Estrogen dependence of synthesis and secretion of apolipoprotein B-containing lipoproteins in the chicken hepatoma cell line, LMH-2A

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Abstract The chicken hepatoma cell line LMH-2A, which permanently overexpresses the chicken estrogen receptor, was used to study the synthesis and secretion of lipoproteins in response to treatment with estrogen. In the absence of the hormone, only small amounts of apolipoprotein B (apoB) and no apolipoprotein VLDL II (apoII) were found in cell extracts. After treatment of cells with moxestrol, a stable estrogen derivative, for 24 to 48 h, a dramatic increase in the quantities of these lipoproteins was observed both in cell extracts and in the medium. As determined by pulse-chase experiments, both proteins also showed enhanced rates of synthesis after estrogen induction, and secretion of the newly synthesized proteins was essentially complete by 6 h. The secreted apoB-containing lipoprotein particles have a density corresponding to that of very low density lipoprotein (VLDL). Furthermore, in estrogen-stimulated cells, the secreted particles also contain apoII, as shown by co-immunoprecipitation of apoII, and apoB. It appears that vitellogenin, the product of another estrogen-regulated gene in egg-laying species, is not synthesized by LMH-2A cells. Taken together, the data suggest that LMH-2A cells provide a new and promising cell system to investigate lipoprotein synthesis, assembly, and secretion in an estrogen-dependent manner.—**Hermann, M., F. Seif, W. J. Schneider, and N. E. Ivessa.** Estrogen dependence of synthesis and secretion of apolipoprotein B-containing lipoproteins in the chicken hepatoma cell line, LMH-2A. *J. Lipid Res.* 1997. **38:** 1308–1317.

Supplementary key words apolipoprotein II • very low density lipoprotein • hormonal regulation • secretory pathway

One of the important roles of the hepatocyte is the synthesis of triacylglycerol which upon secretion into the plasma serves as energy source in peripheral tissues as well as for deposition in adipose tissue and, especially in egg-laying species, in the developing oocyte. This role is mediated by the very low density lipoprotein (VLDL) that is comprised of a core of neutral triglycerides, surrounded by polar phospholipids and cholesterol, and apolipoproteins (1). The main protein component essential for the assembly of VLDL is apolipoprotein B (apoB), a hydrophobic glycoprotein greater than 500 kDa. A second protein component present in avian VLDL is the 9.5 kDa small apolipoprotein VLDL-II (apoII) that is functionally equivalent to the mammalian apoC-III, as it acts as an inhibitor of lipoprotein lipase (2).

The synthesis of components of lipoproteins, the assembly of the lipoprotein particles and their secretion has been studied so far mainly in primary rat hepatocytes (3), in HepG2 cells, a human hepatoma cell line (4, 5), in McArdle-RH7777 rat hepatoma cells (6, 7), and in primary chicken hepatocytes (8, 9). Obviously, studies with primary cells are rather complicated to conduct and may pose problems of reproducibility. Also, for instance, HepG2 cells are known to secrete underlipidated apoB-containing lipoprotein particles (for review see refs. 10, 11). Therefore, the establishment of a novel system(s) for the study of hepatic lipoprotein synthesis would be a valuable tool.

In the chicken, the formation of VLDL and of other yolk precursors has been shown to be under strict hormonal control. Estrogen causes an induction of apoII, vitellogenins I, II, and III, and also of apoB synthesis (12, 13), which is, at least in part, due to the presence of estrogen-responsive elements in the 5′-upstream regions of the respective genes (14, 15). In fact, the avian apoII gene has been widely used for the analysis of estrogen-dependent gene regulation on the level of the apoII mRNA. Recently, these studies were extended to the chicken leghorn male hepatoma (LMH) cell line. This cell line, developed by Kawaguchi et al. (16), is

Abbreviations: apoB, apolipoprotein B; apoII, apolipoprotein VLDL-II; ELC, enhanced chemiluminescence; HRP, horseradish peroxidase; IP, immunoprecipitation; LMH, leghorn male hepatoma; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein; VTG, vitellogenin.

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responsive to estrogen in that estrogen-responsive genes, such as apoII, are induced by the hormone, albeit to levels far below those observed in vivo (17–19). To augment the responsiveness to estrogen, the clonal cell line LMH-2A, which stably expresses the chicken estrogen receptor, has been produced (20).

Here we describe our studies on the regulation by estrogen of the (apo)lipoprotein synthesis in LMH-2A cells. We demonstrate that in these cells apoB and apoII protein synthesis and secretion are induced by estrogen. Furthermore, it is shown that among the apoB- and apoII-containing lipoprotein particles that are secreted from LMH-2A cells, the VLDL class predominates.

EXPERIMENTAL PROCEDURES

Materials

Waymouth's medium MB 752/1 without L-glutamine, L-methionine, sodium bicarbonate, and phenol red, penicillin, streptomycin sulfate, L-glutamine, geneticin (G418 sulfate), PBS, and fetal calf serum (FBS) were purchased from Gibco/BRL. Gelatin, protein A, coupled with horseradish peroxidase, L-methionine, Triton X-100, PMSF, aprotinin, leupeptin, leu-leu-leu, and pepstatin were obtained from Sigma. Sodium deoxycholate was purchased from USB, DE-52 from Whatman, and Protein A-Sepharose CL-4B beads were from Pharmacia. Moxestrol, EN^{3}HANCE and X-ray films were purchased from DuPont/NEN. Protein standards for SDS-PAGE were obtained from Bio-Rad (broad range makers), and [^{14}C]protein standards were from BRL. Met-label ([^{35}S]methionine and [^{35}S]cysteine; >1100 Ci/mmol) was purchased from ARC. Nitrocellulose membranes and the ECL kit were obtained from Amersham.

Preparation of antibodies

Polyclonal antibodies directed against chicken apoB and apoII were raised in adult female New Zealand White rabbits as described (21). For the preparation of anti-apoB antibodies, the apolipoprotein was isolated from purified laying hen VLDL by electroelution from 4.5–18% gradient SDS-polyacrylamide gels on which VLDL apoproteins had been separated. Anti-apoII was prepared against the purified apolipoprotein as described earlier (21). Polyclonal antibodies against chicken vitellogenin were also prepared in rabbits. Vitellogenin was purified from the sera of moxestrol-treated roosters by centrifugation at 140,000 *g* (Beckman 70.1Ti rotor) for 16 h at 4°C followed by chromatography on a DE-52 column as described previously (22). The respective antigen was prepared for injection by mixing equal volumes of the apolipoprotein-containing solution and Freund's complete adjuvant. Antisera were tested by Western blotting, and IgG fractions were purified from sera by affinity chromatography on protein A-Sepharose CL-4B according to Harlow and Lane (23).

Cell culture

The LMH-2A cell line (constructed by stable transfection of LMH cells with a plasmid containing the chicken estrogen receptor cDNA) was generously provided by Dr. David L. Williams, State University of New York, Stony Brook, NY. The LMH-2A cells were cultured in complete Waymouth's MB 752/1 medium without phenol red, supplemented with penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), L-glutamine (2 mM), L-methionine (0.35 mM), G418 (100 μg/ml), and 10% FBS, on gelatin-coated (0.1%) plastic in a humidified environment of 5% CO_2/95% air (20). For estrogen-treated cultures, the synthetic analog of 17β-OH estradiol, moxestrol (20), was dissolved in ethanol at a concentration of 50 μM, kept as a stock solution at − 20°C, and used at a final concentration of 50 nM. This concentration of moxestrol has been demonstrated to be effective in the induction of the apoII mRNA (20).

Cell solubilization

After culturing cells for 24 h and 48 h with or without moxestrol in 100-mm diameter tissue culture dishes, LMH-2A cells and media (5 ml) were recovered for immunoblot analysis. Cell monolayers were washed three times with PBS and scraped from the dishes with a rubber policeman. The cells were centrifuged at 2000 *g* for 5 min, and the cell pellet was solubilized by addition of buffer containing detergent (200 mM Tris maleate, pH 6.5, 2 mM $CaCl_2$, 0.5 mM PMSF, 2.5 μM leupeptin, and 1.4% Triton X-100). The cell extracts were centrifuged at 300,000 *g* (Beckman TKA 100.1 rotor) for 40 min at 4°C. The pellets were discarded and the supernatants were subjected to one-dimensional SDS polyacrylamide gel electrophoresis and Western blot analysis as described below. For the experiment shown in Fig. 1, sera from a chicken, a control rooster, and a rooster that was treated with 17β-estradiol for 48 h (10 mg/kg body weight; dissolved in 1, 2-propanediol and injected intravenously) were used for comparison.

Protein electrophoresis, transfer to nitrocellulose, and Western blot analysis

One-dimensional SDS-PAGE under non-reducing conditions was performed according to Laemmli (24) on 4–20% gradient slab gels (unless indicated otherwise) using a minigel system (Bio-Rad). The samples did not contain reducing agents and were not heated

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prior to application. Gels were calibrated with the following molecular mass standards: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.5 kDa; aprotinin, 6.5 kDa. The gels were transferred to nitrocellulose in tank buffer containing 20% methanol for 1 h at 100 V under cooling. For the immunodetection, the instructions of the ECL kit were followed. The antibodies were used at a protein concentration of 10 μg/ml. Immunoblots were exposed to X-ray film; quantitation of band intensities by scanning densitometry was performed as described below.

Pulse-chase studies and immunoprecipitations

After culturing cells for 24 h and 48 h with or without moxestrol in 60-mm diameter tissue culture dishes, LMH-2A cells were washed twice with PBS and incubated for 30 min in methionine- and serum-free Waymouth's medium (± moxestrol) to deplete the intracellular methionine pool. After this incubation the medium was removed and the cells were pulse-labeled for 60 min in labeling medium (Waymouth's medium ± moxestrol) containing 250 μCi/ml Met-label ([^{35}S]methionine and [^{35}S]cysteine). After labeling, the medium was removed and the cells were incubated for up to 6 h in chase medium (complete Waymouth's medium ± moxestrol) containing 10 mM L-methionine. Cells and media (3 ml) were recovered for immunoprecipitation (IP) analysis followed by one-dimensional SDS-PAGE. In general, IPs under non-stringent conditions were performed as follows. For the IP of cell-associated material, the cells were treated with 1 ml lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 62.5 mM sucrose; 0.5% Triton X-100; 0.5% sodium deoxycholate; protease inhibitors: 1 μg/ml leupeptin; 1 μg/ml leu-leu-leu; 1 μg/ml pepstatin; 10 U/ml aprotinin; 0.5 mM PMSF). Cells were scraped from the plates using a rubber policeman, sonicated for 15 sec, and the lysates were spun in an Eppendorf centrifuge at maximal speed for 15 min. For each IP, 250 μl of the supernatant was used, 250 μl additional lysis buffer was added, and the lysates were incubated with the respective antibody at a concentration of 50 μg/ml and protein A-Sepharose beads for 16 h at 4°C. For IPs from cell culture supernatants, the media were adjusted to pH 7.4 with 5 × PBS, pH 7.4, and centrifuged for 15 min. IPs were performed with 300 μl supernatant as described for cell-associated material. Identical results were obtained when the IPs of the media were performed in the presence of the same concentrations of detergents as used for the IPs of cell-associated proteins. The protein A-Sepharose beads were washed three times with IP buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 0.5% Triton X-100; 0.1% SDS; protease inhibitors as above) and twice with PBS for cell-associated material, and five times with PBS for the supernatants, respectively. Finally, the beads were suspended in Laemmli sample buffer containing 150 mM 2-mercapto-ethanol, and heated to 95°C for 5 min. After electrophoresis on 4–20% polyacrylamide gradient gels, the gels were fixed (10% acetic acid, 30% methanol), treated for fluorography with EN^{3}HANCE solution, dried, and exposed to X-ray films. The X-ray films were analyzed by scanning densitometry on a densitometer (Molecular Dynamics) using the ImageQuant software. By using X-ray films with different exposure times it was assured that only signals that are in the linear range of sensitivity of the film were considered. For the quantitation of band intensities, differences in the aliquot volumes used for the IPs, in the sample volumes loaded on the gels, and in the exposure times of the X-ray films of samples that had to be compared were taken into account.

For the sequential immunoprecipitation of the apolipoproteins (apoB and apoII) from the medium of moxestrol-treated LMH-2A cells, the material obtained in the first immunoprecipitation performed as described above (referred to as the non-stringent method) was released from the protein A-Sepharose beads by addition of 100 μl lysis buffer (25 mM Tris-HCl, pH 7.4; 95 mM NaCl; 3 mM EDTA; 2% SDS; protease inhibitors as above) and vortexing. The beads were sedimented by a short spin, and the supernatant was mixed with 400 μl of wash buffer (25 mM Tris-HCl, pH 7.4; 95 mM NaCl; 3 mM EDTA; 1.25% Triton X-100; 0.2% SDS; protease inhibitors as above). The second IP (that due to the presence of the high concentration of SDS is referred to as stringent method) was performed with the respective antibody at a concentration of 50 μg/ml and protein A-Sepharose beads for 16 h at 4°C. The protein A-Sepharose beads were washed three times with wash buffer and twice with PBS, suspended in Laemmli sample buffer containing 2-mercapto-ethanol, and heated to 95°C for 5 min before subjecting the eluted material to SDS-PAGE.

Gradient analysis

After culturing LMH-2A cells for 24 h with or without moxestrol in 100-mm diameter tissue culture dishes, the medium (4 ml) was adjusted to a density of 1.31 g/ml with NaBr and applied to the bottom of a 14 × 95 mm tube (Beckman). A step gradient was formed by overlaying the sample solution manually with 3.8, 3.3, and 0.8 ml of NaBr solutions with the densities of 1.063, 1.019, and 1.006 g/ml, respectively. The centrifugation was performed in a SW-40Ti rotor (Beckman) at 38,000 rpm for 24 h at 14°C. One-ml fractions were collected

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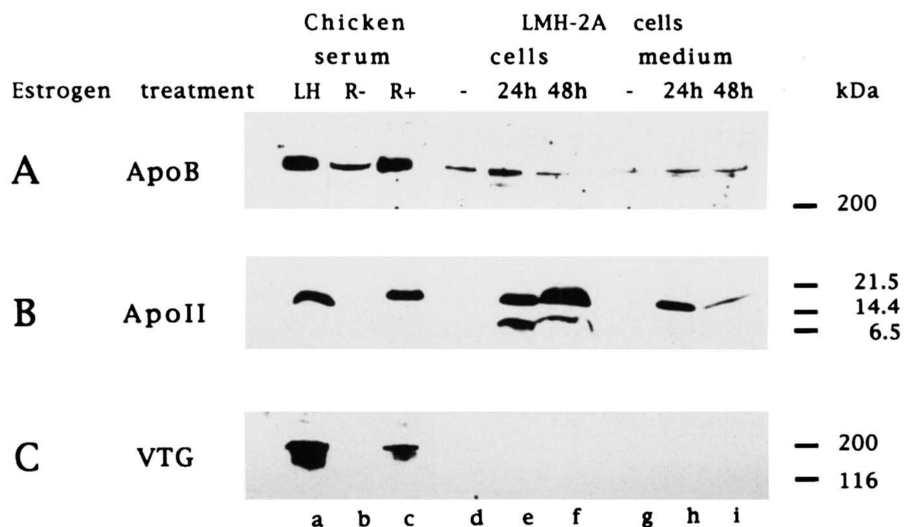

Fig. 1. Estrogen-dependent expression of apoB, apoII, and VTG in the laying hen, rooster, and in LMH-2A cells. Sera of laying hens (LH, lane a), control (R−, lane b) and estrogen-treated (R+, lane c) roosters were diluted in PBS 1:30 for apoB, 1:1000 for apoII, and 1:300 for VTG analysis. Cell extracts were prepared in a buffer containing Triton X-100 from LMH-2A cells that were not treated (lane d) or treated with moxestrol (50 nM) for 24 h (lane e) or 48 h (lane f). The media of these LMH-2A cell cultures were also analyzed (lanes g–i). For Western blot analysis the diluted chicken and rooster sera (10 μl) and the LMH-2A cell extracts (20 μg protein) were loaded on 4–20% SDS polyacrylamide gradient gels. For the analysis of the culture media, 50, 5, and 25 μg protein were loaded to detect apoB, apoII, and VTG, respectively; 4–10% SDS polyacrylamide gradient gels were used to separate apoB, and 4–20% gradient gels for the other proteins. Incubations were performed with polyclonal rabbit antibodies directed against the respective chicken proteins (panel A: apoB; panel B: apoII; panel C: VTG) and an enhanced chemiluminescent detection protocol as described in Experimental Procedures.

and equal aliquots were analyzed by immunoprecipitation with anti-apoB and anti-apoII antibodies, respectively, SDS-PAGE, and fluorography.

Other methods

Protein concentrations were determined as described by Lowry et al. (25). The protein concentration of samples containing Triton X-100 was determined using a modified Lowry procedure as described previously (26) with bovine serum albumin as standard.

RESULTS

In this report we describe the estrogen-dependent synthesis and secretion of apolipoproteins in the chicken hepatoma cell line LMH-2A. As a basis for these studies we first determined the expression of the apolipoproteins apoB and apoII as well as of vitellogenin in the serum of the laying hen and of untreated and estrogen-treated roosters by Western blotting. As shown in **Fig. 1,** apoB is highly abundant in serum of the laying hen (Fig. 1A, lane a), but only marginally present in that of a rooster (lane b). The amount of apoB is increased, however, upon treatment of a rooster with estrogen for 48 h (lane c), as was also described previously for the apoB mRNA in the liver of estrogenized roosters (13). Very low amounts of apoB are found in cell extracts and media from control LMH-2A cells (Fig. 1A, lanes d and g). The amount of this apolipoprotein is increased 2.5-fold in cells treated with moxestrol for 24 h (lane e), but decreased by approx. 40% after 48 h of exposure to moxestrol (lane f). Furthermore, the secretion of apoB into the culture media is also increased 2.2-fold and 2.1-fold under these conditions, respectively, (lanes h and i) indicating that the process of apoB secretion has already reached an equilibrium after 24 h of moxestrol treatment. It should be noted that our anti-apoB antibody directed against the chicken protein does not recognize the bovine protein contained in the serum (not shown).

ApoII is produced by the liver of the laying hen during the egg-laying cycle and thus present in the serum (Fig. 1B, lane a), but not in that of a rooster (lane b). After the administration of estrogen for 48 h, the expression of apoII is highly induced in the rooster (lane c) (see also refs. 27, 28). A similar induction of apoII synthesis and secretion is observed in moxestrol-treated LMH-2A cells (compare lanes d to f and g to i in Fig. 1B). It is notable that in cell extracts of moxestrol-treated LMH-2A cells both the monomer and the disul-

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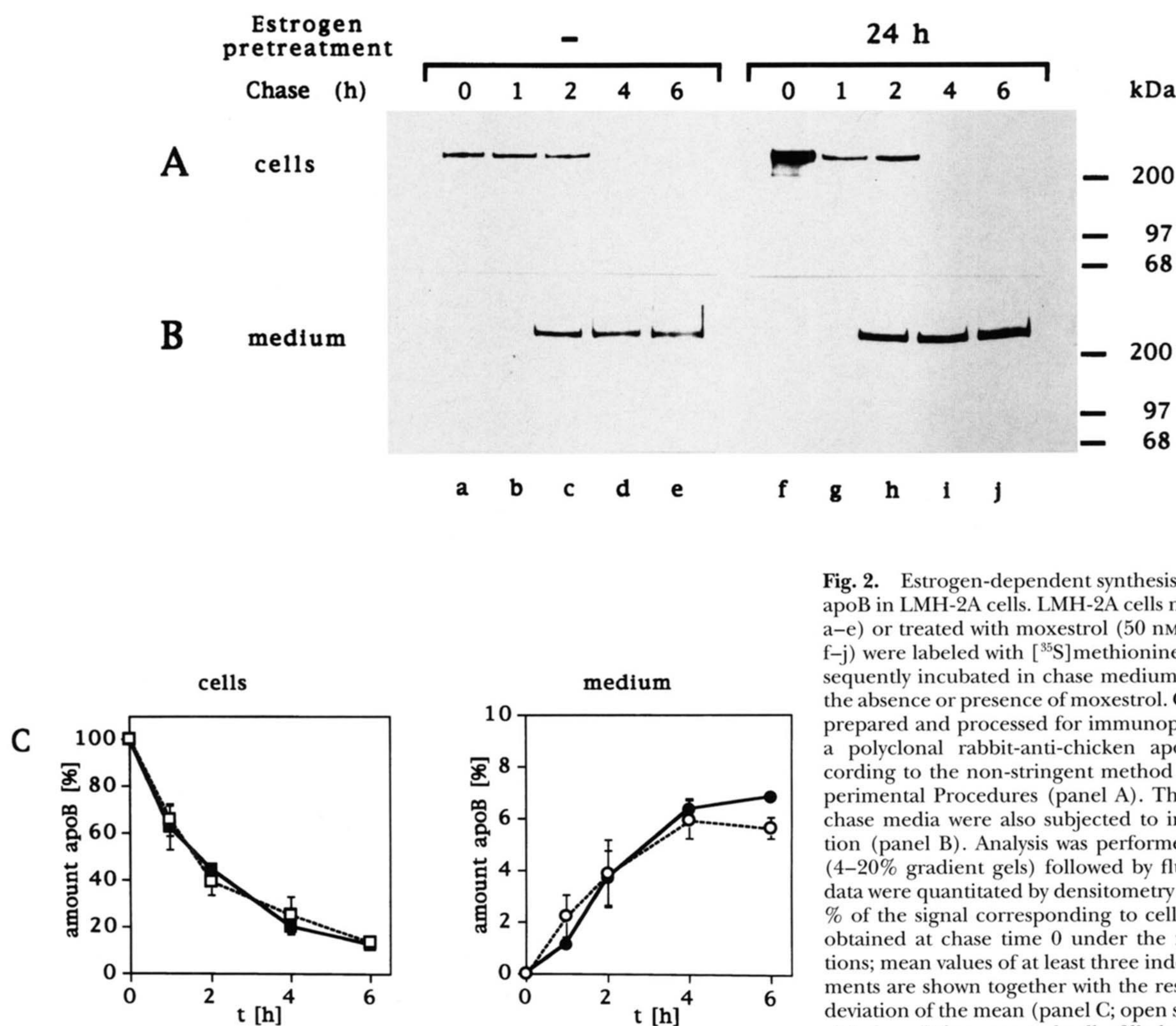

Fig. 2. Estrogen-dependent synthesis and secretion of apoB in LMH-2A cells. LMH-2A cells not treated (lanes a–e) or treated with moxestrol (50 nM) for 24 h (lanes f–j) were labeled with [^{35}S]methionine for 1 h and subsequently incubated in chase medium for up to 6 h in the absence or presence of moxestrol. Cell extracts were prepared and processed for immunoprecipitation with a polyclonal rabbit-anti-chicken apoB antibody according to the non-stringent method described in Experimental Procedures (panel A). The corresponding chase media were also subjected to immunoprecipitation (panel B). Analysis was performed by SDS-PAGE (4–20% gradient gels) followed by fluorography. The data were quantitated by densitometry and expressed as % of the signal corresponding to cell-associated apoB obtained at chase time 0 under the respective conditions; mean values of at least three independent experiments are shown together with the respective standard deviation of the mean (panel C; open squares, cell-associated apoB from control cells; filled squares, cell-associated apoB from moxestrol-treated cells; open circles, apoB in media from control cells; filled circles, apoB in media from moxestrol-treated cells). Note that 3-fold more apoB is synthesized in moxestrol-treated cells when compared to controls.

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fide-linked dimer of apoII are detectable under non-reducing conditions.

It is well established that the phospholipoglycoprotein vitellogenin is produced in the liver of the laying hen during the egg-laying cycle and that vitellogenin expression can be induced in roosters after the administration of estrogen (12; and Fig. 1C, lanes a to c). However, vitellogenin synthesis and secretion were not detectable in LMH-2A cells under the conditions used for the moxestrol-mediated induction of apoB and apoII described above (lanes d to i).

To investigate the time course of synthesis and secretion of apoB and apoII in LMH-2A cells under control and moxestrol-induced conditions, pulse-chase experiments were performed followed by immunoprecipitation of the respective proteins from cell lysates and media. The synthesis of apoB is increased up to 3-fold after incubation of LMH-2A cells with moxestrol (compare lanes a and f in **Fig. 2A**). ApoB disappears from the cells with a half-time of 108 min in control cells and 104 min in moxestrol-treated cells (Fig. 2A and C), and a small fraction of the apoB synthesized becomes detectable in the medium (Fig. 2B). Secretion levels off at 6 h of chase, when approx. 6% of the apoB synthesized during the pulse period are secreted. The kinetics of apoB secretion are similar in untreated and moxestrol-treated cells (Fig. 2C). Reprecipitation of the same samples showed that the initial immunoprecipitations from both cell lysates and media, respectively, yielded more than 90% of apoB present in the original samples.

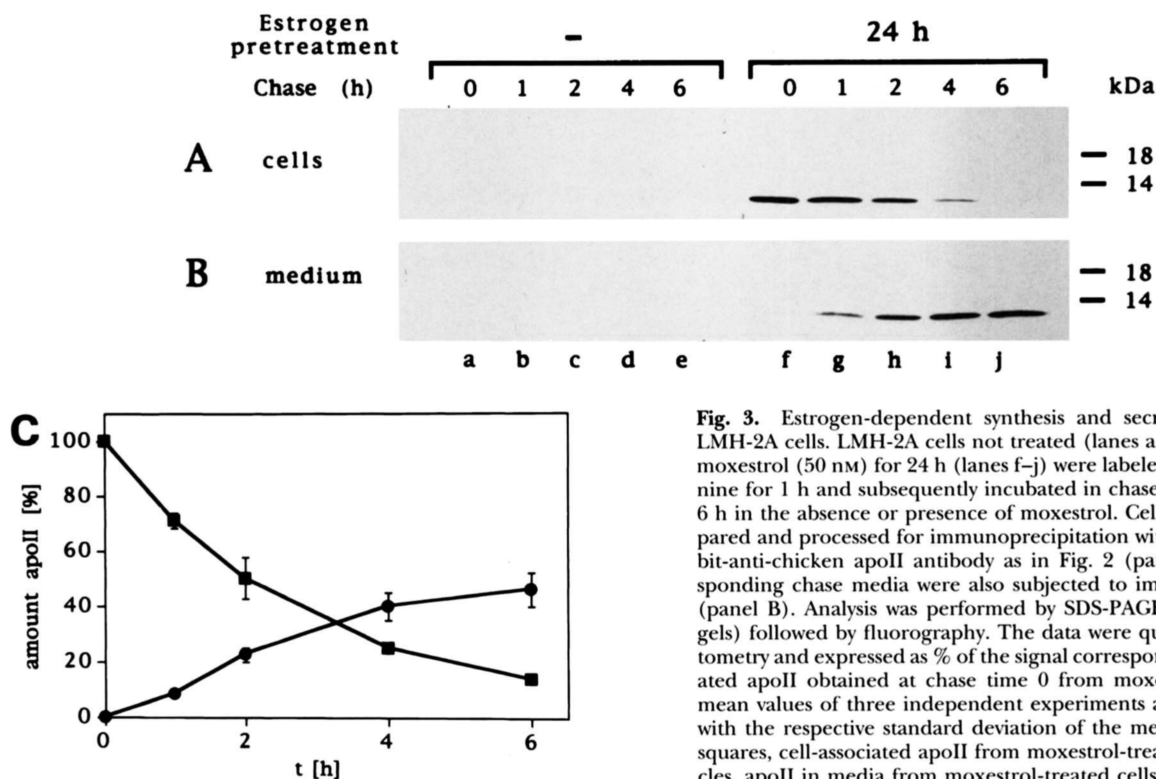

Fig. 3. Estrogen-dependent synthesis and secretion of apoII by LMH-2A cells. LMH-2A cells not treated (lanes a–e) or treated with moxestrol (50 nM) for 24 h (lanes f–j) were labeled with [^{35}S]methionine for 1 h and subsequently incubated in chase medium for up to 6 h in the absence or presence of moxestrol. Cell extracts were prepared and processed for immunoprecipitation with a polyclonal rabbit-anti-chicken apoII antibody as in Fig. 2 (panel A). The corresponding chase media were also subjected to immunoprecipitation (panel B). Analysis was performed by SDS-PAGE (4–20% gradient gels) followed by fluorography. The data were quantitated by densitometry and expressed as % of the signal corresponding to cell-associated apoII obtained at chase time 0 from moxestrol-treated cells; mean values of three independent experiments are shown together with the respective standard deviation of the mean (panel C; filled squares, cell-associated apoII from moxestrol-treated cells; filled circles, apoII in media from moxestrol-treated cells).

Therefore, we feel that the calculated relative values shown in the figure are correct.

As evident from the Western blotting experiments, apoII synthesis and secretion occur only after induction by estrogen. In agreement with this finding, apoII is detectable only in immunoprecipitates of cell lysates obtained from moxestrol-treated cultures (**Fig. 3A,** compare lanes a and f). Similar to apoB, apoII secretion reaches a plateau at 6 h of chase (Fig. 3B). A significantly larger portion of the synthesized apoII (approx. 50%), when compared to apoB, is secreted; the half time of disappearance of apoII from the cells was calculated to be 122 min (Fig. 3C).

To characterize the apoB- and apoII-containing lipoprotein particles that are secreted from moxestrol-treated LMH-2A cells, culture media from labeled cells were analyzed by density gradient centrifugation followed by immunoprecipitation of the respective protein from each fraction (**Fig. 4**). In media from both control and moxestrol-treated cells, the majority of apoB is recovered in the top fraction with a density range of less than 1.006 g/ml (Fig. 4A and B), typical of VLDL particles. A small amount of apoB is also found in fractions with densities corresponding to that of LDL. As already observed in the Western blotting (Fig. 1) and immunoprecipitation experiments (Fig. 2), in moxestrol-treated cells apoB synthesis and thus VLDL secretion are increased (compare lane 1 in Fig. 4A and B). Most of apoII is also detected in the top fraction of the gradient prepared from media of moxestrol-treated cells, although a small amount of apoII migrates also to higher densities (Fig. 4C).

In immunoprecipitates obtained from labeled secreted products with the anti-apoII antibody, we noticed an additional band that appeared to migrate in the position of apoB (Fig. 4C). To determine more directly whether apoII is present on apoB-containing particles secreted from moxestrol-treated LMH-2A cells, a co-immunoprecipitation experiment was performed (**Fig. 5**). For this purpose, media from radioactively labeled moxestrol-treated LMH-2A cells were immunoprecipitated under non-stringent conditions (see Experimental Procedures) with anti-apoB or anti-apoII antibodies, respectively (lanes c and d). Precipitation with anti-apoII under these conditions resulted in coprecipitation of apoB (lane d). Furthermore, duplicate immunoprecipitates of those shown in lanes c and d were dissolved and reprecipitated under stringent conditions (see Experimental Procedures) with either the anti-apoII (lane e) or the anti-apoB (lane f) antibodies. While apoB could be immunoprecipitated with anti-apoB from dissolved precipitates first obtained with anti-apoII, the reverse was not the case. Both proteins, however, were immunoprecipitated directly from the

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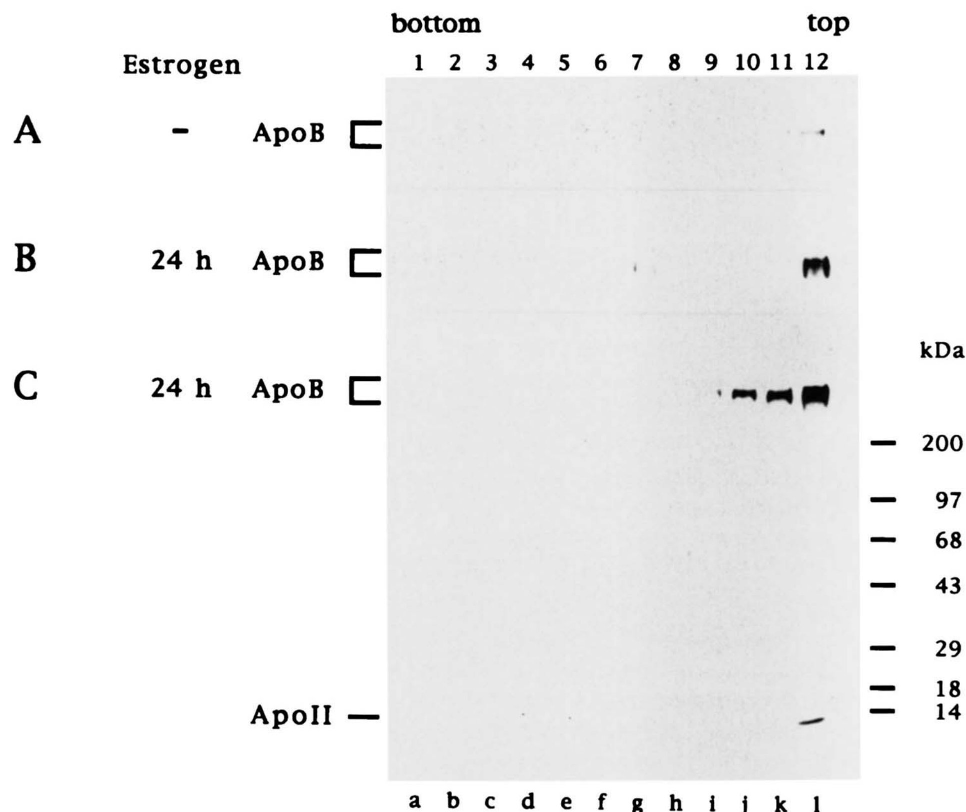

Fig. 4. ApoB- and apoII-containing lipoprotein particles secreted from LMH-2A cells have a buoyant density characteristic of VLDL. LMH-2A cells not treated (panel A) or treated with moxestrol (50 nM) for 24 h (panels B and C) were labeled with [^{35}S]methionine for 1 h and subsequently incubated in lipoprotein-deficient chase medium for 6 h in the absence or presence of moxestrol. The media were adjusted to a density of 1.31 g/ml with NaBr, and flotation analysis was performed on a step gradient of the densities 1.063, 1.019, and 1.006 g/ml at 178,000 g_{av} for 24 h at 14°C. Twelve fractions were collected and analyzed by immunoprecipitation with polyclonal rabbit-anti-chicken apoB (panels A and B) and apoII (panel C) antibodies, respectively, followed by SDS-PAGE (4–20% gradient gels) and fluorography.

media under the stringent conditions applied in the reprecipitation steps (lanes a and b).

DISCUSSION

We have obtained strong evidence that the chicken hepatoma cell line LMH-2A is capable of producing and secreting VLDL-like particles that contain both apoB and apoII. This is in agreement with the earlier finding that the apoII mRNA is induced in LMH-2A cells treated with the hormone (20), and in the liver of estrogenized roosters (27, 28). In mammals, an induction of apoB by estrogen has been observed only at extremely high hormone concentrations (29). In contrast, in chicken liver the apoB gene is highly estrogen-responsive, resulting in 5- to 7-fold increased amounts of the mRNA 24 h after hormone administration (13). Our studies indicate that estrogen-treated LMH-2A cells synthesize and secrete 2- to 3-fold more apoB protein as compared to controls after 24 h. The production of VTG in the chicken is also tightly regulated by estrogen (12, 14, 30). However, LMH cells apparently have lost the capacity to produce VTG, as even after estrogen exposure, neither VTG II gene expression (17) nor protein synthesis (Fig. 1C) was detectable.

Quantitative analysis of apoB secretion from LMH-2A cells revealed that the estrogen effect on cellular synthesis is maximal at about 24 h, and that newly synthesized apoB disappears from the cells within approximately 6 h. Interestingly, only about 6% of the apoB molecules are secreted. We assume that the remainder must be degraded intracellularly, in analogy to the situation reported for apoB in HepG2 cells (e.g., see refs. 4, 5, 31). The portion of apoB secreted from LMH-2A cells is lower than that from HepG2 cells, for which an efficiency of secretion of about 18% of the apoB synthesized within 90 min has been reported (32). In another study it was determined that 13% of the apoB initially

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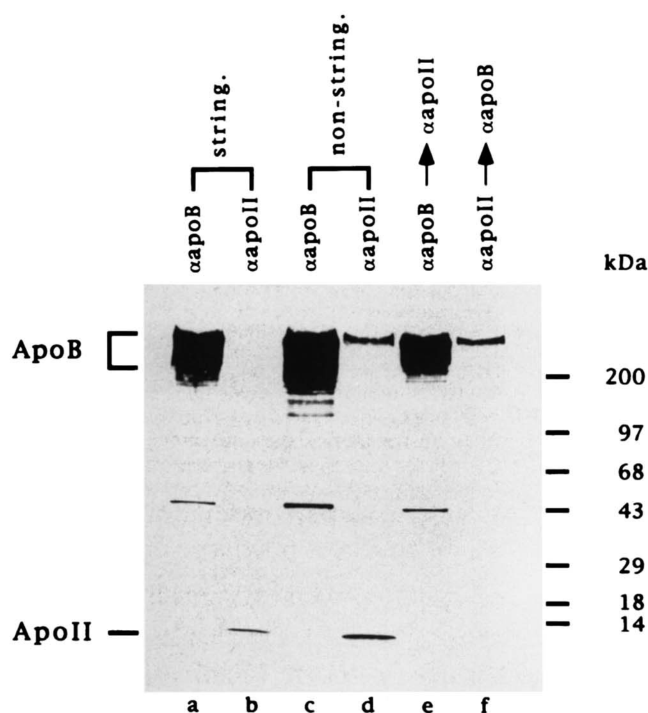

Fig. 5. The VLDL particles secreted from moxestrol-treated LMH-2A cells contain both apoB and apoII. LMH-2A cells treated with moxestrol (50 nM) for 24 h were labeled with [^{35}S]methionine for 1 h and subsequently incubated in chase medium for 6 h in the presence of the drug. The media were adjusted to conditions for stringent immunoprecipitation in the presence of SDS (lanes a and b) or for non-stringent IP (lanes c and d) and immunoprecipitated with polyclonal rabbit-anti-chicken apoB (lanes a and c) or anti-apoII (lanes b and d). In lane e, a first IP was performed under non-stringent conditions with anti-apoB, followed by a second IP under stringent conditions with anti-apoII. In lane f, a first IP was performed under non-stringent conditions with anti-apoII, followed by a second IP under stringent conditions with anti-apoB. The IPs were analyzed by SDS-PAGE (4–20% gradient gels) followed by fluorography.

present intracellularly are secreted from HepG2 cells within 60 min (33). As both synthesis and secretion of apoB are increased similarly after estrogen treatment of LMH-2A cells, and occur with essentially the same kinetics, processing and lipoprotein synthesis after estrogen induction appear uncompromised. This notion is supported by the fact that the majority of the secreted particles have the density of typical VLDL. HepG2 cells, in contrast, produce triacylglycerol-containing, but underlipidated lipoprotein particles with resulting densities corresponding to LDL rather than to VLDL (34). It has been previously shown that the liver of roosters treated with high doses of estrogen almost exclusively produces apoB-containing lipoprotein particles with a continuous density range from VLDL to LDL (35, 36). LMH-2A cells secrete mostly apoB-containing VLDL particles, although, especially in estrogen-induced cells, LDL-like particles are also detectable (Fig. 4). It appears, therefore, that the lipid-loading of apoB in the chicken hepatoma cell line is tightly controlled, and represents the key to conferring secretion competence to apoB-containing particles. LMH-2A cells may be defined as displaying greater fidelity than HepG2 cells in regards to bona-fide hepatic VLDL synthesis. It has been postulated that hepatic cells contain two separate pools of triacylglycerol that may account for the observation that the lipidation of apoB occurs in two distinct steps; HepG2 cells may be deficient in executing the second step of lipidation, resulting in the secretion of underlipidated apoB-containing lipoproteins (11). It remains to be established whether LMH-2A cells follow a similar two-step mode of apoB lipidation. In that case it may be speculated that secretion competence of apoB is strictly dependent on completion of loading the nascent particle with lipids during the second step. Nevertheless, it will be interesting to see whether the secreted portion of the total synthesized apoB can be increased by oleate addition to the culture medium of LMH-2A cells. For HepG2 cells it was demonstrated that such treatment results in increased lipid availability (33, 37).

The activation of the apoII gene in hepatocytes is strictly dependent on estrogen (19). In contrast to the low relative amount of apoB secreted, the proportion of apoII that is secreted from estrogen-treated LMH-2A cells is almost 50%. In primary chicken hepatocytes, the transit times for apoB and apoII through the secretory pathway have been determined to be 69 and 92 min, respectively (38). The half-times of loss of these apolipoproteins from estrogen-treated LMH-2A cells (104 min for apoB and 122 min for apoII) are thus somewhat longer than those observed with the primary cells. A significant part of the assembly of VLDL particles occurs in a post-ER compartment, most likely the Golgi apparatus (8), although the precise mechanism, in particular that underlying the coordination of lipid association with the synthesis of the two apolipoproteins apoB and apoII, is not known. Importantly, under mild conditions apoB can be co-immunoprecipitated with apoII from lipoproteins secreted into the medium of estrogen-treated LMH-2A cells (Fig. 4, lane l; Fig. 5), suggesting that at least a portion of the secreted particles harbors both apolipoproteins. Consecutive immunoprecipitations of secreted products biosynthetically labeled with [^{35}S]methionine by antibodies to apoII and apoB required more stringent conditions, and were only partially successful (Fig. 5). This is possibly due to diminished antibody recognition of the antigens (particularly of apoII) after dissolution of precipitates in SDS-containing buffers. It must also be noted that on a molar basis, apoII contains approx. 80-fold less labeled amino acids than apoB, significantly limiting the detectability of the small apolipoprotein (e.g., see Fig. 4, lane l).

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In summary, these studies demonstrate that due to their responsiveness to estrogen and their capacity to produce VLDL-like particles, LMH-2A cells provide a useful cell system for investigations on the mechanism and regulation of lipoprotein synthesis, assembly, and secretion.

We are indebted to Dr. David L. Williams, State University of New York, Stony Brook, NY, for providing us with the LMH-2A cell line. We appreciate the help of Romana Kukina with the cell culture, of Angelika Kranawetter with the illustrations, and of Andrea Ocko in preparation of the manuscript. This work was supported by grants from the Hochschuljubiläumsstiftung of the City of Vienna, the Anton Dreher-Stiftung of the University of Vienna School of Medicine (to M.H.), and the Austrian Science Foundation (FWF S07108, to W.J.S.).

Manuscript received 10 January 1997 and in revised form 18 March 1997.

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