described in Plonné et al. (26). The density profile of secreted newly synthesized apoB-100 and apoB-48 was analyzed with 4 ml of medium, as described above for cells in suspension culture.

To monitor the intracellular degradation of newly synthesized apoB-100 and apoB-48 hepatocytes were preincubated for 30 min in methionine-free medium followed by 20 min with [^{35}S]methionine. This "pulse" was followed by a "chase" initiated by replacing the labeling medium with medium containing no

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radioactivity but 0.1 mM cold methionine. After 0, 1, 2, and 4 h the medium was harvested, the cells were lysed, and apoB was immunoprecipitated and analyzed after SDS-PAGE.

Determination of the synthesis and secretion of newly synthesized triglycerides, free cholesterol, and cholesteryl esters was the same as that described above for cells in suspension culture, except that the incubation time was 4 h. As was proven in prior experiments the incorporation of [^{35}S]methionine, [^{3}H]glycerol, and [^{3}H]mevalonolactone into apoB, or lipid, was linear over the 4-h incubation period (data not shown).

Analysis of microsomal lipids

Total microsomes were prepared from livers of suckling and adult rats, as described previously (34). Lipids were extracted and separated by high performance TLC (HPTLC), and the masses were determined by laser densitometry with a Camlab (Cambridge, UK) densitometer (35, 36).

Immunoblotting

Homogenates or microsomes were mixed with reducing SDS sample buffer to a final protein concentration of 1 μg/μl, boiled for 5 min, and 20 μg of protein from each sample was separated by SDS-PAGE on 4–20% gradient gels. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane and blocked for 1 h followed by overnight incubation at 4°C with the anti-apoB antiserum (1:500 dilution). The membranes were washed and incubated with horseradish peroxidase conjugated to goat IgG as secondary antibody (1:10,000 dilution). This was detected with Western blotting Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

Isolation of total hepatic RNA and determination of apoB and microsomal triacylglycerol transfer protein (MTP) mRNA levels

Total hepatic RNA was extracted from livers, freshly isolated hepatocytes, and primary culture hepatocytes from suckling and adult rats by the guanidine thiocyanate method as described by Chomczynski and Sacchi (37). A 275-bp fragment of rat apoB cDNA (nucleotides 6512–6786) was cloned into the *Hin*dIII-*Eco*RI site of pCR Script SK+ (Stratagene, La Jolla, CA) to synthesize the apoB RNA probes with [α-^{32}P]UTP (3,000 Ci/mmol; Amersham Buchler). RNA solution hybridization was performed according to the protocol of the RNase protection assay system from Promega (Madison, WI). MTP mRNA levels were quantified by RNase protection assay as previously described (38, 39). Results were corrected for variation in the mRNA content of total RNA samples by quantitation of poly(A) RNA, using oligo(dT)$_{18}$ hybridization (40). All mRNA values are expressed as attomoles of mRNA per microgram of total RNA normalized to 20 ng of poly(A) RNA per μg of total RNA. Poly(A) RNA levels were not found to differ significantly between samples taken at different life stages.

MTP activity assay

The MTP activity was measured with a fluorometric MTP activity assay kit from Calbiochem (Calbiochem-Novabiochem, Bad Soden, Germany) according to the manufacturer instructions. Freshly isolated or harvested cultured hepatocytes were washed three times with ice-cold PBS and finally sonicated in an appropriate volume of homogenization buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin (20 μg/ml)]. Homogenate protein (100 μg) was made up to 20 μl with homogenization buffer and combined with 480 μl of assay buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA] containing 10 μl of donor molecule and 10 μl of acceptor molecule. In addition, a blank sample was prepared containing 20 μl of homogenization buffer instead of the sample. After incubation for 5 h at 37°C the fluorescence intensity was measured (excitation, 465 nm; emission, 535 nm) and the fluorescence intensity of the samples was corrected by subtracting the blank fluorescence intensity.

RESULTS

ApoB and lipid compositions of livers from adult and suckling rats

The concentrations of apoB-100 and apoB-48 (micrograms of apoB per milligram of total protein) were higher in microsomal fractions than in whole homogenates (**Fig. 1A**), as would be expected as these are secretory proteins. The apoB-48 concentration was similar in samples from adult and suckling rats, whereas apoB-100 was at a higher concentration in livers of suckling rats compared with that from adult rats. This is consistent with our previous finding, that apoB mRNA editing is higher in hepatocytes from adult animals (85%) than in hepatocytes from suckling rats (30%), resulting in a preponderance of hepatic apoB-100 during the suckling period (20). The mRNA levels for apoB in freshly isolated hepatocytes from adult

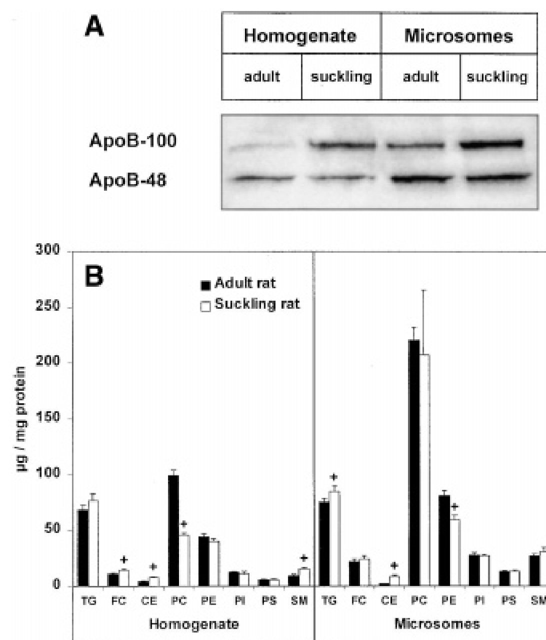

Fig. 1. ApoB and lipid compositions of homogenates and microsomal fractions prepared from livers of adult rats (90 days old) and suckling rats (17 days old). A: Immunoblotting analysis of apoB-100 and apoB-48. Equal amounts (20 μg of protein) of homogenates and microsomes were subjected to SDS-PAGE and proteins were transferred onto PVDF membranes for immunoblotting with rabbit antisera against rat apoB as described in Materials and Methods. B: Lipid analysis of homogenates and microsomal fractions. The lipids were extracted and analyzed by HPTLC. FC, Free cholesterol; CE, cholesteryl esters; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin. Data are expressed as means ± SD (n = 4). $^{+}$ Significant difference at the $P < 0.05$ level versus the adult value.

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rats (22.8 ± 6.2 pg of apoB mRNA per μg of total RNA; n = 10) and suckling rats (20.5 ± 5.1 pg of apoB mRNA per μg of total RNA; n = 6) were similar. Differences in apoB were not, therefore, a consequence of differences in transcription.

The lipid composition of the livers from adult and suckling rats exhibited some differences (Fig. 1B). Phosphatidylcholine was two times lower in liver homogenates from adult rats, whereas sphingomyelin, cholesteryl esters, and free cholesterol were, respectively, 2-fold, 1.6-fold, and 1.4-fold higher in liver homogenates from suckling rats. The most prominent difference in the lipid composition was a 5-fold higher cholesteryl ester content per milligram of protein in microsomes from suckling rats in comparison with those from adult animals. Apart from slightly enhanced triglyceride and slightly diminished phosphatidylethanolamine amounts the quantities of all other microsomal lipids were identical at the two stages of development.

Net synthesis of apoB and lipid in hepatocytes from adult and suckling rats in suspension culture

Freshly isolated hepatocytes incubated in suspension culture synthesized and secreted apoB, triglyceride, cholesteryl esters, and free cholesterol (**Fig. 2**). However, there were marked differences between cells isolated from suckling and adult rats. Although net secretion of apoB-100 was similar, net secretion of apoB-48 by hepatocytes from suckling rats was reduced about 2.5-fold compared

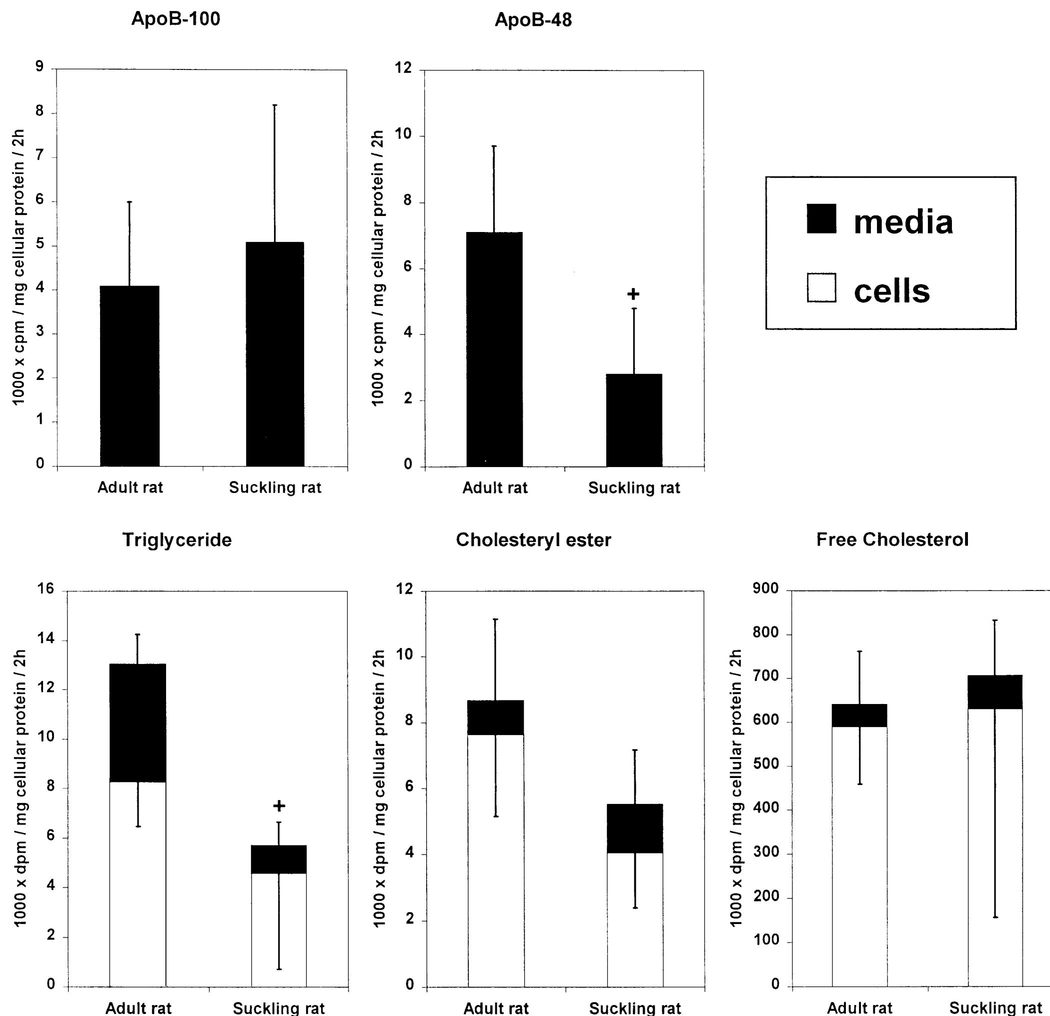

Fig. 2. Incorporation of radioactivity into apoB-100, apoB-48, triglyceride, cholesteryl esters, and free cholesterol synthesized by hepatocytes of adult rats (90 days old) and suckling rats (17 days old) in suspension culture. Freshly isolated hepatocytes from adult and suckling rats were incubated in Williams' medium E (1.2% BSA) in the presence of [^{35}S]methionine (apoB-100 and apoB-48), [^{3}H]glycerol (triglyceride), or [^{3}H]mevalonolactone (cholesteryl esters and free cholesterol) for 2 h. The radioactivity of secreted apoB-100 and apoB-48 was measured in the total lipoprotein fraction after ultracentrifugation of the medium at density d = 1.21 g/ml as described in Materials and Methods. The lipids of cells and media were extracted and separated by TLC. After visualization by iodine vapor the corresponding spots for triglycerides, cholesteryl esters, and free cholesterol were scraped off and the radioactivity was measured by β counting. Data are expressed as means ± SD (n = 3 or 4). $^{+}$Significant difference at the $P < 0.05$ level versus the adult value of secreted radioactivity.

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with that from adult rats. Triglyceride net synthesis and secretion were reduced in hepatocytes from suckling rats by 50% and 75%, respectively, compared with hepatocytes from adult rats. Cholesteryl ester net synthesis, but not secretion, was also reduced in cells from suckling rats and cholesterol net synthesis was not altered.

Net synthesis of apoB and lipid in hepatocytes from adult and suckling rats in primary culture

ApoB-100 net synthesis and secretion tended to be about 30% lower in hepatocytes from suckling rats than in those from adults whereas apoB-48 net synthesis and secretion appeared to be lower by 50% and 62%, respectively, in hepatocytes from suckling rats compared with those from adults (**Fig. 3**). However, the differences in apoB were not statistically significant. Triglyceride net synthesis and secretion in hepatocytes from suckling rats were reduced significantly and accounted for only 20% and 7%, respectively, of that in hepatocytes from adult rats. The net synthesis of cholesteryl esters and free cholesterol in hepatocytes from suckling rats was reduced to 65% of that in cultures of adult hepatocytes. Net secretion of free cholesterol was not different, but the net secretion of cholesteryl esters was about three times lower in hepatocytes from suckling rats than in those from adult rats. In suspension culture hepatocytes of sucklings

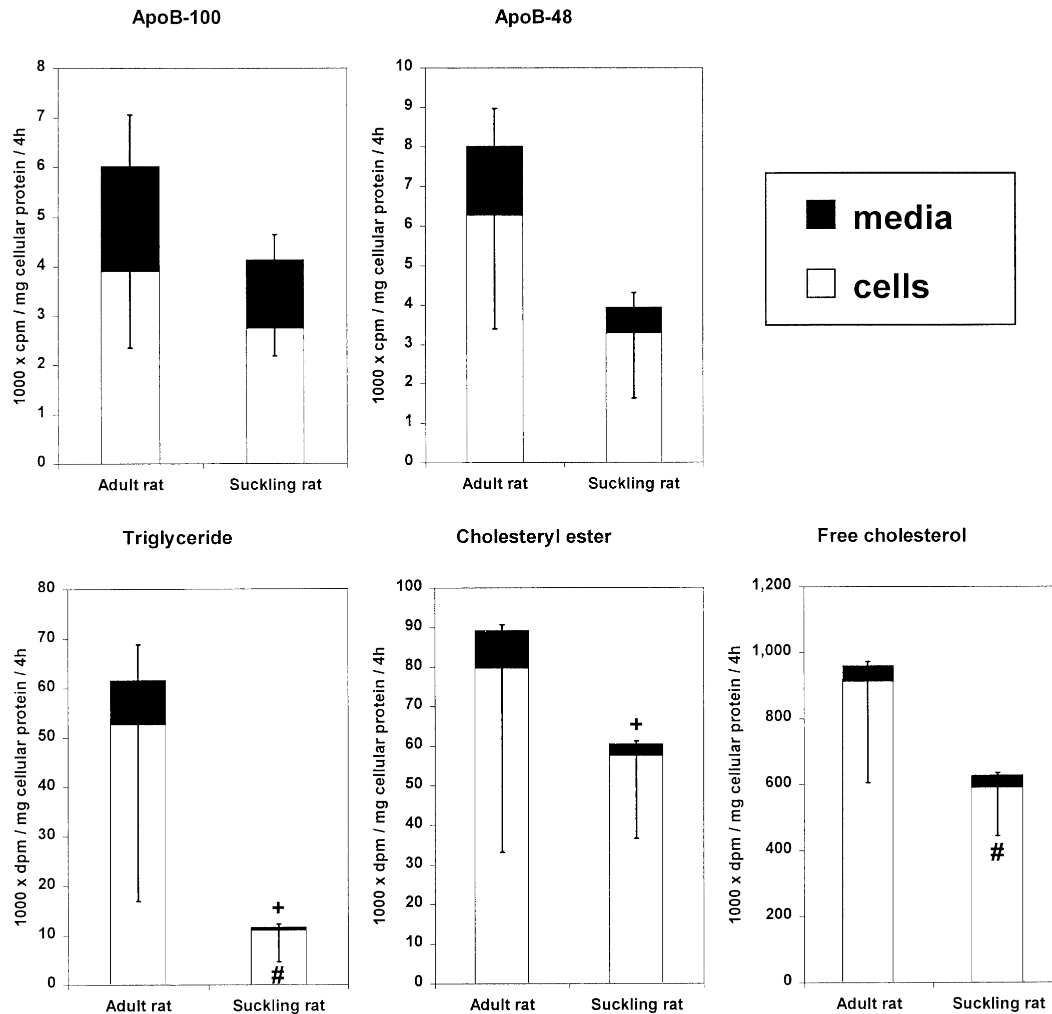

Fig. 3. Incorporation of radioactivity into apoB-100, apoB-48, triglyceride, cholesteryl esters, and free cholesterol synthesized by hepatocytes of adult rats (90 days old) and suckling rats (17 days old) in primary culture. Hepatocytes from adult and suckling rats were cultured on collagen-coated dishes in Williams' medium E (10% FCS) for 24 h. Thereafter, the medium was changed to Williams' medium E (1.2% BSA) and the cells were incubated in the presence of [^{35}S]methionine (apoB-100 and apoB-48), [^{3}H]glycerol (triglyceride), or [^{3}H]mevalonolactone (cholesteryl esters and free cholesterol) for a further 4 h. After incubation cells and media were collected and the incorporated radioactivities were determined. ApoB was immunoprecipitated from media and cell lysates. After separation by SDS-PAGE the apoB-100 and apoB-48 bands were excised and the radioactivity was measured by β counting. The lipids of cells and media were extracted and separated by TLC. After visualization by iodine vapor the corresponding spots for triglycerides, cholesteryl esters, and free cholesterol were scraped off and the radioactivity was measured by β counting. Data are expressed as means ± SD (n = 3 or 4). $^{+,\#}$ Significant difference at the $P < 0.05$ level versus the adult value of secreted (plus sign) and cellular (pound sign) radioactivity.

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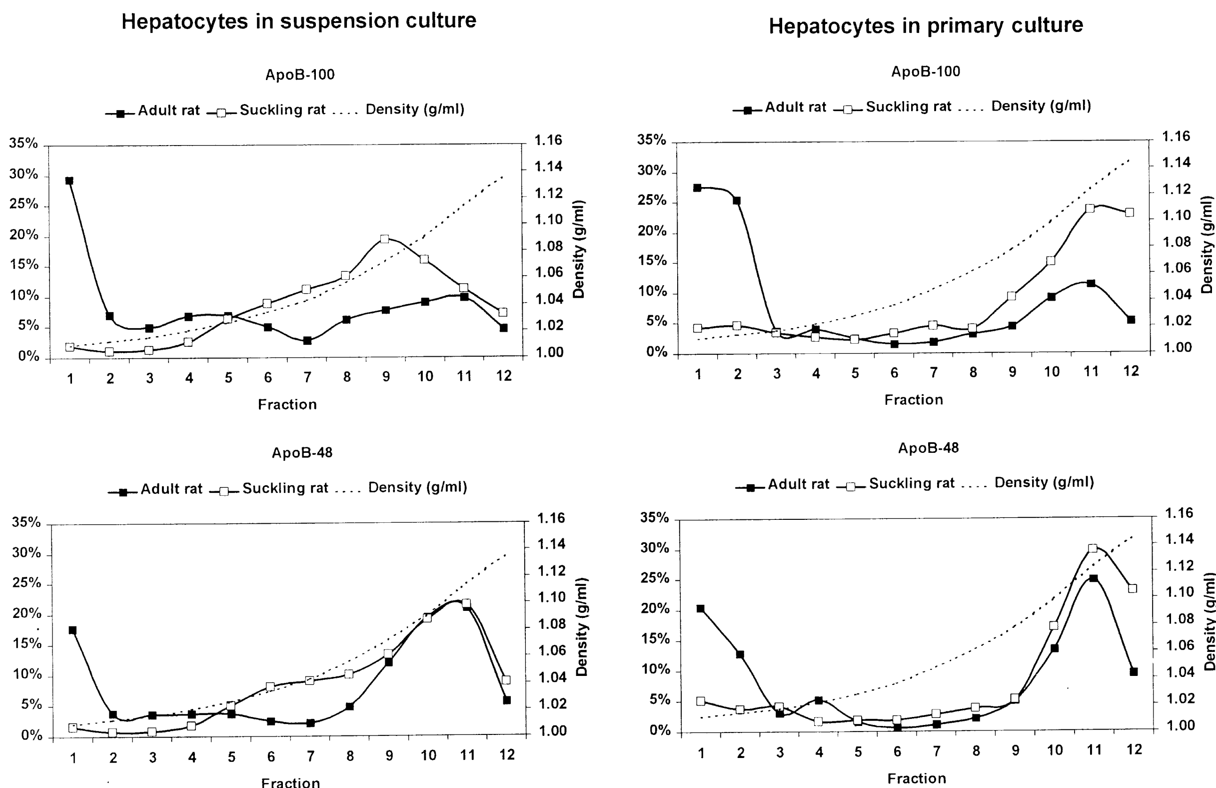

Fig. 4. Density profiles of secreted, newly synthesized apoB-100 and apoB-48 from of adult rat (90 days old) and suckling rat (17 days old) hepatocytes in suspension culture and in primary culture. For suspension culture (left) freshly isolated hepatocytes from adult and suckling rats were incubated in Williams' medium E (1.2% BSA) in the presence of [^{35}S]methionine for 2 h. For primary culture (right) hepatocytes were grown on collagen-coated dishes in Williams' medium E (10% FCS, 0.6% BSA) for 24 h. Thereafter, the medium was changed to methionine-free Williams' medium E (1.2% BSA) and the cells were incubated in the presence of [^{35}S]methionine for a further 4 h. After incubation the medium was subjected to ultracentrifugation at density d = 1.21 g/ml. The total lipoprotein fraction (i.e., the supernatant) was collected and further separated into 12 fractions by means of density gradient ultracentrifugation. The apolipoproteins of each fraction were separated by SDS-PAGE and the radioactivity of the excised apoB-100 and apoB-48 bands was measured by β counting. Values are given as the percentage of apoB radioactivity in each fraction. Each profile represents the average of three separate experiments. For clarity the standard deviations are not shown.

secreted 1.2, 1.4, and 1.5 times more apoB-100, cholesteryl esters, and free cholesterol radioactivities, respectively, than hepatocytes of adult animals. Net secretion of apoB-48 and triglycerides by hepatocytes of suckling rats accounted for only 39% and 25%, respectively, of that in hepatocytes of adult rats. Primary cultured hepatocytes of sucklings secreted 66%, 38%, 7%, 32%, and 78%, respectively, of the apoB-100, apoB-48, triglycerides, cholesteryl esters, and free cholesterol radioactivities compared with those secreted by hepatocytes from adults. Thus, the secretory capacity of hepatocytes from suckling rats dropped relatively more than that of hepatocytes from adult rats when the cells were grown in primary culture for 24 h. However, a marked difference between apoB and triglyceride net secretion by hepatocytes from suckling rats was found in both suspension culture and primary culture. Therefore, compared with hepatocytes of adult rats, hepatocytes of suckling rats secreted less newly synthesized triglyceride than newly synthesized apoB regardless of the culture system used. In primary culture, the net secretion of newly synthesized cholesteryl esters was also lower in hepatocytes of suckling rats than in those of adults.

The newly synthesized apoB-100 secreted by adult rat hepatocytes incubated in suspension culture or primary culture was mainly in lipoproteins of the density of VLDL, with smaller amounts in LDL and HDL (**Fig. 4**). The density profile of apoB-48 secreted by hepatocytes from adult rats was similar except that the peak in the HDL density range was generally higher. In contrast, hepatocytes from suckling rats secreted apoB in particles of LDL-HDL density in suspension culture and HDL density in primary culture, with little of the density of VLDL. Thus, hepatocytes from suckling rats do not assemble mature VLDL but secrete apoB in small lipoprotein particles with a density in the LDL-HDL range. This property is maintained in primary culture for at least 24 h, making this model suitable for further investigations.

Metabolic effects of dexamethasone and oleate in primary cultured hepatocytes

Because the level of glucocorticoids is extremely low during the suckling period and increases strikingly after weaning (2, 3) we have examined whether dexamethasone affects the production of apoB-containing lipoproteins in primary cultured hepatocytes. Addition of dexa-

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methasone to the culture medium increased the total apoB-100 and apoB-48 net synthesis, with a greater effect on hepatocytes of suckling rats (3-fold) than on hepatocytes of adults (1.5-fold), and slightly increased the percentage of secreted apoB-100 and apoB-48 by about 6–15% at both developmental stages (**Fig. 5**). Dexamethasone tended to decrease the total net synthesis of triglycerides (0.6 times), cholesteryl esters (0.3 times), and free cholesterol (0.8 times) in hepatocytes of adult rats, although the differences were not statistically significant. Dexamethasone had no effect on total net synthesis of lipids in hepatocytes of suckling rats. However, dexamethasone increased the percentage of newly synthesized lipids secreted by hepatocytes from both adult and suckling rats, in comparison with the control without dexamethasone.

Addition of oleate to the culture medium slightly stimu-

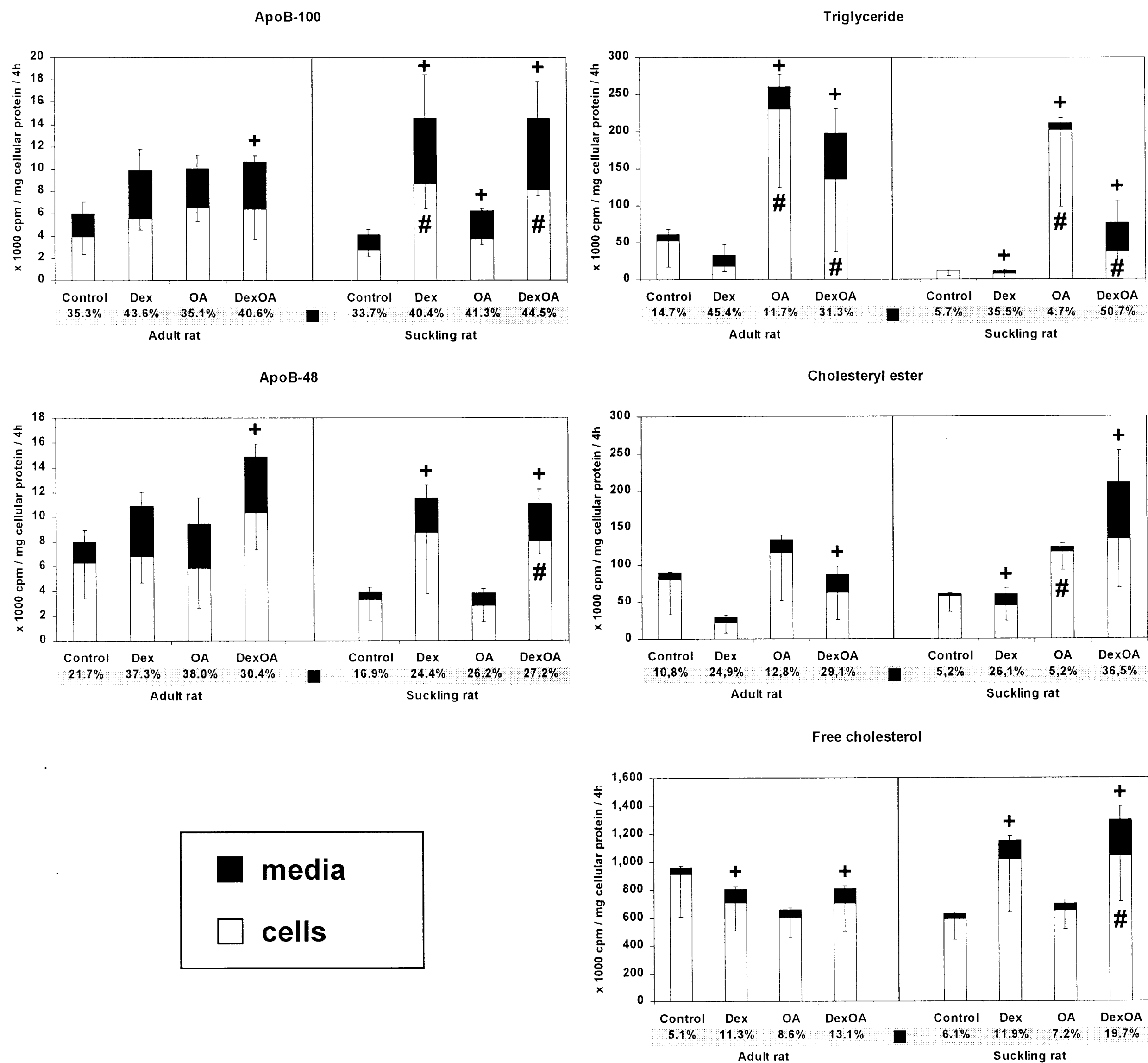

Fig. 5. Incorporation of radioactivity into apoB-100, apoB-48, triglyceride, cholesteryl esters, and free cholesterol synthesized by primary culture hepatocytes of adult rats (90 days old) and suckling rats (17 days old) without additives (control) or with 100 nM dexamethasone (Dex), 0.4 mM oleate (OA), or 100 nM dexamethasone plus 0.4 mM oleate (DexOA). Hepatocytes from adult and suckling rats were cultured on collagen-coated dishes in Williams' medium E (10% FCS, 0.6% BSA) without additives (control) or with 100 nM dexamethasone (Dex), 0.4 mM oleate (OA), or 100 nM dexamethasone plus 0.4 mM oleate (DexOA) for 24 h. Thereafter, the medium was changed to Williams' medium E (1.2% BSA) and the cells were incubated with the same additives in the presence of [^{35}S]methionine (apoB-100 and apoB-48), [^{3}H]glycerol (triglyceride), or [^{3}H]mevalonolactone (cholesteryl esters and free cholesterol) for a further 4 h. After incubation cells and media were collected and the incorporated radioactivities were determined. ApoB was immunoprecipitated from media and cell lysates. After separation by SDS-PAGE the apoB-100 and apoB-48 bands were excised and the radioactivity was measured by β counting. The lipids of cells and media were extracted and separated by TLC. After visualization by iodine vapor the corresponding spots for triglycerides, cholesteryl esters, and free cholesterol were scraped off and the radioactivity was measured by β counting. Data are expressed as means ± SD (n = 3 or 4). $^{+,\#}$ Significant difference at the $P < 0.05$ level versus the control value of secreted (plus sign) and cellular (pound sign) radioactivity. The numbers along the x axis represent the percentage of secreted radioactivity.

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lated total apoB-100 net synthesis by 1.6-fold and 1.5-fold in hepatocytes from adult rats and suckling rats, respectively, and had no effect on total apoB-48 net synthesis. Triglyceride net synthesis was markedly stimulated after oleate treatment by 4-fold and 24-fold in hepatocytes from adults and suckling rats, respectively. Apart from increased net synthesis of cholesteryl esters by 2-fold in hepatocytes of suckling rats, there were no additional significant changes in lipid net synthesis after oleate treatment. The percentage of newly synthesized lipid secreted by hepatocytes from adult or suckling rats was not affected by addition of oleate.

The effects of dexamethasone plus oleate in combination were additive. Stimulation of triglyceride net synthesis by oleate was reduced if dexamethasone was also added to the medium. This effect was more distinct in hepatocytes of sucklings than in those of adults. However, the total triglyceride net synthesis was still higher in dexamethasone plus oleate-treated cells than in controls without additives. The combined treatment caused a clear increase in the percentage of secreted apoB and lipids, which was more pronounced in hepatocytes of suckling rats than in those of adults. The greatest stimulation of net secretion after simultaneous dexamethasone and oleate treatment was observed in hepatocytes from suckling rats, in which triglyceride and cholesteryl ester net secretion was 55 and 25 times higher, respectively, than in the corresponding controls.

Overall, the data reveal that in spite of the massively stimulated triglyceride net synthesis by oleate, only 12% and 5% of the newly synthesized triglyceride, respectively, was secreted by hepatocytes of adult and suckling rats. Not until dexamethasone was added together with oleate was the percentage of secreted triglyceride increased significantly to 31% (hepatocytes of adult rats) and 51% (hepatocytes of suckling rats). A similar effect was found for cholesteryl esters.

After treatment of hepatocytes with dexamethasone, the VLDL peaks of apoB-100 and apoB-48 found in controls disappeared almost completely in hepatocytes of adult rats and the bulk of secreted apoB-containing lipoproteins floated in the LDL-HDL density range (**Fig. 6**). Treatment with dexamethasone did not change the density profile of apoB secreted by hepatocytes of suckling rats compared with controls; the predominant amounts of

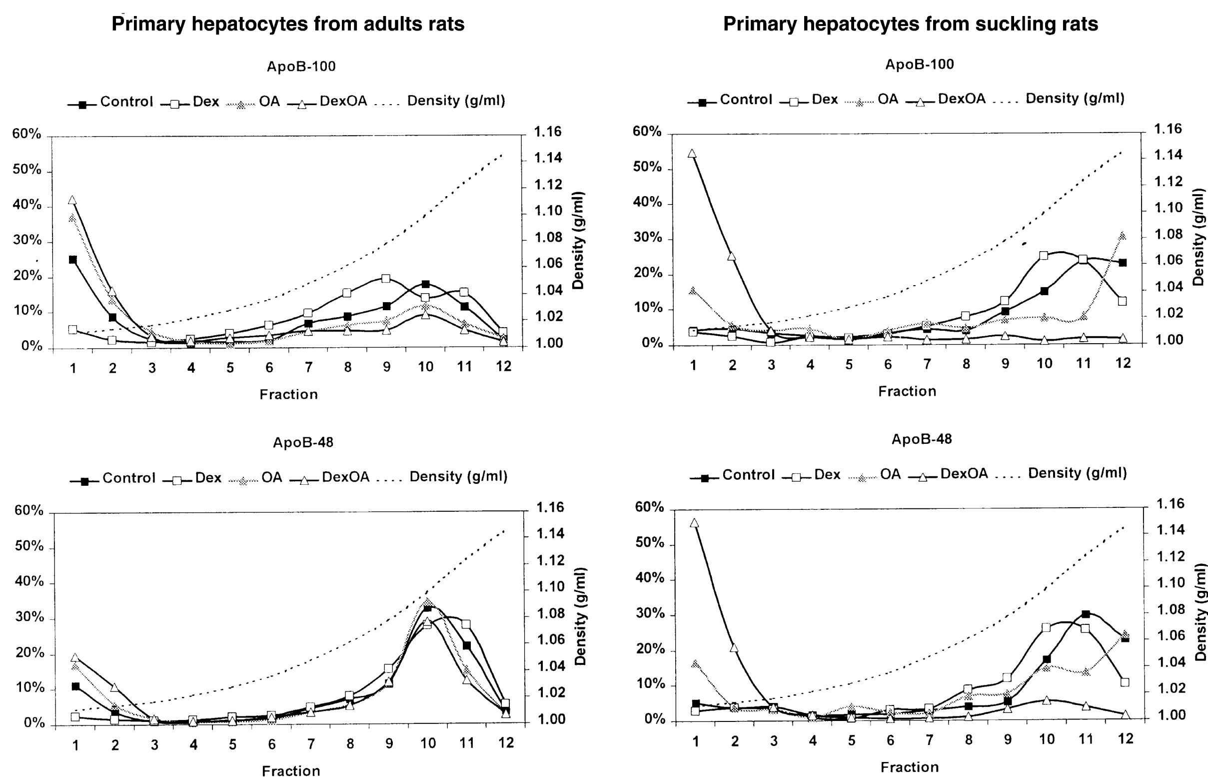

Fig. 6. Density profiles of secreted, newly synthesized apoB-100 and apoB-48 from primary culture hepatocytes of adult rats (90 days old) and suckling rats (17 days old) without additives (control) or with 100 nM dexamethasone (Dex), 0.4 mM oleate (OA), or 100 nM dexamethasone plus 0.4 mM oleate (DexOA). Hepatocytes from adult and suckling rats were cultured on collagen-coated dishes in Williams' medium E (10% FCS, 0.6% BSA) without additives (control) or with 100 nM dexamethasone (Dex), 0.4 mM oleate (OA), or 100 nM dexamethasone plus 0.4 mM oleate (DexOA) for 24 h. Thereafter, the medium was changed to Williams' medium E (1.2% BSA) and the cells were incubated with the same additives in the presence of [^{35}S]methionine for a further 4 h. After incubation the medium was subjected to ultracentrifugation at density d = 1.21 g/ml. The total lipoprotein fraction (i.e., the supernatant) was collected and further separated into 12 fractions by means of density gradient ultracentrifugation. The apolipoproteins of each fraction were separated by SDS-PAGE and the radioactivity of the excised apoB-100 and apoB-48 bands was measured by β counting. Values are given as the percentage of apoB radioactivity in each fraction. Each profile represents the average of three separate experiments. For clarity the standard deviations are not shown.

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radiolabeled apoB were found in the HDL density range. In contrast, oleate enhanced the VLDL peak of secreted apoB in cells isolated from rats at either stage of development. However, despite a clearly visible VLDL peak in hepatocytes of suckling rats the main portion of secreted apoB-100 and apoB-48 was still at LDL-HDL densities. This pattern was dramatically changed after addition of dexamethasone and oleate to the culture medium of hepatocytes from suckling rats, resulting in the secretion of apoB-100 and apoB-48-containing lipoproteins exclusively as VLDL and IDL. In hepatocytes of adult rats the combined effect of dexamethasone and oleate was not as pronounced as in hepatocytes from suckling rats and the density profile of apoB-100 and apoB-48 was only slightly different from that after oleate alone.

Intracellular apoB degradation

Because apoB secretion is known to be regulated to a great extent by intracellular apoB degradation, we carried out pulse-chase experiments to study this process in cultured hepatocytes from adult and suckling rats (**Fig. 7**). In control hepatocytes of adult rats, 38% of the initial radio-

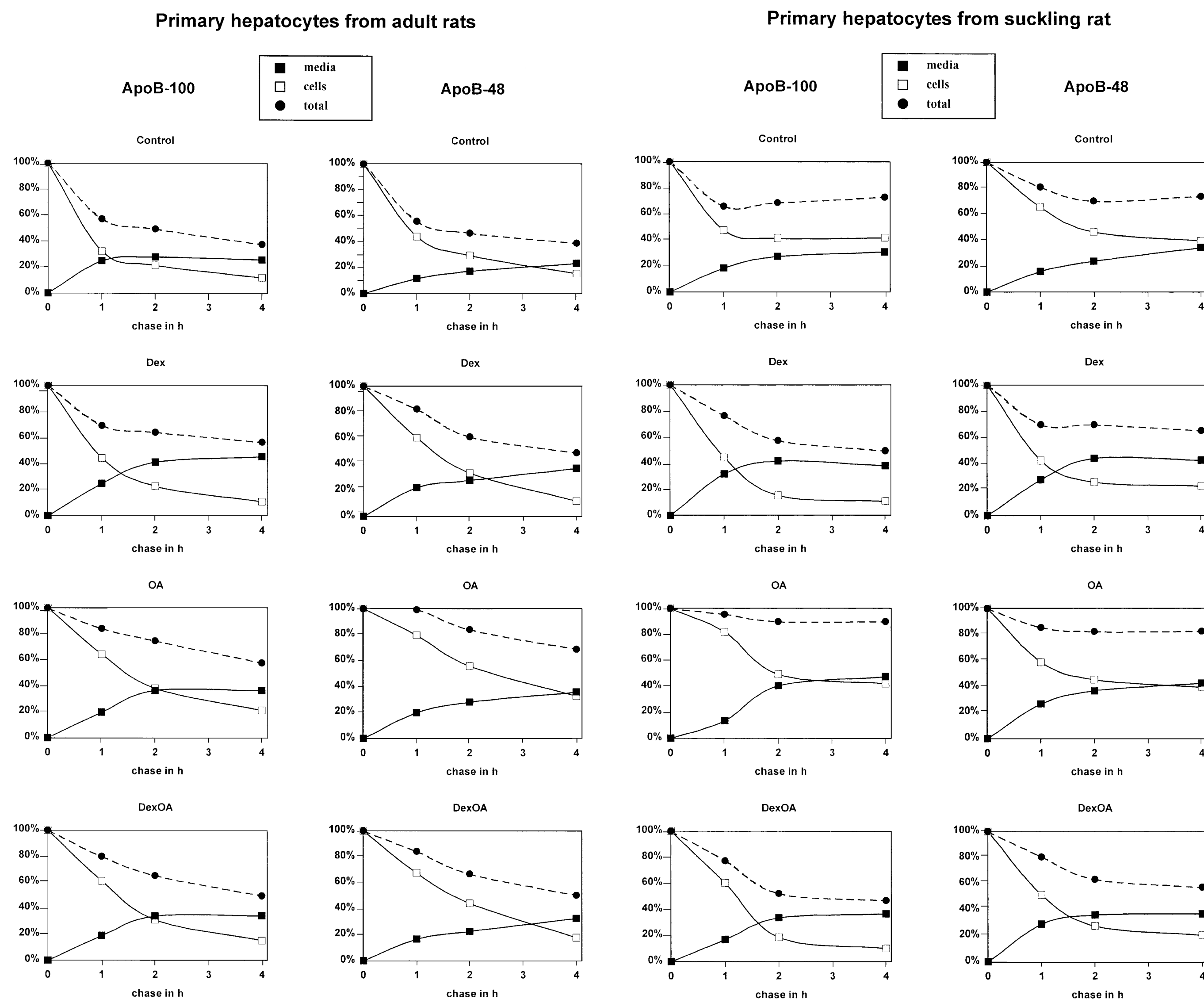

Fig. 7. Pulse-chase analysis of apoB-100 and apoB-48 in primary cultures of adult rat (90 days old) and suckling rat (17 days old) hepatocytes treated without additives (control) or with 100 nM dexamethasone (Dex), 0.4 mM oleate (OA), or 100 nM dexamethasone plus 0.4 mM oleate (DexOA). Hepatocytes from adult and suckling rats were cultured on collagen-coated dishes in Williams' medium E (10% FCS, 0.6% BSA) without additives (control) or with 100 nM dexamethasone (Dex), 0.4 mM oleate (OA), or 100 nM dexamethasone plus 0.4 mM oleate (DexOA) for 24 h. Thereafter, the medium was changed to methionine-free Williams' medium E (1.2% BSA) containing the same additives and the cells were pulse labeled with [^{35}S]methionine for 20 min and chased for 0, 1, 2, and 4 h. After the chase apoB in the culture medium and in the cell lysate was immunoprecipitated and subjected to SDS-PAGE, and the radioactivity in the excised apoB-100 and apoB-48 bands was measured by β counting. Results are given as the percentage of radioactivity in cellular apoB at $t = 0$ h. The total radioactivity was calculated as the sum of the relative cellular and secreted apoB radioactivities. The data represent the means of two or three independent experiments. For clarity the standard deviations are not shown.

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labeled apoB-100 was recovered at the end of the 4-h chase (12% in cells and 26% in medium), indicating that 62% of newly synthesized apoB-100 was intracellularly degraded during this time. Dexamethasone decreased the intracellular degradation of apoB-100 to 43% without changing the cellular recovery (11%) but increasing the secreted portion to 46% of the initial radioactivity at the beginning of the chase. After oleate treatment of hepatocytes from adult rats, the intracellular apoB-100 degradation was 42%, and the recovered radioactivity was higher in both the cells (21%) and the medium (37%) by about 10% in comparison with controls. The simultaneous addition of dexamethasone and oleate had an effect intermediate between each of these additives alone. Fifty percent of apoB-100 was degraded, with 15% and 34% of the initial radioactivity recovered in cells and medium, respectively. The results of the pulse-chase experiments for apoB-48 were similar to those for apoB-100. At the 4-h chase point 39% of the initial apoB-48 radioactivity was recovered in cells (16%) plus medium (23%), indicating an intracellular degradation of 61%. The apoB-48 degradation was decreased to 51%, 31%, and 50% after treatment with dexamethasone, oleate, and dexamethasone plus oleate, respectively. Like apoB-100, apoB-48 was recovered to a greater extent exclusively in the medium after dexamethasone treatment. In contrast, oleate increased the recovery of apoB-48 in cells and medium by similar amounts.

In control hepatocytes from suckling rats 74% of radioactive apoB-100 was recovered at the end of the chase, indicating that only 27% of newly synthesized apoB-100 was degraded, 35% less than in control hepatocytes of adults. After the 4-h chase, 31% of the initial radioactivity was secreted but most of the nondegraded apoB-100 was found in the cells (42% of the initial apoB-100 radioactivity). After dexamethasone treatment the radioactivity recovered in the cells dropped to 11% and that in medium slightly increased to 39%, giving a total recovery of 50% at the 4-h chase point, a situation similar to that of dexamethasone-treated hepatocytes from adult rats. The total recovery of initial apoB-100 radioactivity was highest in hepatocytes of sucklings after oleate treatment and was 90% of initial radioactivity (42% secreted and 48% cellular). After dexamethasone plus oleate treatment, the recovered cellular and secreted apoB-100 radioactivity was almost the same as after dexamethasone alone. Like in hepatocytes of adults the behavior of apoB-48 was similar to that of apoB-100. The recovery of radiolabeled apoB-48 amounted to 74% in control cells. The stimulation of intracellular degradation of apoB-48 by dexamethasone was not as pronounced as for apoB-100 but increased from 26% in controls to 35% in treated hepatocytes. The inhibitory effect of oleate on apoB-48 degradation was comparable to that on apoB-100, giving a recovery of 82% at the end of the chase. Like for adult hepatocytes, the state after dexamethasone plus oleate treatment was an average of the states resulting from treatment with each alone.

In general, on the basis of mathematical modeling (data not shown), the pulse-chase curves suggest a two-compartment system consisting of an "early" and a "late" intracellular degradation pool for both apoB-100 and apoB-48 in hepatocytes of adult and suckling rats. The lower apoB degradation in control hepatocytes of sucklings in comparison with hepatocytes of adults could be explained by negligible apoB degradation principally in the late compartment. Dexamethasone seems to inhibit the apoB degradation in the early degradation compartment at both stages of development but has different effects on the degradation in the late pool. The degradation in the late pool seemed to be unaltered in hepatocytes of adults but was clearly stimulated in hepatocytes of sucklings by dexamethasone. Furthermore, dexamethasone tended to accelerate apoB secretion in hepatocytes of sucklings more than in hepatocytes of adults. The most distinct inhibition of intracellular apoB degradation found after oleate treatment was restricted to the early compartment in hepatocytes of both adults and sucklings. In addition, oleate tended to slow down apoB secretion.

MTP mRNA concentration and MTP activity

To determine whether MTP might contribute to developmental and dexamethasone and/or oleate-induced changes in apoB assembly and secretion we measured MTP mRNA concentrations and MTP activities. Although the MTP mRNA concentration was nearly twice as high in livers from adult rats than in those from suckling rats (**Fig. 8**) the MTP activity was similar in freshly isolated hepatocytes from adult and suckling rats (**Fig. 9**). If hepatocytes were cultured for 24 h MTP mRNA concentration dropped in hepatocytes from suckling rats, reaching a 10 times lower level than in cultured hepatocytes from adult animals (Fig. 8). However, apart from a decreased MTP

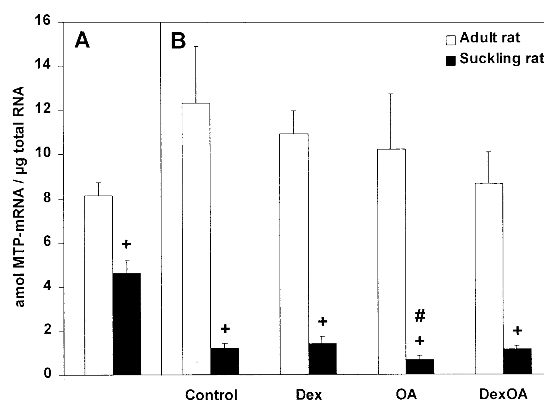

Fig. 8. MTP mRNA concentrations in livers and primary cultures of hepatocytes from adult rats (90 days old) and suckling rats (17 days old). Total RNA was isolated by the guanidine thiocyanate method from livers and from hepatocytes cultured on collagen-coated dishes in Williams' medium E (10% FCS, 0.6% BSA) without additives (control) or with 100 nM dexamethasone (Dex), 0.4 mM oleate (OA), or 100 nM dexamethasone plus 0.4 mM oleate (DexOA) for 24 h. The MTP mRNA levels were quantified by RNase protection assay as described in Materials and Methods. Data are expressed as means ± SD (n = 4). $^{+,\#}$ Significant difference at the $P < 0.05$ level versus the adult value (plus sign) or versus the corresponding control value (pound sign).

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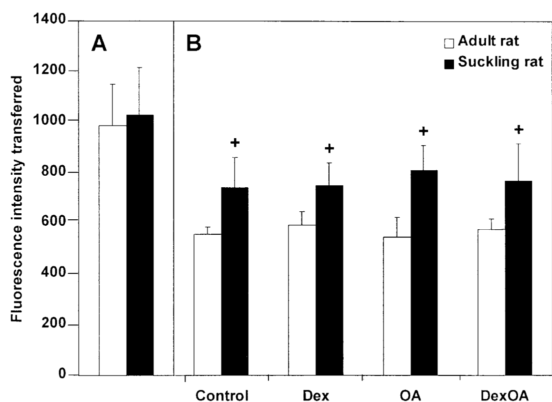

Fig. 9. MTP activities in homogenates of freshly isolated (A) and primary culture (B) hepatocytes from adult and suckling rats. Freshly isolated hepatocytes and hepatocytes cultured on collagen-coated dishes in Williams' medium E (10% FCS, 0.6% BSA) without additives (control) or with 100 nM dexamethasone (Dex), 0.4 mM oleate (OA), or 100 nM dexamethasone plus 0.4 mM oleate (DexOA) for 24 h were homogenized by sonication. Homogenate protein (100 μg) was subjected to the MTP activity assay as described in Materials and Methods. Data are expressed as mean ± SD (n = 4 or 5). $^+$ Significant difference at the $P < 0.05$ level versus the adult value.

mRNA concentration of about 50% in hepatocytes of sucklings after treatment with oleate, there were no significant changes in MTP mRNA concentrations after treatment with dexamethasone, oleate, or the combination of both. The differences in MTP mRNA concentration of cultured hepatocytes (Fig. 8) were not reflected in different MTP activities (Fig. 9). MTP activities were somewhat higher in cultured hepatocytes from suckling rats than in those from adult animals under all conditions (Fig. 9). None of the treatments affected MTP activity levels, either in hepatocytes from adults or in hepatocytes from sucklings.

DISCUSSION

The results presented in this study provide evidence that hepatocytes from suckling rats do not assemble mature VLDL but secrete apoB as lipoprotein particles in the LDL-HDL density range. Because hepatocytes from adult rats produce VLDL under the same experimental conditions it appears that VLDL assembly and secretion are developmentally regulated. Although our studies were performed in vitro the findings probably reflect the in vivo conditions because our initial studies were carried out with freshly isolated hepatocytes, which closely resemble hepatocytes in vivo. Furthermore, the density profiles of apoB-containing lipoproteins secreted by freshly isolated hepatocytes were similar to those of apoB-containing lipoproteins secreted by cells in primary culture (Fig. 4). In addition, VLDL secreted by hepatocyte cultures was structurally similar to VLDL secreted by perfused livers and to VLDL found in plasma (41). Thus our data give strong support to the idea that the high plasma LDL concentration during suckling of the rat (20–22) is the result of direct hepatic LDL secretion due to incomplete VLDL assembly. Furthermore, they extend the findings of Coleman et al. (16), who suggested a defect in hepatic VLDL assembly/secretion during the suckling period of the rat on the basis of triglyceride and apoB synthesis and secretion data. However, because lipoproteins and lipoprotein subfractions were not studied by these authors their conclusions remained speculative. Interestingly, we observed that the immaturity in VLDL production could be overcome in vitro if hepatocytes of sucklings were treated with dexamethasone plus oleate but not with either alone (Fig. 6). This indicates that the separate induction of apoB net synthesis by dexamethasone and of triglyceride net synthesis by oleate (Fig. 5) is not sufficient to trigger VLDL assembly in hepatocytes from suckling rats. Rather, VLDL assembly requires additional factors, which are underexpressed during suckling and can be stimulated by a synergistic effect of dexamethasone and oleate in vitro. The assembly of apoB-containing lipoprotein has been proposed to occur via a two-step mechanism (42–47). The first step occurs cotranslationally and involves the transfer of apoB from the rough endoplasmic reticulum membrane to the lumen and the formation of partially lipidated particles having the density and size of HDL. In the second step, the bulk of lipids is added to the lipid-poor VLDL precursors, generating the mature VLDL-sized particles. Accordingly the initial apoB lipidation, that is, the assembly of dense apoB-containing lipoproteins in the first step, would be a necessary requirement for the bulk addition of lipids and the production of VLDL in the second step of apoB assembly. Because hepatocytes from sucklings secreted apoB radioactivity in quantities similar to that of hepatocytes from adults, but as dense lipoprotein particles in the LDL-HDL density range rather than in the VLDL density range, one can draw the conclusion that the initial lipidation step of apoB is well developed whereas the second step is undeveloped during suckling. Only after addition of both dexamethasone and oleate do hepatocytes from sucklings secrete VLDL dense apoB-containing lipoproteins, indicating that the second step of VLDL assembly was predominantly induced by this treatment. The molecular mechanisms of the second step in VLDL assembly are poorly understood. However, results from several studies have suggested that in the smooth endoplasmic reticulum triglyceride-rich and apoB-free lipid particles fuse with the apoB-containing VLDL precursors to form complete VLDL (48–50). It is now well established that MTP is absolutely necessary for the first step of apoB lipoprotein assembly to form stable but dense apoB lipoprotein particles (51). However, it is not yet clear whether MTP is involved in the second step of VLDL assembly (52, 53). Despite lower MTP mRNA concentrations in livers and cultured hepatocytes from suckling rats than in those from adult animals (Fig. 8) we have found the same MTP activity in freshly isolated hepatocytes from adult and suckling rats and even somewhat higher MTP activities in cultured hepatocytes from sucklings in comparison with adults (Fig. 9). A discrepancy between the regulation of MTP mRNA and MTP activity levels was also reported by Lin, Gordon,

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and Wetterau (54), who have shown that this was due to the long half-life of MTP protein, which amounted to 4.4 days. In their studies a decrease in MTP mRNA levels after insulin administration had no effect on MTP activity within 24 h, as in our studies none of the treatments (dexamethasone, oleate, and dexamethasone plus oleate) affected MTP activity levels in cultured hepatocytes. The distinct differences in the density of secreted apoB-containing lipoproteins between suspended hepatocytes from adult and suckling rats at essentially the same MTP activities, together with the extreme density shift of secreted apoB after dexamethasone plus oleate treatment of cultured hepatocytes from suckling rats without changes in MTP activity, reveal that MTP is not a limiting factor in the second main lipidation step of VLDL assembly in rat hepatocytes. Consistently, in 10-day-old suckling rats, in which VLDL production is also markedly reduced, MTP levels were found to be unaltered in comparison with adult animals (55). Taken together, the data imply that hepatic MTP is present in excess during rat development and therefore the incomplete VLDL assembly during the suckling period is not associated with low MTP.

There is now ample evidence that only a small portion of newly synthesized triglyceride is secreted directly as VLDL, whereas the main part passes through an intracellular triglyceride storage pool from which it is derived for VLDL assembly via lipolysis/re-esterification (56–58). Our finding that livers from suckling and adult rats contain the same masses of triglycerides (Fig. 1) suggests that hepatocytes from sucklings cannot mobilize their triglyceride stores for VLDL secretion. In addition, even though oleate treatment stimulated total triglyceride net synthesis it did not increase the percentage of newly synthesized triglyceride that was secreted after 4 h (Fig. 5; 12% and 5% for hepatocytes from adults and sucklings, respectively). Hepatic microsomal lipase (28, 59, 60), which was low in livers of suckling rats but was increased during weaning (28), might be responsible for the differences in triglyceride mobilization and VLDL secretion between hepatocytes from adult and suckling rats and could be the subject of further investigations.

Another enzyme known to play an important role in regulating processes in the VLDL assembly/secretion pathway is cholesterol 7α-hydroxylase (61). Transcriptional stimulation of cholesterol 7α-hydroxylase induces triglyceride net synthesis and secretion of large triglyceride-rich VLDL whereas inhibition of cholesterol 7α-hydroxylase transcription decreases triglyceride secretion (62). Interestingly, the expression of cholesterol 7α-hydroxylase mRNA is low during suckling and increases after weaning (63), coinciding with the increasing ability of hepatocytes for triglyceride secretion. However, whether cholesterol 7α-hydroxylase is important for developmental control of VLDL assembly in rat hepatocytes remains to be elucidated.

Beside VLDL assembly intracellular apoB degradation is also clearly under developmental control as shown by the pulse-chase experiments (Fig. 7). These experiments revealed that there may be early and late degradation sites for apoB-100 and apoB-48 in hepatocytes of adult and suckling rats, which were also shown in most other primary hepatocytes examined (64–67). The low apoB degradation in hepatocytes of suckling rats is mainly due to the lack of apoB degradation in the late degradation site, which was stimulated by dexamethasone to nearly the adult level. On the other hand, dexamethasone inhibited apoB degradation in the early degradation site at both stages of development. Interestingly enough, cholesterol 7α-hydroxylase, which can be stimulated by dexamethasone, blocked the ubiquitin-dependent proteasome degradation of apoB-53 in Chinese hamster ovary cells by reducing ubiquitin conjugation (68). The most distinct inhibition of apoB degradation was found after oleate treatment of hepatocytes from both adult and suckling rats (Fig. 7). Oleate apparently protects apoB from degradation in the early degradation site, thus increasing the stability of cellular apoB. This was also found for primary hamster and rabbit hepatocytes (69) and for Hep-G2 cells, in which oleate facilitated translocation of newly synthesized apoB across the endoplasmic reticulum membrane (70, 71).

In conclusion, hepatocytes from suckling rats are unable to assemble mature VLDL; rather, they secrete apoB as primordial lipoprotein particles in the LDL-HDL density range. This is in part due to the low hepatocellular triglyceride synthesis during the suckling period. However, even after massive stimulation of triglyceride synthesis by oleate, hepatocytes from suckling rats were still unable to efficiently assemble VLDL for triglyceride secretion. Only after simultaneous addition of dexamethasone and oleate to the culture medium were hepatocytes from suckling rats capable of producing mature VLDL. Although we cannot explain this phenomenon on the basis of our data it is noteworthy that strong synergistic effects of dexamethasone and oleate are also described for stimulating the expression of the transcription factors peroxisome proliferator-activated receptor α and retinoid X receptor α, known to play a key role in regulating the transcription of essential proteins of lipid and lipoprotein metabolism, such as cholesterol 7α-hydroxylase, apoA-I, apoA-II, apoC-III, HMG-CoA synthase, acyl-CoA oxidase, and fatty acid-binding protein (72–76). Our data reveal that MTP is not a limiting factor in VLDL assembly during rat development. Rather, they imply that the MTP-dependent first step in VLDL assembly, that is, the production of primordial, dense apoB-containing lipoproteins, is equally developed at both stages of development. The second step, the addition of bulk lipids to form mature VLDL, is still immature during the suckling period and requires other, still unknown factors beside MTP.

Our studies of VLDL assembly during the suckling period of the rat, a state characterized by hypertriglyceridemia and hyposinsulinism, might be of general interest. It is well known that the severely atherogenic small, dense LDL (phenotype B) preferentially appear in association with hypertriglyceridemia and insulin resistance (77–80). However, the mechanisms of the formation of the small, dense LDL subfraction are still unknown. In kinetic studies Packard et al. (81) have found that a substantial amount of LDL apoB was directly produced by the liver

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bypassing the VLDL precursor pathway. Our findings support the concept that the liver can secrete LDL directly under certain circumstances that might be possible also for a small, dense LDL subfraction.

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REFERENCES

1. Girard, J., P. Ferré, J-P. Pégorier, and P-H. Duée. 1992. Adaptations of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition. *Physiol. Rev.* **72:** 507–562.
2. Greengard, O. 1975. Steroids and the maturation of rat tissues. *J. Steroid Biochem.* **6:** 639–642.
3. Henning, S. J. 1978. Plasma concentrations of total and free corticosterone during development in the rat. *Am. J. Physiol.* **235:** E451–E456.
4. Lee, P. C., and E. Lebenthal. 1983. Role of corticoids independent of food intake in premature increase of pancreatic enzyme activities following early weaning in rats. *J. Nutr.* **113:** 1381–1387.
5. Raabe, M., L. M. Flynn, C. H. Zlot, J. S. Wong, M. M. Véniant, R. L. Hamilton, and S. G. Young. 1998. Knockout of the abetalipoproteinemia gene in mice: reduced lipoprotein secretion in heterozygotes and embryonic lethality in homozygotes. *Proc. Natl. Acad. Sci. USA.* **95:** 8686–8691.
6. Mayor, F., and J. M. Cuezva. 1985. Hormonal and metabolic changes in the perinatal period. *Biol. Neonate.* **48:** 185–196.
7. Marceau, N., H. Baribault, and I. Leroux-Nicollet. 1985. Dexamethasone can modulate the synthesis and organization of cytokeratins in cultured differentiating rat hepatocytes. *Can. J. Biochem. Cell Biol.* **63:** 448–457.
8. Ferré, P., J. F. Decaux, T. Issad, and J. Girard. 1986. Changes in energy metabolism during the suckling and weaning period in the newborn. *Reprod. Nutr. Dev.* **26:** 619–631.
9. Angel, J. F., and D. W. Back. 1985. Weaning and metabolic regulation in the rat. *Can. J. Physiol. Pharmacol.* **63:** 538–545.
10. Wells, M. A. 1985. Fatty acid metabolism and ketone formation in the suckling rat. *Fed. Proc.* **44:** 2365–2368.
11. Decaux, J. F., P. Ferré, D. Robin, P. Robin, and J. Girard. 1988. Decreased hepatic fatty acid oxidation at weaning in the rat is not linked to a variation of malonyl-CoA concentration. *J. Biol. Chem.* **263:** 3284–3289.
12. Iritani, N., H. Fukuda, and Y. Matsumura. 1993. Lipogenic enzyme gene expression in rat liver during development after birth. *J. Biochem.* **113:** 519–525.
13. Garcia, M. V., J. A. Aguilera, A. Gil, and P. A. Sanchez. 1996. Changes in plasma lipoproteins and liver lipids in neonatal rats. *Comp. Biochem. Physiol.* **113:** 789–793.
14. Frost, S. C., W. A. Clark, and M. A. Wells. 1983. Studies on fat digestion, absorption, and transport in the suckling rat. IV. In vivo rates of triacylglycerol secretion by intestine and liver. *J. Lipid Res.* **24:** 899–903.
15. Imaizumi, K., Y. F. Lu, and M. Sugano. 1987. Characterization of serum apolipoprotein patterns in rats during suckling and post-weaning periods. *Biochim. Biophys. Acta.* **917:** 269–278.
16. Coleman, R. A., E. B. Haynes, T. M. Sand, and R. A. Davis. 1988. Developmental coordinate expression of triacylglycerol and small molecular weight apoB synthesis and secretion by rat hepatocytes. *J. Lipid Res.* **29:** 33–42.
17. Wu, J. H., C. F. Semenkovich, S. H. Chen, W. H. Li, and L. Chan. 1990. Apolipoprotein B mRNA editing. Validation of a sensitive assay and developmental biology of RNA editing in the rat. *J. Biol. Chem.* **265:** 12312–12316.
18. Inui, Y., A. M. Hausman, N. Nanthakumar, S. J. Henning, and N. O. Davidson. 1992. Apolipoprotein B messenger RNA editing in rat liver: developmental and hormonal modulation is divergent from apolipoprotein A-IV gene expression despite increased hepatic lipogenesis. *J. Lipid Res.* **33:** 1843–1856.
19. Funahashi, T., F. Giannoni, A. M. DePaoli, S. F. Skarosi, and N. O. Davidson. 1995. Tissue-specific, developmental and nutritional regulation of the gene encoding the catalytic subunit of the rat apolipoprotein B mRNA editing enzyme: functional role in the modulation of apoB mRNA editing. *J. Lipid Res.* **36:** 414–428.
20. Lorentz, A., D. Plonné, H. P. Schulze, and R. Dargel. 1996. Dexamethasone enhanced by insulin, but not by thyroid hormones stimulates apolipoprotein B mRNA editing in cultured rat hepatocytes depending on the developmental stage. *FEBS Lett.* **391:** 57–60.
21. Fernando-Warnakulasuriya, G. J., M. L. Eckerson, W. A. Clark, and M. A. Wells. 1983. Lipoprotein metabolism in the suckling rat: characterization of plasma and lymphatic lipoproteins. *J. Lipid Res.* **24:** 1626–1638.
22. Perin Ansart, M. C., D. Vacher, and G. A. Girard. 1988. Lipoproteins of the newborn rat. Reciprocal development of low density lipoproteins and apoprotein E-rich high density lipoproteins. *J. Dev. Physiol.* **10:** 321–334.
23. Plonné, D., L. Winkler, H. Franke, and R. Dargel. 1992. The visceral yolk sac—an important site of synthesis and secretion of apolipoprotein B containing lipoproteins in the feto-placental unit of the rat. *Biochim. Biophys. Acta.* **1127:** 174–185.
24. Franke, H., D. Plonné, L. Winkler, and R. Dargel. 1992. Synthesis, secretion and immunoelectron microscopic demonstration of apolipoprotein B-containing lipoprotein particles in the visceral rat yolk sac. *Histochemistry.* **97:** 283–292.
25. Plonné, D., H. Heller, U. Kahlert, and R. Dargel. 1995. Quantitative and qualitative characterization of apolipoprotein B containing lipoproteins produced by the visceral rat yolk sac in two different in vitro systems: organ culture and isolated epithelial cells in suspension culture. *Biochim. Biophys. Acta.* **1256:** 71–80.
26. Plonné, D., A. Stacke, K. U. Weber, U. Endisch, and R. Dargel. 1996. The pattern of apolipoprotein B100 containing lipoprotein subclasses produced by the isolated visceral rat yolk sac depends on developmental stage and fatty acid availability. *Biochim. Biophys. Acta.* **1299:** 54–66.
27. Benoist, F., and T. Grand-Perret. 1996. ApoB-100 secretion by HepG2 cells is regulated by the rate of triglyceride biosynthesis but not by intracellular lipid pools. *Arterioscler. Thromb. Vasc. Biol.* **16:** 1229–1235.
28. Lehner, R., Z. Cui, and D. E. Vance. 1999. Subcellullar localization, developmental expression and characterization of a liver triacylglycerol hydrolase. *Biochem. J.* **338:** 761–768.
29. Dürer, U., H. Franke, R. Dargel, and J. Ude. 1986. A post-embedding immunoelectron-microscopic demonstration of apolipoprotein-B-containing lipoprotein particles in hepatocytes of fetal rats. *Histochemistry.* **84:** 263–270.
30. Seglen, P. O. 1976. Preparation of isolated rat liver cells. *Methods Cell Biol.* **13:** 29–83.
31. Zimmermann, T., H. Franke, M. Peuker, and R. Dargel. 1992. Quantitative studies on fatty acid metabolism in isolated parenchymal cells from normal and cirrhotic livers in rats. *J. Hepatol.* **15:** 10–16.
32. Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
33. Sparks, J. D., and C. E. Sparks. 1990. Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. *J. Biol. Chem.* **265:** 8854–8862.
34. Plonné, D., I. J. Cartwright, W. Linss, R. Dargel, J. M. Graham, and J. A. Higgins. 1999. Separation of the intracellular secretory compartment of rat liver and isolated rat hepatocytes in a single step using self-generating gradients of iodixanol. *Anal. Biochem.* **276:** 88–96.
35. Cartwright, I. J., and J. A. Higgins. 1995. Intracellular events in the assembly of very-low-density-lipoprotein lipids with apolipoprotein B in isolated rabbit hepatocytes. *Biochem. J.* **310:** 897–907.
36. Cartwright, I. J., J. A. Higgins, J. Wilkinson, S. Bellavia, J. S. Kendrick, and J. M. Graham. 1997. Investigation of the role of lipids in the assembly of very low density lipoproteins in rabbit hepatocytes. *J. Lipid Res.* **38:** 531–545.
37. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
38. Bennett, A. J., M. A. Billett, A. M. Salter, and D. A. White. 1995. Regulation of hamster hepatic microsomal triglyceride transfer protein mRNA levels by dietary fats. *Biochem. Biophys. Res. Commun.* **212:** 473–478.
39. Bennett, A. J., M. A. Billett, A. M. Salter, E. H. Mangiapane, J. S. Bruce, K. L. Anderton, C. B. Marenah, N. Lawson, and D. A. White. 1995. Modulation of hepatic apolipoprotein B, 3-hydroxy-3-

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methylglutaryl-CoA reductase and low-density lipoprotein receptor mRNA and plasma lipoprotein concentrations by defined dietary fats. Comparison of trimyristin, tripalmitin, tristearin and triolein. *Biochem. J.* **311:** 167–173.
40. Harley, C. B. 1987. Hybridization of oligo(dT) to RNA on nitrocellulose. *Gene Anal. Tech.* **4:** 17–22.
41. Davis, R. A. 1986. Lipoprotein synthesis and secretion by cultured hepatocytes. *Methods Enzymol.* **129:** 272–283.
42. Borén, J., S. Rustaeus, and S-O. Olofsson. 1994. Studies on the assembly of apolipoprotein B-100- and B-48-containing very low density lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **269:** 25879–25888.
43. Swift, L. L. 1995. Assembly of very low density lipoproteins in rat liver: a study of nascent particles recovered from the rough endoplasmic reticulum. *J. Lipid Res.* **36:** 395–406.
44. Kendrick, J. S., J. Wilkinson, I. J. Cartwright, S. Lawrence, and J. A. Higgins. 1998. Regulation of the assembly and secretion of very low density lipoproteins by the liver. *Biol. Chem.* **379:** 1033–1040.
45. Rustaeus, S., P. Stillemark, K. Lindberg, D. Gordon, and S-O. Olofsson. 1998. The microsomal triglyceride transfer protein catalyzes the post-translational assembly of apolipoprotein B-100 very low density lipoprotein in McA-RH7777 cells. *J. Biol. Chem.* **273:** 5196–5203.
46. Rustaeus, S., K. Lindberg, P. Stillemark, C. Claesson, L. Asp, T. Larsson, J. Borén, and S-O. Olofsson. 1999. Assembly of very low density lipoprotein: a two-step process of apolipoprotein B core lipidation. *J. Nutr.* **129:** 463S.
47. Stillemark, P., J. Borén, M. Andersson, T. Larsson, S. Rustaeus, K. A. Karlsson, and S-O. Olofsson. 2000. The assembly and secretion of apolipoprotein B-48-containing very low density lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **275:** 10506–10513.
48. Alexander, C. A., R. L. Hamilton, and R. J. Havel. 1976. Subcellular localization of B apoprotein of plasma lipoproteins in rat liver. *J. Cell Biol.* **69:** 241–263.
49. Hamilton, R. L., J. S. Wong, C. M. Cham, L. B. Nielsen, and S. G. Young. 1998. Chylomicron-sized lipid particles are formed in the setting of apolipoprotein B deficiency. *J. Lipid Res.* **39:** 1543–1557.
50. Higgins, J. A. 1988. Evidence that during very low density lipoprotein assembly in rat hepatocytes most of the triacylglycerol and phospholipid are packaged with apolipoprotein B in the Golgi complex. *FEBS Lett.* **232:** 405–408.
51. Gordon, D. A., and H. Jamil. 2000. Progress towards understanding the role of microsomal triglyceride transfer protein in apolipoprotein-B lipoprotein assembly. *Biochim. Biophys. Acta.* **1486:** 72–83.
52. Gordon, D. A., H. Jamil, R. E. Gregg, S-O. Olofsson, and J. Borén. 1996. Inhibition of the microsomal triglyceride transfer protein blocks the first step of apolipoprotein B lipoprotein assembly but not the addition of bulk core lipids in the second step. *J. Biol. Chem.* **271:** 33047–33053.
53. Wang, Y., R. S. McLeod, and Z. Yao. 1997. Normal activity of microsomal triglyceride transfer protein is required for the oleate-induced secretion of very low density lipoproteins containing apolipoprotein B from McA-RH7777 cells. *J. Biol. Chem.* **272:** 12272–12278.
54. Lin, M. C., D. Gordon, and J. R. Wetterau. 1995. Microsomal triglyceride transfer protein (MTP) regulation in HepG2 cells: insulin negatively regulates MTP gene expression. *J. Lipid Res.* **36:** 1073–1081.
55. Brett, D. J., R. J. Pease, J. Scott, and G. F. Gibbons. 1995. Microsomal triglyceride transfer protein activity remains unchanged in rat livers under conditions of altered very-low-density lipoprotein secretion. *Biochem. J.* **310:** 11–14.
56. Gibbons, G. F., and D. Wiggins. 1995. The enzymology of hepatic very-low-density lipoprotein assembly. *Biochem. Soc. Trans.* **23:** 495–500.
57. Gibbons, G. F., and D. Wiggins. 1995. Intracellular triacylglycerol lipase: its role in the assembly of hepatic very-low-density lipoprotein (VLDL). *Adv. Enzyme Regul.* **35:** 179–198.
58. Lankester, D. L., A. M. Brown, and V. A. Zammit. 1998. Use of cytosolic triacylglycerol hydrolysis products and of exogenous fatty acid for the synthesis of triacylglycerol secreted by cultured rat hepatocytes. *J. Lipid Res.* **39:** 1889–1895.
59. Lehner, R., and R. Verger. 1997. Purification and characterization of a porcine liver microsomal triacylglycerol hydrolase. *Biochemistry.* **36:** 1861–1868.
60. Lehner, R., and D. E. Vance. 1999. Cloning and expression of a cDNA encoding a hepatic microsomal lipase that mobilizes stored triacylglycerol. *Biochem. J.* **343:** 1–10.
61. Davis, R. A. 1999. Cell and molecular biology of the assembly and secretion of apolipoprotein B-containing lipoproteins by the liver. *Biochim. Biophys. Acta.* **1440:** 1–31.
62. Angelin, B., M. Eriksson, and M. Rudling. 1999. Bile acids and lipoprotein metabolism: a renaissance for bile acids in the post-statin era? *Curr. Opin. Lipidol.* **10:** 269–274.
63. Massimi, M., S. R. Lear, S. L. Huling, A. L. Jones, and S. K. Erickson. 1998. Cholesterol 7alpha-hydroxylase (CYP7A): patterns of messenger RNA expression during rat liver development. *Hepatology.* **28:** 1064–1072.
64. Sparks, J. D., and C. E. Sparks. 1994. Insulin regulation of triacylglycerol-rich lipoprotein synthesis and secretion. *Biochim. Biophys. Acta.* **1215:** 9–32.
65. Cartwright, I. J., A. M. Hebbachi, and J. A. Higgins. 1993. Transit and sorting of apolipoprotein B within the endoplasmic reticulum and Golgi compartments of isolated hepatocytes from normal and orotic acid-fed rats. *J. Biol. Chem.* **268:** 20937–20952.
66. Wang, C. N., T. C. Hobman, and D. N. Brindley. 1995. Degradation of apolipoprotein B in cultured rat hepatocytes occurs in a post-endoplasmic reticulum compartment. *J. Biol. Chem.* **270:** 24924–24931.
67. Cartwright, I. J., and J. A. Higgins. 1996. Intracellular degradation in the regulation of secretion of apolipoprotein B-100 by rabbit hepatocytes. *Biochem. J.* **314:** 977–984.
68. Du, E. Z., J. F. Fleming, S. L. Wang, G. M. Spitsen, and R. A. Davis. 1999. Translocation-arrested apolipoprotein B evades proteasome degradation via a sterol-sensitive block in ubiquitin conjugation. *J. Biol. Chem.* **274:** 1856–1862.
69. Taghibiglou, C., D. Rudy, S. C. Van Iderstine, A. Aiton, D. Cavallo, R. Cheung, and K. Adeli. 2000. Intracellular mechanisms regulating apoB-containing lipoprotein assembly and secretion in primary hamster hepatocytes. *J. Lipid Res.* **41:** 499–513.
70. Macri, J., and K. Adeli. 1997. Studies on intracellular translocation of apolipoprotein B in a permeabilized HepG2 system. *J. Biol. Chem.* **272:** 7328–7337.
71. Wu, X., N. Sakata, K. M. Lele, M. Zhou, H. Jiang, and H. N. Ginsberg. 1997. A two-site model for apoB degradation in HepG2 cells. *J. Biol. Chem.* **272:** 11575–11580.
72. Lemberger, T., R. Saladin, M. Vazquez, F. Assimacopoulos, B. Staels, B. Desvergne, W. Wahli, and J. Auwerx. 1996. Expression of the peroxisome proliferator-activated receptor alpha gene is stimulated by stress and follows a diurnal rhythm. *J. Biol. Chem.* **271:** 1764–1769.
73. Sterchele, P. F., H. Sun, R. E. Peterson, and H. J. Vanden. 1996. Regulation of peroxisome proliferator-activated receptor-alpha mRNA in rat liver. *Arch. Biochem. Biophys.* **326:** 281–289.
74. Kersten, S., J. Seydoux, J. M. Peters, F. J. Gonzalez, B. Desvergne, and W. Wahli. 1999. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J. Clin. Invest.* **103:** 1489–1498.
75. Kersten, S., B. Desvergne, and W. Wahli. 2000. Roles of PPARs in health and disease. *Nature.* **405:** 421–424.
76. Fruchart, J. C., P. Duriez, and B. Staels. 1999. Peroxisome proliferator-activated receptor-alpha activators regulate genes governing lipoprotein metabolism, vascular inflammation and atherosclerosis. *Curr. Opin. Lipidol.* **10:** 245–257.
77. Griffin, B. A., D. J. Freeman, G. W. Tait, J. Thomson, M. J. Caslake, C. J. Packard, and J. Shepherd. 1994. Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk. *Atherosclerosis.* **106:** 241–253.
78. Festa, A., R. J. D'Agostino, L. Mykkänen, R. P. Tracy, C. N. Hales, B. V. Howard, and S. M. Haffner. 1999. LDL particle size in relation to insulin, proinsulin, and insulin sensitivity. The Insulin Resistance Atherosclerosis Study. *Diabetes Care.* **22:** 1688–1693.
79. Haffner, S. M., L. Mykkänen, D. Robbins, R. Valdez, H. Miettinen, B. V. Howard, M. P. Stern, and R. Bowsher. 1995. A preponderance of small dense LDL is associated with specific insulin, proinsulin and the components of the insulin resistance syndrome in non-diabetic subjects. *Diabetologia.* **38:** 1328–1336.
80. Griffin, B. A. 1999. Lipoprotein atherogenicity: an overview of current mechanisms. *Proc. Nutr. Soc.* **58:** 163–169.
81. Packard, C. J., T. Demant, J. P. Stewart, D. Bedford, M. J. Caslake, G. Schwertfeger, A. Bedynek, J. Shepherd, and D. Seidel. 2000. Apolipoprotein B metabolism and the distribution of VLDL and LDL subfractions. *J. Lipid Res.* **41:** 305–318.

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