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Progesterone Inhibits Cholesterol Biosynthesis in Cultured Cells

ACCUMULATION OF CHOLESTEROL PRECURSORS*

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Cells acquire cholesterol through endogenous synthesis and through receptor-mediated uptake of cholesterol-rich low density lipoprotein (LDL). Esterification of LDL-derived cholesterol is catalyzed by acyl-CoA:cholesterol acyltransferase (ACAT) in the endoplasmic reticulum (ER). Progesterone inhibits esterification, and, although the mechanism of inhibition is not completely understood, this inhibition results from progesterone's ability to inhibit the activity of multiple drug resistance (MDR) P-glycoproteins (P. DeBry and J. E. Metherall, submitted for publication). In the current manuscript, we demonstrate that progesterone inhibits cholesterol biosynthesis resulting in the accumulation of a number of sterol precursors. In Chinese hamster ovary (CHO) cells, high concentrations (100 μM) of progesterone completely blocked cholesterol production, resulting in the accumulation of lanosterol and a lanosterol precursor. Lower concentrations (40 μM) of progesterone cause plasma membrane accumulation of several sterol products. The majority of these sterols are precursors of cholesterol since they were efficiently converted to cholesterol upon removal of progesterone from the culture medium. Although very high concentrations (>200 μM) of progesterone killed CHO cells, their growth was restored by the addition of cholesterol to the growth medium, indicating that progesterone toxicity resulted from cholesterol auxotrophy. The effect of progesterone was not unique to CHO cells; progesterone also inhibited cholesterol biosynthesis in all human cell lines tested. These observations suggest that a common progesterone-sensitive pathway is involved in both cholesterol biosynthesis and the processing of LDL-derived cholesterol.

Active mechanisms of cholesterol transport and sorting are required for maintaining proper distributions and levels of cholesterol within the cell. Approximately 90% of cellular cholesterol is found in the plasma membrane (1, 2), and most of the remainder is found in endocytic vesicles derived from the plasma membrane (3). The endoplasmic reticulum (ER)[1] and other organelle membranes are relatively devoid of cholesterol. Despite this distribution, a number of important enzymatic reactions involving sterols occur at the ER. Sterols must be transported from the plasma membrane to the ER to function as substrates for these reactions. The mechanisms of this transport are poorly understood.

When excess cholesterol accumulates in the plasma membrane, cholesterol is transported to the ER where it is esterified by acyl-CoA:cholesterol acyltransferase (ACAT). ACAT catalyzes the transfer long-chain fatty acyl residues from acyl-CoA to the β-hydroxyl group of cholesterol. This esterification reaction contributes to cholesterol homeostasis; while free cholesterol is toxic, cholesteryl esters can accumulate to relatively high levels as cytosolic lipid droplets. Metabolic labeling studies demonstrate that the cholesterol substrate pool for ACAT derives from the plasma membrane (4) and that, under most conditions, ACAT is not saturated with cholesterol substrate (5, 6). These findings indicate that regulation of cholesterol esterification occurs at the level of cholesterol substrate availability and suggest that cholesterol transport to the ER may be a regulated process.

Progesterone has long been known to inhibit esterification of low density lipoprotein (LDL)-derived cholesterol (7). This inhibition does not involve direct inhibition of ACAT activity since it has no effect on the esterification of cholesterol that has been solubilized in detergent (8). Rather, progesterone appears to inhibit delivery of LDL-derived cholesterol to ACAT. Progesterone inhibits the movement of LDL-derived cholesterol from the lysosome to the plasma membrane (9) and the movement of cholesterol from the plasma membrane to the ER (8). Progesterone's effects on esterification appear to be mediated through its ability to inhibit the activity of one or more of the multidrug resistance (MDR) families of P-glycoproteins.[2]

The movement of sterols from the plasma membrane to the ER is also required for cholesterol biosynthesis. Enzymes involved in the late stages of cholesterol biosynthesis reside in the ER. However, newly synthesized substrates for these enzymes (lanosterol and zymosterol) are found in the plasma membrane (11, 12). These substrates are rapidly and efficiently transported to the ER for conversion to cholesterol (13, 14).

In the current report, we demonstrate that progesterone inhibits cholesterol biosynthesis, resulting in the accumulation of a number of cholesterol precursors. We suggest that this inhibition results from progesterone's ability to prevent plasma membrane-derived sterol precursors from reaching ER-resident enzymes. These findings suggest that cholesterol precursors utilize a common transport system to reach the ER as does cholesterol in the process of cholesterol esterification. In the accompanying paper (15), we provide evidence that the effect of progesterone on cholesterol biosynthesis requires the multidrug resistance family of P-glycoproteins.

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[1] The abbreviations used are: ER, endoplasmic reticulum; ACAT, acyl-CoA:cholesterol acyltransferase; LDL, low density lipoprotein; CHO, Chinese hamster ovary; LPPS, newborn calf lipoprotein-deficient serum; FCS, fetal calf serum; 7-DHC, 7-dehydrocholesterol; BSA, bovine serum albumin.

[2] P. DeBry and J. E. Metherall, submitted for publication.

EXPERIMENTAL PROCEDURES

Materials—CHO cells (CHO-7) are a previously described subline (16) of CHO-K1 cells. Newborn calf lipoprotein-deficient serum ($d >$ 1.215 g/ml; cholesterol content of 33–61 μg/ml), human LDL (d 1.019–1.063 g/ml), and rabbit β-very low density lipoprotein were prepared by ultracentrifugation as described previously (17). Progesterone was purchased from Sigma. RU 486 was the kind gift of Raymond Daynes (University of Utah). AY-9944 was obtained from Sharon Burns (Wyeth-Ayerst, Princeton, NJ), and SKF 104976 was provided by Julia Christie (Smith Kline Beecham, King of Prussia, PA). Cholesterol was obtained from Alltech Chemicals, and methyl-β-cyclodextrin was obtained from Sigma. DL-Mevalonic acid lactone was purchased from Fluka Chemical Co. and was converted to the sodium salt as described (18). [2-^{14}C]Acetic acid (52–55 mCi/mmol) and [1α,2α-^{3}H]cholesterol (47 Ci/mmol) were purchased from Amersham. Other materials were obtained from previously reported sources (16, 19, 20).

Cell Growth—All cells were grown in monolayer at 37 °C in an atmosphere of 5% CO_2. CHO cells were maintained in medium A (a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's minimum essential medium containing 100 units/ml penicillin and 100 μg/ml streptomycin) containing 5% (v/v) newborn calf lipoprotein deficient serum (LPPS). HeLa and Chang Liver cells, initially grown in medium B (Dulbecco's modified Eagle's minimum essential medium with 100 units/ml penicillin, 100 μg/ml streptomycin) containing 10% (v/v) fetal calf serum or 10% newborn calf serum, respectively, were gradually adapted to growth in medium A containing 5% LPPS. Caco-2 and Hep G2 cells did not grow well in medium A and, consequently, were maintained in medium B containing 10% fetal calf serum. At least 2 days prior to assay, all cell lines were switched to growth in medium A containing 5% LPPS. Sterols and progesterone were added to the culture medium in ethanol; the final ethanol concentration was identical for all conditions in each experiment and did not exceed 0.5% (v/v).

Assays—Alkaline phosphodiesterase (21), lactate dehydrogenase (22), and hexosaminidase (23) activities were measured as described in the indicated references. The incorporation of [^{14}C]acetate by cell monolayers into sterols and fatty acids was measured as described (18) with the following modifications: 1) following extraction, the sterol residue was dissolved in 30 μl of heptane and resolved by thin layer chromatography (TLC) on Silica Gel G plates in petroleum ether:diethyl ether: acetic acid (60:40:1 v/v), 2) the TLC sheets were dried and subjected to autoradiography for 3–14 days using Amersham hyperfilm, and 3) protein assays were performed using the method of Bradford (24). No autoradiographic signal was observed at these exposure times from the [^{3}H]cholesterol used as a recovery control (data not shown). Following autoradiography, the positions of the recovery-derived cholesterol and lanosterol were visualized by staining in iodine vapor. For quantitation, the regions of the TLC sheet that corresponded to autoradiographic signals were scraped and counted in a liquid scintillation counter.

Cell Fractionation—Cell fractionation was performed as described previously by Lange and Muraski (25) with minor modification. Briefly, cell monolayers were scraped into ice-cold phosphate-buffered saline. Cell pellets were washed in cold phosphate-buffered saline and resuspended in 5 mM sodium phosphate, pH 7.5, containing 0.25 M sucrose. The suspension was centrifuged at 1,700 $\times$ g, and the recovered pellet was resuspended in 0.5 mM sodium phosphate, pH 7.5, containing 0.25 M sucrose. After centrifugation of the suspension at 3,000 $\times$ g, the recovered pellet was resuspended in 5 volumes of 0.5 mM sodium phosphate, pH 7.5, containing 0.25 M sucrose. Following a 10-min incubation on ice, sodium phosphate was added to a final concentration of 5 mM, and $MgCl_2$ was added to a final concentration of 0.3 mM. Protease inhibitors were added to final concentrations of 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin. The suspension was then homogenized by 25 strokes with a Dounce homogenizer using a tight-fitting pestle. The suspension was cleared of unlysed cells and nuclei by two sequential spins at 1,700 $\times$ g. After centrifugation of the suspension at 10,000 $\times$ g, a high density pellet (P10) fraction and a supernatant (S10) fraction were recovered. A microsomal pellet (P100) fraction and cytosolic supernatant (S100) fraction were generated after centrifugation of the S10 fraction at 100,000 $\times$ g. The P10 and P100 pellets were resuspended in a buffer containing 100 mM Tris-Cl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.2 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin.

RESULTS

Progesterone Inhibits Cholesterol Biosynthesis in CHO Cells—To test the effects of progesterone on cholesterol biosynthesis, we monitored the incorporation of [^{14}C]acetate into [^{14}C]cholesterol in CHO cells (Fig. 1). Following a 2-h labeling, sterols were isolated and separated using thin layer chromatography (TLC). All samples were saponified in order to hydrolyze any cholesterol esters that may have formed. In the absence of progesterone, CHO cells (*lane 1*) produced mainly cholesterol, with small amounts of lanosterol and two unidentified sterols (designated A and B). While the levels of lanosterol and the other two sterols remained relatively constant over time (*lanes 2* and *3*), the level of cholesterol continued to accumulate over 18 h. These observations are consistent with cholesterol being the major product of the pathway and lanosterol being an intermediate. The sterol products designated A and B also remained relatively constant over time, suggesting that these compounds are also intermediates in cholesterol production. Addition of 10 μM progesterone to the culture medium reduced the rate of cholesterol synthesis by nearly 3-fold (*lanes 4–6*). Addition of 40 μM progesterone (*lanes 7–9*) further reduced cholesterol production and resulted in the accumulation of a unique set of labeled sterols (designated C, D, E, F, and G). Progesterone added at 100 μM (*lanes 10–12*) completely abolished cholesterol production, resulting in the formation of lanosterol and two unidentified sterols (D and E). Despite the complete lack of cholesterol production, the cells still incorporated acetate into sterols (lanosterol, D, and E) at a relatively high rate. Lanosterol accumulated over time suggesting that, under these conditions, lanosterol is a product of the pathway. Furthermore, the complete block in cholesterol production did not inhibit acetate incorporation into sterols; lanosterol accumulated at a rate that approximated half the rate of cholesterol production in untreated cells, and the unidentified sterol (D) accumulated at a rate that exceeded that of cholesterol in untreated cells.

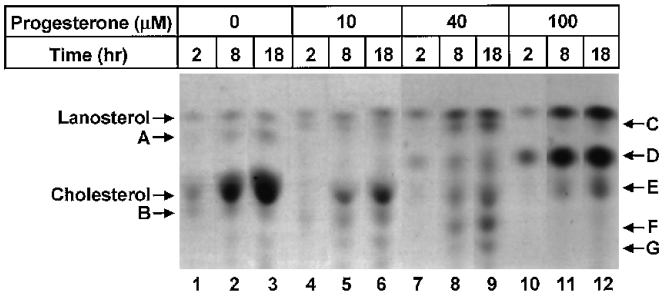

FIG. 1. **Effect of progesterone on the incorporation of [^{14}C]acetate into cellular sterols in CHO cells.** CHO cells were plated at 5 $\times$ 10^4 cells/well in a 24-well Linbro plate in medium A containing 5% (v/v) newborn calf lipoprotein-deficient serum. On day 3, cells were refed 0.5 ml of medium A containing 2 mg/ml bovine serum albumin (BSA) and the indicated additions of progesterone. After incubation at 37 °C for 2 h, [^{14}C]acetate (9.98 dpm/pmol) was added to a final concentration of 0.5 mM and cells were incubated at 37 °C for the indicated time. Additions were made in a staggered fashion so that cells were harvested simultaneously for measurement of [^{14}C]sterol synthesis. Radiolabeled sterols were extracted and resolved by thin layer chromatography as described under "Experimental Procedures." TLC plates were exposed to Amersham Hyperfilm for 4 days at −70 °C, and the bands corresponding to lanosterol and cholesterol were identified by comparing the iodine-stained TLC sheet with the autoradiogram.

To test if the lack of cellular cholesterol resulted from increased release of sterols into the medium, we monitored the appearance of [^{14}C]sterols in the medium for extended periods of time following the addition of progesterone (Fig. 2*A*). In untreated cells, virtually all of the sterols produced were found intracellularly (*upper left*); very few sterols were found in the culture medium (*upper right*). Progesterone treatment resulted in a very slight increase in the production of sterols into the medium. This minor increase in sterols released into the media

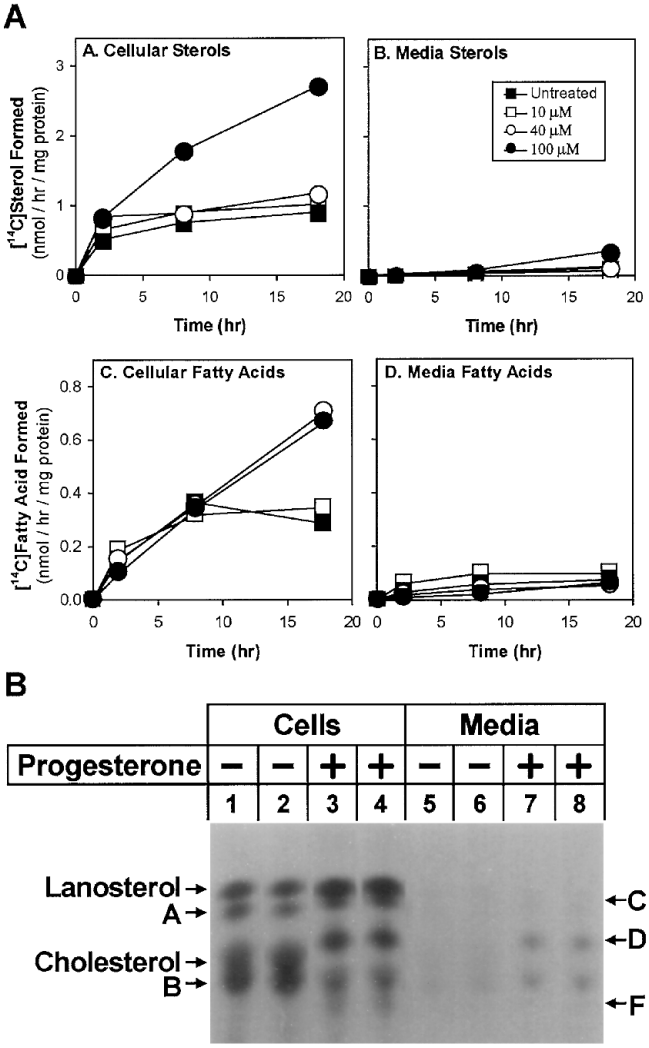

FIG. 2. **Incorporation of [^{14}C]acetate into cellular and media lipids in progesterone-treated cells.** Cells were plated and grown through day 2 as described in the legend to Fig. 1. On day 3, duplicate dishes were refed 0.5 ml of medium A containing 2 mg/ml BSA and the indicated additions of progesterone. After incubation at 37 °C for 2 h, [^{14}C]acetate (95.4 dpm/pmol) was added to a final concentration of 0.5 mM, and the cells were incubated at 37 °C for the indicated time. Additions were made in a staggered fashion so that cultures were harvested simultaneously. In *A*, cultures were assayed for [^{14}C]sterol (*top*) and ^{14}C-fatty acid (*bottom*) content in both cells (*left*) and culture medium (*right*). Sterols and fatty acids were extracted and resolved by TLC as described under "Experimental Procedures." Bands corresponding to lanosterol, cholesterol, and products A-F (see text) were pooled and counted in a liquid scintillation counter for determination of total [^{14}C]sterol content. In *B*, individual sterols produced in the absence or presence of 40 μM progesterone are shown in an autoradiograph of a TLC sheet, as described in the legend to Fig. 1.

does not explain the drastic decrease in cholesterol produced within cells. At 40 μM progesterone, approximately 4% of the sterols were found in the medium as compared to 1% in untreated cells. Two of the products, B and D, were preferentially released into the medium (Fig. 2*B*), suggesting that this release was not due to general cell death or lysis. As a control, most fatty acids produced from [^{14}C]acetate remained within the cell (*lower left*) while very few reached the medium (*lower right*).

Sterols That Accumulate in Progesterone-treated Cells Are Precursors of Cholesterol—We used metabolic inhibitors and kinetic studies to investigate the nature of the sterols produced in progesterone-treated cells. Following a 2-h labeling, CHO cells produced mainly cholesterol as well as some lanosterol and products A and B (Fig. 3*A*, *lanes 1* and *7*). Treatment with 40 μM progesterone resulted in the production of lanosterol, and products designated C, D, E, and F (*lane 2*). We used 40 μM progesterone in these studies because this concentration produced the most complex pattern of labeled metabolites. To investigate the nature of these metabolites, we monitored their production in the presence of two inhibitors that interfere with specific steps in the conversion of lanosterol to cholesterol.

The conversion of lanosterol to cholesterol is a complicated process requiring at least five enzymatic modifications; some of the key intermediates in this process are shown in Fig. 3*B*. The order of these modifications is not well established, and, in fact, the order may not be strictly processive. Saturation at the C-24(25) position distinguishes the intermediates shown on the left from those on the right. Many, if not all, of the enzymes involved in modifications to the steroid nucleus recognize both C-24(25) saturated and unsaturated substrates, resulting in two parallel pathways for conversion; one pathway processing C-24(25) saturated intermediates (*left*) and the other processing C-24(25) unsaturated intermediates (*right*).

The first step in modification of the steroid nucleus involves three demethylations; one at position 14 and two at position 4. The removal of these methyl groups is catalyzed by the enzyme lanosterol demethylase. Reduction at the C-24(25) position most likely occurs following the removal of a single methyl group at position 14. SKF 104976 is a specific inhibitor of lanosterol demethylase and thereby prevents demethylation of lanosterol and dihydrolanosterol. Treatment of CHO cells with SKF 104976 completely blocked the conversion of lanosterol to cholesterol and resulted in the specific accumulation of lanosterol (*lane 3*). Treatment of CHO cells with SKF 104976 in the presence of progesterone resulted in the production of only lanosterol and the product designated D (*lane 6*). These findings suggest that product D is a precursor of lanosterol, and that products C, E, and F are products of lanosterol.

The final step in the modification of the steroid nucleus involves reduction of the C-7(8) double bond. This step is catalyzed by 7-dehydrocholesterol (7-DHC) reductase and results in the conversion of cholest-5,7,24-trien-3β-ol to desmosterol and 7-DHC to cholesterol. AY-9944 is an inhibitor of 7-DHC reductase. Treatment of CHO cells with AY-9944 resulted mainly in the accumulation of products B and F (*lane 2*). Our observation that product F comigrates with authentic 7-DHC (data not shown), has resulted in our tentative assignment of product F as 7-DHC and product B as cholest-5,7,24-trien-3β-ol. AY-9944 had no effect on the spectrum of labeled intermediates produced in the presence of progesterone (compare *lanes 4* and *5*). These findings demonstrate that all of the products generated in progesterone-treated cells are derived from sterol intermediates upstream of 7-DHC reductase. Interestingly, while one of these products appears to be 7-DHC itself, there is little or no cholest-5,7,24-trien-3β-ol produced, suggesting that reduction of the C-24(25) position must occur efficiently in the presence of progesterone.

The metabolic inhibition experiments described above demonstrate that the abnormal products observed in the presence of progesterone must be direct precursors of cholesterol or products derived from these precursors. To determine if these metabolites were in fact precursors of cholesterol, we monitored their conversion to cholesterol following removal of progesterone from the medium. CHO cells were labeled for 1 h with [^{14}C]acetate in either the absence (Fig. 4, *lane 1*) or presence (*lanes 2–7*) of 40 μM progesterone. The label was removed and the cells were chased with cold acetate for different lengths of time. As previously shown, progesterone treatment nearly completely inhibited cholesterol production (*lane 2*). These products diminished rapidly; within a few hours of chase, after

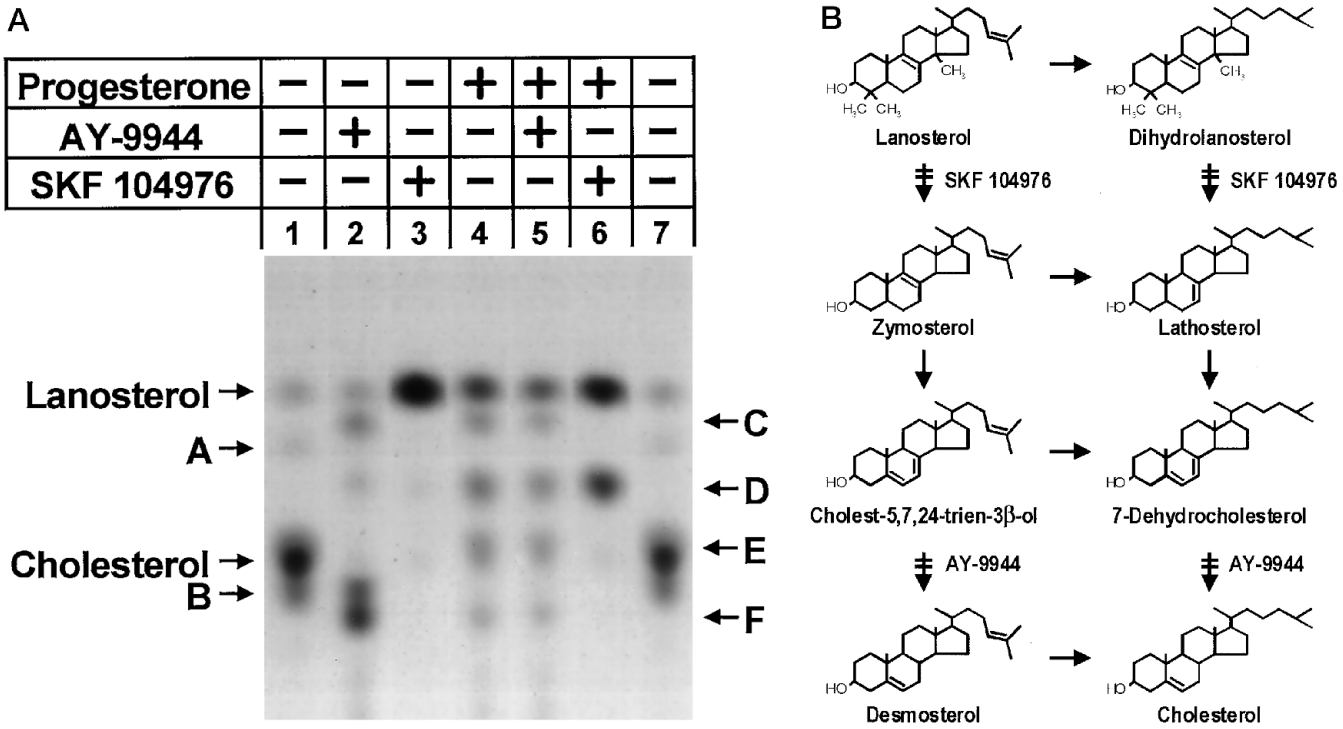

FIG. 3. **Effect of cholesterol synthesis inhibitors on incorporation of [^{14}C]acetate into cellular sterols in progesterone-treated cells.** Cells were plated and grown through day 2 as described in the legend to Fig. 1. On day 3, duplicate dishes were refed medium A containing 2 mg/ml BSA and the indicated additions of 30 nM SKF 104976, 1 μM AY-9944, and 40 μM progesterone. After incubation at 37 °C for 2 h, [^{14}C]acetate (95.4 dpm/pmol) was added to a final concentration of 0.5 mM and cells were incubated at 37 °C for an additional 2 h. Cells were harvested, and sterols were extracted and resolved by TLC as described under "Experimental Procedures." TLC plates were exposed to Kodak XAR film for 4 days at −70 °C. The indicated locations of recovery-derived lanosterol and cholesterol were determined by staining with iodine vapor.

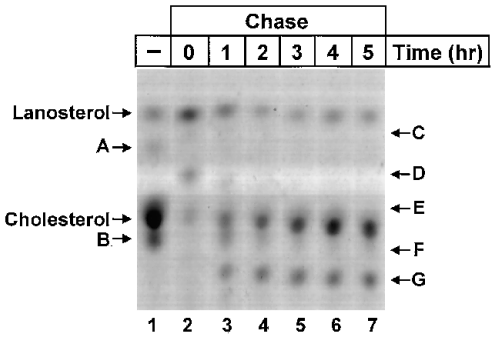

FIG. 4. **Pulse-chase analysis of incorporation of [^{14}C]acetate into cellular sterols in the absence or presence of progesterone.** Cells were plated and grown through day 2 as described in the legend to Fig. 1. On day 3, duplicate dishes were refed 0.5 ml of medium A containing 2 mg/ml BSA in either the absence (−) or presence (+) of 40 μM progesterone. After incubation at 37 °C for 2 h, [^{14}C]acetate (9.9 dpm/pmol) was added to a final concentration of 0.5 mM, and the cells were incubated at 37 °C for an additional 1 h. Cells were then washed 3 times with phosphate-buffered saline and refed 2 ml of medium A containing 5% LPPS and 2 mM sodium acetate. Cells were further incubated at 37 °C for the indicated amount of time. Additions were made in a staggered fashion so that cells were harvested simultaneously for measurement of [^{14}C]sterol content as described in the legend to Fig. 1.

removal of progesterone (*lanes 3–7*). This disappearance correlated with the appearance of two new bands that corresponded to cholesterol and a novel product, designated G. In addition, some lanosterol remained resistant to metabolic conversion. These results demonstrate that the block in cholesterol synthesis caused by progesterone is reversible and that many of the sterols produced are precursors of cholesterol.

Sterols Accumulate in Plasma Membrane Fractions in Progesterone-treated CHO Cells—Since progesterone is known to block transport of LDL-derived cholesterol from the plasma membrane to the ER (8), we investigated the subcellular location of the sterols that accumulate in progesterone-treated cells. CHO cells were labeled for 2 h with [^{14}C]acetate in the presence or absence of progesterone. Cell pellets were homogenized and fractionated by differential centrifugation. Sterols isolated from the fractions were resolved by TLC. The autoradiogram from one experiment is shown in Fig. 5, and quantitative analyses of several experiments are presented in Table I. The majority of cholesterol (87%) produced in untreated cells was found in the pellet of a 10,000 × *g* spin (P10; *lane 1*), consistent with its localization to the plasma membrane. Smaller amounts (10%) were found in the microsomal fraction (P100; *lane 2*), and very little (3%) was found in the cytosolic fraction (S100; *lane 3*). Lanosterol showed a similar distribution with 81% found in the P10, 14% in the P100, and 5% in the S100. Treatment with 40 μM progesterone inhibited cholesterol synthesis and resulted in the accumulation of products C-F (*lanes 4–6*). Again, these products were found predominantly in the pellet of a 10,000 × *g* spin (P10; *lane 4*) with only small amounts in the microsomal (P100; *lane 5*) and cytosolic (S100; *lane 6*) fractions. For example, 79% of the lanosterol produced was found in the P10, 16% in the P100, and 6% in the S100. This distribution was not statistically different from the distribution observed in untreated cells.

As a control for the mixing of sterols between various fractions during homogenization, we simultaneously and routinely monitored the distribution of [^{3}H]cholesterol in cells labeled with [^{3}H]cholesterol at 4 °C. Under these conditions, labeled cholesterol is only present in the plasma membrane and fails to reach intracellular membranes (26). Cells labeled at 4 °C with [^{3}H]cholesterol were mixed with cells labeled at 37 °C with [^{14}C]acetate, prior to homogenization. The [^{3}H]cholesterol distribution was very similar to that observed for the [^{14}C]acetate-labeled sterols, with 87–88% of the [^{3}H]cholesterol found in the

P10, 9% in the P100, and 4% in the S100. As further controls, we also monitored the distribution of enzyme markers known to reside predominantly within specific subcellular compartments. Nearly 70% of the alkaline phosphodiesterase activity, an enzyme known to reside in the plasma membrane, was found in the P10. Approximately 55% of the hexosaminidase activity, an enzyme known to reside in the lysosome, similarly fractionated with the P10, indicating that this fraction was also rich in lysosomes. A significant amount of hexosaminidase activity (36%) was found in the S100, most likely due to breakage of the lysosome during homogenization; significantly, little or no cholesterol was found in this fraction. Lactate dehydrogenase, a cytosolic enzyme marker, was found predominantly in the S100 fraction. These findings demonstrate that the endogenously produced sterols that accumulate in the presence of progesterone cofractionate with sterols known to reside in the plasma membrane and that the distribution of these sterols is distinctly different from that of lysosomal enzyme markers.

Progesterone Induces Cholesterol Auxotrophy in CHO Cells—The concentration of progesterone required to inhibit cholesterol synthesis is relatively high, and we were concerned that the effects of progesterone on sterol metabolism might result from general metabolic changes associated with progesterone toxicity. Any metabolic changes of this type must be specific since fatty acid production and conversion of acetate to lanosterol were not affected by progesterone treatment. To test for progesterone cytotoxicity, we monitored long-term cell growth in the presence of increasing concentrations of progesterone (Fig. 6*A*). Low concentrations of mevalonate were included in the medium to provide substrate for the non-sterol products of the pathway (27). Progesterone concentrations above 100 μM completely prevented growth of CHO cells. The finding that at these concentrations, progesterone completely blocked cholesterol synthesis raised that possibility that progesterone was preventing cell growth by limiting cholesterol availability. To test this, we evaluated the ability of exogenously added cholesterol to rescue growth of progesterone-treated cells (*panel B*). Concentrations of cholesterol above 2 μg/ml were capable of restoring growth to cells treated with 200 μM progesterone. These findings indicate that progesterone causes cholesterol auxotrophy in CHO cells, presumably due to the inhibition of cholesterol synthesis.

Progesterone Inhibits Cholesterol Synthesis in Other Cell Types—Treatment of a series of human cell lines demonstrated that the effect of progesterone on cholesterol biosynthesis was not unique to CHO cells (Fig. 7). Human cell lines were adapted for growth in lipoprotein-poor serum to increase their reliance on endogenously synthesized cholesterol and thereby increase the efficiency with which [^{14}C]acetate was incorporated into sterols. Cells were labeled in either the absence or presence of 100 μM progesterone, and radiolabeled sterols were extracted and resolved by TLC. CHO cells produced equal concentrations of lanosterol and cholesterol in the 2-h labeling (*lane 1*). Progesterone treatment completely inhibited cholesterol production and resulted in the accumulation of lanosterol and product D (*lane 2*). HeLa cells more efficiently incorporated acetate into cholesterol (*lane 3*); nearly 80% of the sterols produced were cholesterol. Progesterone completely blocked cholesterol production, again resulting in the production of lanosterol and product D (*lane 4*). Similar results were observed in Hep G2 (*lanes 5* and *6*), Caco-2 (*lanes 7* and *8*) and Chang Liver cells (*C.Liv.*; *lanes 9* and *10)*. We have also found that progesterone inhibits cholesterol biosynthesis in cultured human fibroblasts, freshly isolated lymphocytes, and transformed human fibroblasts (data not shown). These observations indicate that progesterone inhibits cholesterol biosynthesis in many, if not all, cell types.

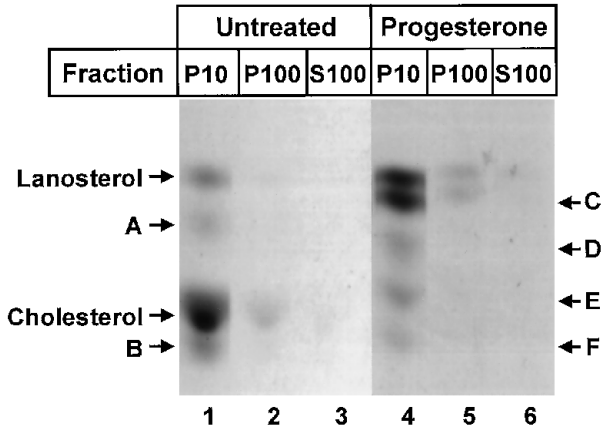

FIG. 5. **Subcellular location of sterols.** Cells were plated, grown, and labeled on day 3 with [^{14}C]acetate for 2 h at 37 °C as described in the legend to Fig. 1. Cells were harvested, homogenized and fractionated into P10, P100, and S100 fractions as described under "Experimental Procedures." Radiolabeled sterols were extracted from each fraction and resolved by TLC as described in the legend to Fig. 1. TLC plates were exposed to Amersham Hyperfilm for 7 days at −70 °C. The bands corresponding to lanosterol and cholesterol were identified by comparing the iodine-stained TLC sheet with the autoradiogram.

TABLE I

Differential centrifugation: subcellular distribution of radiolabeled sterols and marker assays

Cells were plated, grown through, and labeled on day 3 with [^{14}C]acetate at 37 °C as described in the legend to Fig. 1. Parallel cultures were labeled with 0.5 μM [^{3}H]cholesterol (135.6 cpm/pmol) at 4 °C for 1 h. The parallel cultures were combined at 4 °C, homogenized, and subjected to differential centrifugation to generate a 10,000 × *g* pellet (P10), a 100,000 × *g* pellet (P100), and a 100,000 × *g* supernatant (S100). Each fraction was analyzed for labeled sterols and marker enzyme activities as described under "Experimental Procedures." Total values, in the presence and absence of progesterone, respectively, were 31.6 and 8.8 pmol/h [^{14}C]cholesterol, 4.6 and 14.8 pmol/h [^{14}C]lanosterol, 693 and 697 μg/dish protein, 4.6 and 4.8 milliunits/dish alkaline phosphodiesterase, 2185 and 2197 milliunits/dish lactate dehydrogenase, and 66 and 71 milliunits/dish hexosaminidase. ND, none detected.

	Untreated			Progesterone		
	P10	P100	S100	P10	P100	S100
	% total					
Radiolabeled sterols						
[^{14}C]Cholesterol	87	10	3	ND	ND	ND
[^{14}C]Lanosterol	81	14	5	79	16	6
Marker assays						
Protein	48	18	34	44	17	39
[^{3}H]Cholesterol	88	9	4	87	9	4
Alkaline phosphodiesterase	69	8	22	71	7	21
Lactate dehydrogenase	20	17	63	22	2	76
Hexosaminidase	57	7	36	55	9	36

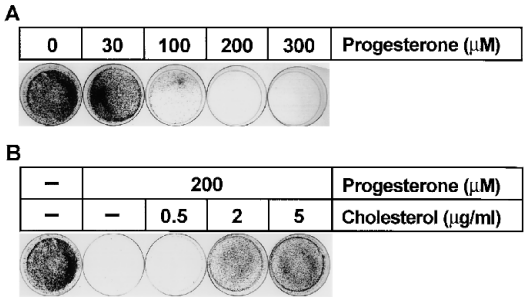

FIG. 6. **Growth of CHO cells in progesterone.** On day 0, CHO cells were plated at 3×10^4 cells/60-mm dish in medium A. On day 1, cells were refed medium A containing 5% LPPS and the indicated additions of progesterone and cholesterol. Cells were refed every 2–3 days with medium of identical composition. On day 12, cells were washed, fixed, and stained with crystal violet.

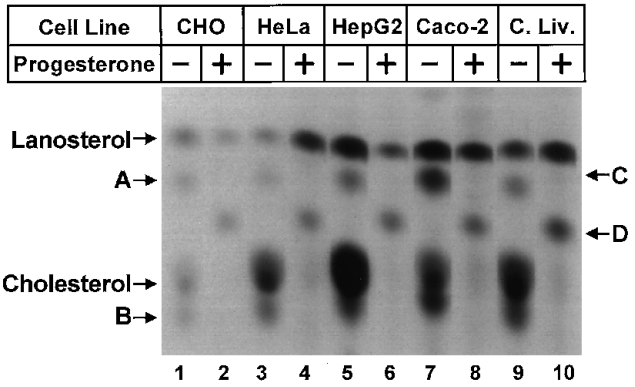

FIG. 7. **Effect of progesterone in human cell lines.** Cells were plated on day 0 in their respective growth media. On day 1, all cells were refed medium A containing 5% LPPS. On day 3, duplicate dishes were refed 0.5 ml of medium A containing 2 mg/ml bovine serum albumin in either the absence (−) or presence (+) of 100 μM progesterone. After incubation at 37 °C for 2 h, [^{14}C]acetate (95.4 dpm/pmol) was added to a final concentration of 0.5 mM, and cells were incubated at 37 °C for an additional 2 h. Cells were harvested, and sterols were extracted and resolved by TLC as described under "Experimental Procedures." TLC plates were exposed to Kodak XAR film for 4 days at −70 °C. The indicated locations of recovery-derived lanosterol and cholesterol were determined by staining with iodine vapor.

DISCUSSION

In the current report, we demonstrate that progesterone inhibits cholesterol biosynthesis by preventing conversion of sterol intermediates to cholesterol. We demonstrate that the ability of progesterone to inhibit cholesterol synthesis is a general phenomenon, seen not only in CHO cells but in many different human cell lines, as well as in human primary tissues. Therefore, it is likely that this progesterone-sensitive process is a general aspect of normal cell function and not a unique characteristic of differentiated cells.

Since progesterone is known to block cellular cholesterol transport, we postulate that progesterone blocks cholesterol biosynthesis by inhibiting intracellular transport of cholesterol precursors. Enzymes involved in the conversion of lanosterol to cholesterol reside in the ER (13, 14). Progesterone may prevent sterol precursors from reaching these ER-resident enzymes, thereby preventing their conversion to cholesterol. Consistent with this hypothesis is our observation that the sterols that accumulate in progesterone-treated cells accumulate in cell fractions containing the plasma membrane.

The concentration of progesterone required to inhibit cholesterol synthesis is relatively high and is similar to the concentration previously shown to be required to inhibit cholesterol esterification.[2] Such high concentrations prevent growth of CHO cells in prolonged culture. Since cell growth can be rescued by addition of cholesterol to the medium, it appears that progesterone toxicity results from inhibition of cholesterol biosynthesis; progesterone induces cholesterol auxotrophy. The increase in sterol production observed in the presence of high concentrations of progesterone (see Figs. 1 and 2*A*) provides further evidence that progesterone induces cholesterol auxotrophy. Normal mechanisms of feedback suppression allow cells to up-regulate cholesterol production as cellular cholesterol levels fall (28). We find that in cells treated with 100 μM progesterone, total sterol production increases, resulting in an increased accumulation of lanosterol and products C and D. These findings suggest that the cells have up-regulated the enzymes controlling the early steps in the biosynthetic pathway and demonstrate that the cells recognize the decreased cholesterol production caused by progesterone. Furthermore, these findings indicate that regulatory metabolites involved in feedback suppression must be downstream of the metabolites that accumulate in progesterone-treated cells. Further studies will be required to determine the exact mechanism of this derepression, since the regulation of the pathway involves many distinct but coordinated mechanisms (28).

Most of the sterols that accumulate in the presence of progesterone have the capacity to continue through the pathway to cholesterol, once progesterone is removed from the media. These studies provide evidence that many of the sterols produced in progesterone-treated cells are cholesterol precursors. There are two notable exceptions to this relationship. First, a novel minor product (product G) is produced upon removal of progesterone, suggesting that some metabolites irreversibly leave the cholesterol biosynthetic pathway to form a novel product. Although the exact nature of this product is not known, it may be one of the 4-carboxysterols recently shown to accumulate in a mutant CHO cholesterol auxotroph (10). Second, a portion of lanosterol that is produced in progesterone-treated cells is refractory to conversion. Presumably, this lanosterol has entered a metabolic pool no longer capable of being converted to cholesterol. The nature of this pool is not known; however, it does not appear to result from fatty acid esterification of lanosterol (data not shown).

Mammalian cells utilize two distinct sources of cholesterol: LDL-derived cholesterol and endogenously synthesized cholesterol. Cells maintain exquisite control over the level of free cholesterol by coordinately regulating these two pathways through transcriptional regulation of genes required in both processes (28). The current observations suggest that a single, progesterone-sensitive process is required for cells both to synthesize cholesterol and to esterify LDL-derived cholesterol. Regulation of this process could provide an additional mechanism for coordinately regulating these pathways. In the process of cholesterol esterification, delivery of cholesterol substrate to the ER is rate-limiting (6). Since the same progesterone-sensitive process seems to be involved in cholesterol synthesis, it seems possible that, under some circumstances, delivery of sterol precursors to the ER may be limiting to cholesterol biosynthesis.

Progesterone is unlikely to be a physiologic regulator of sterol synthesis since the concentration of progesterone required to inhibit cholesterol synthesis exceeds normal physiologic levels. Other steroids might be more potent regulators of the process and may represent physiologic regulators of this process. In the accompanying manuscript (15), we investigate the effects of additional steroid hormones on cholesterol biosynthesis and demonstrate that, like cholesterol esterification,[2] multidrug resistance P-glycoprotein activity is required for cholesterol biosynthesis.

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