The studies presented herein were designed to determine the source(s) of cholesterol used for steroid biosynthesis in a clonal strain of cultured Leydig tumor cells (designated MA-10).

Our results show that, when the cells are placed in medium devoid of extracellular cholesterol and acutely stimulated with human chorionadotropin, most of the cholesterol used for steroid biosynthesis is derived from intracellular stores. Upon prolonged stimulation, when the intracellular stores are depleted, the cells meet the enhanced demand for cholesterol by increasing de novo synthesis. The presence of low density lipoprotein has little or no effect on the amount of steroid synthesized during acute stimulation, but it enhances the amount of steroid produced during prolonged stimulation by directly providing cholesterol substrate to the cells.

There are three mammalian tissues: adrenals, ovaries, and testes, where steroid biosynthesis is enhanced by polypeptide hormones (adrenocorticotropic and luteinizing or hCG, respectively). When steroidogenesis is stimulated, the enhanced demand for cholesterol may be met by using one or more of the following three sources: (a) intracellular stores; (b) de novo synthesis; and (c) lipoproteins.

Recent studies on the rat and mouse adrenals (1-3), and in cultured adrenocortical cells derived from a mouse adrenocortical tumor (Y-1) (4), normal bovine adrenal cortex (5), normal rat adrenal cortex (6), and normal human fetal adrenals (7) showed that most of the cholesterol used for steroid biosynthesis is derived from lipoproteins. Similar observations have been made in luteinized rat ovaries (8, 9), as well as in cultured rat granulosa/luteal cells (10) and cultured bovine granulosa cells (11).

The source of cholesterol used for steroid biosynthesis in the testes (Leydig cells) has not been fully elucidated. The following observations, however, suggest that the source of substrate used for steroid biosynthesis in this tissue may be different than in the adrenals or ovaries: 1) Morris and Chaitkov (12) determined the relative contribution of plasma and endogenous cholesterol to the cholesterol content of several rat tissues and concluded that, while most (92-95%) of the adrenal cholesterol was derived from the plasma, only 38-43% of the testicular cholesterol was derived from the plasma. 2) Andersen and Dietsch (13) compared the effects of drug-induced hypcholesterolemia on steroid levels and cholesterol biosynthesis in the three steroidogenic tissues of the rat. Their results showed that the adrenals and ovaries of hypocholesterolemic rats had reduced levels of free and esterified cholesterol and enhanced cholesterol biosynthesis. The testes, however, were not affected. 3) Studies from this laboratory (14-16) showed that Leydig cells derived from a transplanted mouse tumor differ from adrenal cells and granulosa/luteal cells in having larger stores of free than esterified cholesterol. Like normal rat Leydig cells, the levels of cholesterol in the tumor (in vivo) and cholesterol or steroid biosynthesis were not affected by drug-induced hypcholesterolemia (16). 4) A recent report by Charreau et al. (17) showed that, unlike the adrenals and ovaries (1-3, 8, 9), the HMG-CoA reductase activity of normal rat Leydig cells was not affected by drug-induced hypocholesterolemia.

The studies presented herein were designed to determine the sources of cholesterol utilized for steroid biosynthesis by cultured Leydig tumor cells (MA-10). The MA-10 cells are a clonal strain of mouse Leydig tumor cells that respond to lutropin/hCG, cholera toxin, and cAMP with increased progesterone production (18). Our results show that during the initial stages of hCG stimulation, the MA-10 cells primarily use intracellular cholesterol stores for progesterone biosynthesis. Upon prolonged stimulation, the cells use both newly synthesized and exogenous (i.e., lipoprotein-derived) cholesterol for progesterone biosynthesis. The data presented show that of the two lipoproteins tested, LDL is more efficient than HDL in providing the cells with cholesterol. The effects of LDL on steroidogenesis can be entirely explained on the basis of the delivery of cholesterol by the classic LDL pathway (19-21).

**EXPERIMENTAL PROCEDURES**

**Hormones and Supplies—**HCG (Batch CR-121) was obtained from the National Institute of Child Health and Human Development, 8-Br-cAMP, Brij 96 (polyoxyethylene 10 oleyl ether), mevalonolactone, NADP, glucose-6-phosphate dehydrogenase (Type XXIII), sodium oleate, and o-phthalaldehyde were from Sigma. Cholesterol and cholesterol olate were purchased from Steraloids. Cabosil was obtained from Packard Instrument Co. [7-3H(N)]cholesterol (34.6 Ci/mmol), [cholesterol-4-14C]oleate (52.5 mCi/mmol), dl-3-hydroxy-3-methylglutaric acid (HMG-CoA) reductase, mevalonolactone, cholesterol, mevalonolactone, cholesterol, and cholesterol olate were purchased from Sigma. All other reagents were obtained from commercial sources.

**METHODS**

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Cholesterol Metabolism in Leydig Tumor Cells

[glutaryl-3-14C] CoA (50.3 mCi/mmol), [1-14C]oleic acid (89 mCi/ 
mmol), RS-[5-3H(N)]-mevalonolactone (5.7 Ci/mmol), and [1-14C,7- 
3H(N)]-progestosterone (101 Ci/mmol) were from New England Nuclear. 
[1-14C]Lactic acid (56.7 Ci/mmol) was from Amersham Corp. Bio- 
Rex 5 (100-200 mesh, chloride form) was from Bio-Rad. Plastic- 
based silica gel thin layer sheets were from Eastman-Kodak or 
Brinkman. Bovine serum albumin (Fraction V) was from Miles Lab- 
oratories, Inc. All tissue culture supplies were purchased from Gibco 
Laboratories. Aminogluthethimide was a gift from Ciba-Geigy, and 
Compandin (ML-236B) was a gift from Dr. Akira Endo (Tokyo-Noko 
University, Japan).

**Cell culture**—The origin and handling of the MA-10 cells has 
been described (18, 22). All experiments were carried out using a similar 
protocol. Stock cultures were subcultured (split ratio, 1:8) on day 0 
into 6-cm culture dishes containing a total volume of 5 ml of growth 
medium (Waymouth MB-752/1 containing 1.12 gm/liter of NaHCO3, 
20 pg/ml of gentamycin, 20 mm 4-(2-hydroxyethyl)-1-piperazineeth- 
anesulfonic acid, pH 7.4, and 15% horse serum). On day 2, the medium 
was replaced with 4 ml of the above growth medium, or with growth 
medium modified to contain 15% lipoprotein-deficient horse serum. 
All experiments were performed on day 3 as follows: The medium 
was aspirated and the dishes washed 2–3 times (3 ml each time) with 
wash medium. The cell suspensions were then extracted with 4 ml of 
chloroform:methanol (2:1) containing trace amounts of [3H]chole- 
sterol and [14C]cholesterol to determine procedural losses (16). The extracts from four dishes were combined 
and evaporated to dryness under N2. The lipids were redissolved in 
dna:disk between replicate dishes varied by at most 15%

The content of cholesterol in each fraction was determined by 
correcting for endogenous fatty acids bound to the albumin. 
Cells were incubated in 4 ml of serum-free medium with or without 
hCG (40 ng/ml). At the times indicated, 0.1 ml of a solution of [3H] 
oleate-albumin (4000–6000 cpm/nmol) was added to give a final oleate 
concentration of 0.1 mM, and the dishes were incubated for 1 h at 
37 °C. After washing, the lipids were extracted and the extracts from 
two dishes combined and chromatographed as described above. The 
cholesterol ester fraction was identified, cut, and counted (see above). 
The reaction was shown to be linear with time (0–1 h) and zero order 
with respect to substrate concentration.

**Other Methods**—The content of total cholesterol in serum was 
determined by the method of Rudel and Morris (24). Lipoprotein- 
deficient horse serum was prepared by stirring the serum (4 °C) with 
2% (w/v) Cabosil for 24 h (30). After centrifugation (25,000 × g, 1 h, 
at 4 °C) the serum was sterilized by filtration. The concentrations of 
triglycerides determined in horse serum and lipoprotein-deficient horse 
serum were 700 ± 150, respectively. LDL (d 1.019–1.063 g/ml) and HDL 
(d 1.090–1.215 g/ml) were isolated by sequential flotation according to 
standard procedures (31), using solid KBr for density adjustment (32). Lipoprotein fractions were treated 
and stored as described by Goldstein and Brown (33), and dialyzed 
for 3 h against 0.15 M NaCl/5 mM 4-(2-hydroxyethyl)-1-piperazineeth-
anesulfonic acid, pH 7.4, prior to use in the experiments. The ratio of 
cholesterol to protein was 1.36:1 and 1:4.76 for LDL and HDL, 
respectively. Both lipoprotein fractions were judged to be homoge-
neous by lipoprotein electrophoresis (34). The protein content of the 
lipoprotein fractions was measured by the method of Lowry et al. (35) 
and modified by Markwell et al. (36). The cells were disrupted as 
follows:

Progestrone was determined by radioimmunoassay following 
extraction of the medium (16, 18, 20). Cellular DNA and protein 
were measured by the methods of Burton (37) and Bradford (38), respec-
tively. The salt form of Compactin was prepared as described by 
Brown et al. (39).

**RESULTS**

**Utilization of Intracellular Cholesterol Stores**—The MA-10 cells differ from normal rat adrenal or luteal cells in the 
major form of intracellular cholesterol. Thus, while the former 
have 3–10 times more esterified than free cholesterol (1, 18, 
13), the MA-10 cells have 3–4 times more free than esterified 
cholesterol (Fig. 1).

Incubation of MA-10 cells with a saturating concentration of 
hCG (Fig. 1) caused a rapid reduction in cellular stores of 

In order to determine the incorporation of radioactivity into 
intracellular cholesterol, 1-ml aliquots of the medium were 
extracted with 10 ml of ethyl ether. The upper phase was transferred to scintillation 
vials. The lower phase was aspirated and saved. The efficiency of extraction 
determined with [3H]progesterone was essentially quantitative (90–95%) and no corrections for recovery were done.

In another experimental protocol, we tested the effect of hCG on 
[1-14C]acetate incorporation. For these experiments, the dishes were 
ashed (on day 3) and the received 4 ml of medium containing 1 
mg/ml albumin and 10 μg [1-14C]acetate (0.025 μCi/ml) with or 
without hCG (40 ng/ml). At the times indicated, the medium was 
removed, and the dishes washed 5 times. The incorporation of 
radioactivity into intracellular cholesterol and cholesteryl esters, 
and extracellular steroids was then determined as described above.
free and esterified cholesterol. After a short lag period, free cholesterol fell rapidly for 4 h and eventually declined to 56% of control levels by 12 h. Esterified cholesterol decreased in a linear manner for 4 h and declined to 32% of control levels after 12 h of hCG stimulation. The accumulation of progesterone in the medium reached 85% of maximum after 4 h of hCG stimulation and peaked by 8 h. At this time, the amount of cholesterol (free and esterified) lost from cells was 99 ng/μg of DNA. The amount of progesterone accumulated in the medium was 44 ng/μg of DNA.

The reason for the incomplete recovery of intracellular cholesterol as extracellular progesterone is not entirely understood. It should be noted, however, that progesterone can be slowly metabolized to 20α-dihydroprogesterone and other unidentified products by these cells (18). If other steroids produced are taken into account (see Figs. 2 and 3), the recovery increases to 60–70%. This finding does not appear to be peculiar to the MA-10 cells, since others have reported that when freshly isolated rat adrenocortical cells are stimulated with adrenocorticotropic hormone, only 5–10% of the intracellular cholesterol is recovered as extracellular corticosterone (40).

When these experiments were performed in the presence of aminogluthethimide, an inhibitor of the cholesterol side chain cleavage enzyme (41, 42), there were no hCG-induced changes in cellular cholesterol stores and progesterone synthesis was inhibited by 98% (Fig. 1, right).

To demonstrate that the decline in cellular cholesterol stores occurred because cholesterol was used as substrate for steroid biosynthesis, the experiment shown in Fig. 2 was performed. In this experiment, cells were incubated overnight with [1-14C]acetate to prelabel the intracellular cholesterol pools. The cultures were then washed and placed in fresh media and further incubated with or without hCG. The addition of hCG caused the radioactivity associated with cholesteryl esters2 to decline rapidly (t½ = 2 h). The loss of label from this pool was almost complete by 4 h. The radioactivity associated with free cholesterol was lost more slowly (t½ = 4 h) but continued for the entire 12-h experiment. Ether-soluble radioactivity rapidly appeared in the media (t½ = 2 h) and reached 80% of total accumulation by 4 h. By 8–12 h of hCG stimulation, 60–70% of the radioactivity originally associated with the intracellular cholesterol pools was recovered in ether-

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2Alkaline hydrolysis of this fraction (isolated at t = 0, Fig. 2) or t = 4 h, Fig. 4) showed that about 50% of the radioactivity was associated with cholesterol. The radioactivity associated with cholesteryl esters in both of these figures represents the total radioactivity (i.e., cholesterol and fatty acids) present in this fraction.
soluble form in the medium. Aminoglutethimide blocked all of these changes (not shown).

The ether-soluble radioactivity released into the medium was analyzed by thin layer chromatography (Fig. 3). 4 h after hCG stimulation, 86% and 9% of the radioactivity co-migrated with progesterone and 20α-dihydroprogesterone, respectively, and 5% of the radioactivity remained at the origin. By 12 h of stimulation, the major peak was broader and contained 64% of the radioactivity; the amount of radioactivity associated with 20α-dihydroprogesterone increased to 16%, the radioactivity that remained at the origin increased to 13%, and another peak became prominent. Since in the solvent system used cholesterol migrates just slightly behind progesterone (see legend to Fig. 3), it was possible that the shift of the major peak observed in the 12-h sample was due to the release of free cholesterol to the medium. In order to check this possibility, ether extracts of the same samples were analyzed on a second solvent system (cyclohexane:ethyl acetate, 1:1), where the relative migration of cholesterol and progesterone are reversed. In this system, the RF values for 20α-dihydroprogesterone, progesterone, cholesterol, and cholesteryl esters are 0.28, 0.36, 0.41, and 0.59, respectively. The results (not shown) again indicated that most of the radioactivity migrated with progesterone, and that this peak shifted slightly toward the origin at late time points. None of the radioactivity released migrated with cholesterol or cholesteryl esters. These results are consistent with previous results showing that the major steroid produced by the MA-10 cells is progesterone and other unidentified products (18, 43).

Utilization of Newly Synthesized Cholesterol—The effect of hCG on [14C]acetate incorporation into cellular cholesterol stores and into extracellular steroids is shown in Fig. 4. Cells incubated with [14C]acetate in the absence of hCG accumulated 94% of the radioactivity into free and esterified cholesterol. The addition of hCG decreased [14C]acetate incorporation into cholesterol and cholesteryl esters, and increased [14C]acetate incorporation into steroids. The incorporation of radioactivity into steroids showed a short lag, and then proceeded linearly for 8 h. Aminoglutethimide blocked all of the hCG-induced changes (not shown).

The sum of the radioactivity incorporated into cholesterol, cholesteryl esters, and steroids was similar in both groups of cells. These results show that hCG increases the utilization of newly synthesized cholesterol for steroid biosynthesis. It should also be noted that the utilization of newly synthesized cholesterol for steroid biosynthesis proceeded linearly for 8 h (Fig. 4, lower), while the utilization of cholesterol stores proceeded linearly for only 4 h (Fig. 2, lower).

The effect of hCG treatment on de novo synthesis of cholesterol and on cholesterol esterification was examined more directly by measuring the activity of HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis, and the rate of [14C]oleate incorporation into cholesteryl esters. The results of these experiments are shown in Fig. 5. Treatment of cells with hCG induced a 3-fold increase in HMG-CoA reductase activity by 4 h. The enzyme activity then declined steadily toward control levels, such that by 12 h of hCG simulation, it was only 1.3 times higher than control. The rate of cholesterol esterification declined precipitously to 10% of control values by 3-4 h of hCG exposure. However, after reaching this low level, it gradually increased to 35% of control levels by 12 h of stimulation. Inasmuch as both of these effects are blocked by aminoglutethimide (Fig. 5, lower), it is concluded that they are mediated by the depletion of the intracellular cholesterol pools induced by hCG (cf. Figs. 1 and 2).

Taken all together, these results show that, when steroidogenesis is stimulated in the absence of an exogenous source of cholesterol, the MA-10 cells use stored and newly synthesized cholesterol to meet the enhanced demand for steroid production. The time course for the utilization of stored cholesterol suggests that stores are utilized early during steroidogenic stimulation while de novo synthesis of cholesterol is stimulated more slowly and persists longer, hence becoming important at late time points (>4 h).
Utilization of Lipoprotein-Derived Cholesterol—Previous results from other laboratories have shown that lipoproteins provide an important source of substrate for steroid production in the adrenals and ovaries (1-11). Thus, we sought to determine if they also provide cholesterol to the MA-10 cells.

Most experiments on the interaction of lipoproteins with cultured cells are done by first preincubating the cells for 24-48 h in medium supplemented with lipoprotein-deficient human serum. These conditions result in a decrease in the intracellular levels of free and esterified cholesterol, an increase in HMG-CoA reductase activity and LDL receptors, and a decrease in the rate of cholesterol esterification (1-5, 7-11, 19-21).

We cannot use human serum because the MA-10 cells do not thrive in it; moreover, it has adverse effects on the response of the cells to hCG. When the MA-10 cells are incubated in medium supplemented with lipoprotein-deficient horse serum, the changes observed in the metabolism of cholesterol are different than those described above. There is little or no change in free or total cholesterol or HMG-CoA reductase activity, and there is a 1.7-1.8-fold increase in the LDL receptors. These results from other laboratories have shown that lipoproteins influence cholesterol metabolism by decreasing LDL and increasing HMG-CoA reductase activity and the rate of cholesterol esterification (1-5, 7-11, 19-21).

At the end of this incubation, the intracellular levels of free and esterified cholesterol were measured and found to be depleted (when compared to nonstimulated cells) by 30-40% (19-21). This depletion is similar to that observed in the absence of serum. In order to test if lipoproteins enhance steroidogenesis, we preincubated the cells in lipoprotein-deficient serum for 24 h, and then exposed them to hCG in the presence of increasing concentrations of LDL or HDL. The results of a representative experiment are shown in Fig. 6. In five independent experiments, the average (maximal) increase in progesterone production was 1.6-fold for both LDL and HDL. When expressed in terms of cholesterol content, the half-maximal and maximal LDL effects were observed at 7.5 and 20 μg/ml, respectively. HDL did not have a significant effect at concentrations below 50 μg/ml, but it consistently stimulated steroidogenesis at higher concentrations. The magnitude of the effect of lipoproteins on steroidogenesis in the MA-10 cells is lower than that observed in normal adrenal or ovarian cells (7, 10, 45, 47), but similar to that reported for normal Leydig cells (17, 44, 46, 48).

Since LDL appeared to be more active than HDL in stimulating steroidogenesis, all subsequent experiments were done with this lipoprotein.

The results presented in Tables II and III show that in the absence of hCG stimulation, LDL has the same effects in the MA-10 cells as in other cell types (19-21); it decreases HMG-CoA reductase activity (Table II) and increases the rate of cholesterol esterification (Table III). It is important to note, however, that when the cells are stimulated with hCG, LDL does not suppress the effects of hCG on these processes (Tables II and III), presumably because the cholesterol delivered to the cells is being used for progesterone biosynthesis. These results were complemented by measuring the effects of Compactin, a competitive inhibitor of HMG-CoA reductase activity (39, 49), on the ability of LDL to enhance steroid production. To do this, we placed cells in lipoprotein-deficient medium alone or supplemented with LDL, and stimulated them with hCG in the presence or absence of Compactin. One group of dishes was harvested 4 h after addition of the compounds; the other group was washed (to remove the progesterone that had accumulated in the medium), and hCG, Compactin, and LDL (if appropriate) added again. These dishes were collected after a further 4-h incubation. This procedure was repeated in medium containing Compactin, and on days 3 and 6 of culture, the experiments were repeated in medium without Compactin.

The effects of lipoprotein-deficient serum on cholesterol metabolism in the MA-10 cells are shown in Figs. 6-9. The results were statistically analyzed using Student's t test.

Table 1

<table>
<thead>
<tr>
<th>Cholesterol Metabolism in Leydig Tumor Cells</th>
<th>Complete serum</th>
<th>Lipoprotein-deficient serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cholesterol (ng/μg DNA)</td>
<td>254 ± 24</td>
<td>228 ± 7</td>
</tr>
<tr>
<td>Esterified cholesterol (ng/μg DNA)</td>
<td>53 ± 5</td>
<td>96 ± 18^a</td>
</tr>
<tr>
<td>Total cholesterol (ng/μg DNA)</td>
<td>307 ± 28</td>
<td>324 ± 35</td>
</tr>
<tr>
<td>HMG-CoA reductase activity (pmol/min × mg)</td>
<td>478 ± 56</td>
<td>419 ± 44</td>
</tr>
<tr>
<td>Oleic acid incorporation, into cholesteryl oleate (pmol/μg DNA × h)</td>
<td>12.4 ± 1.1</td>
<td>20.6 ± 1.03^a</td>
</tr>
</tbody>
</table>

^a Significant difference for complete serum group at p < 0.01.

Fig. 6. Effect of lipoproteins on hCG-stimulated steroidogenesis. On day 2 each dish received 4 ml of lipoprotein-deficient medium. After a 24-h incubation at 37 °C, the medium was replaced with 4 ml of lipoprotein-deficient medium containing the indicated concentrations of LDL (top) or HDL (bottom). HCG (40 ng/ml) was then added to all the dishes. Progesterone was measured in the medium after a 8-h incubation at 37 °C. The different symbols show the results obtained with two different preparations of lipoproteins (duplicate dishes/point, variation less than 10%). The lines were drawn through the average of the two points shown. Lipoprotein concentrations are expressed in terms of cholesterol content.
Cholesterol Metabolism in Leydig Tumor Cells

protocol allowed us to determine the relative contributions of cholesterol stores, lipoprotein-derived cholesterol, and de novo synthesis during "short-term" and "long-term" stimulation. The results are presented in Table IV. As expected from the results presented above (cf. Fig. 6), LDL increased progesterone production 1.4-fold during the entire 8-h incubation period. During the first 4 h, the LDL-treated cells produced as much progesterone as the controls. During this time period, Compstatin inhibited steroid production by 27% in the controls, but had no effect on the LDL-treated cells. The effect of Compstatin on the cells incubated in lipoprotein-deficient medium show that the intracellular cholesterol stores contribute the bulk (73%) of substrate for steroidogenesis during the first 4 h of stimulation.

Compstatin inhibited steroid production to a greater degree (in both groups) during the next 4 h. The inhibition (96%) observed in the control cells show that, in the absence of an extracellular source of cholesterol, steroid biosynthesis becomes almost totally dependent on the de novo synthesis of cholesterol. If LDL is present, the cells synthesize 2.5 times more steroid during this incubation, and Compstatin inhibits steroid production only 34%.

Taken all together, these results show that (a) upon prolonged stimulation, hCG-stimulated steroidogenesis is limited by the amount of cholesterol synthesized de novo; (b) this limitation can be overcome by supplying LDL. From the

Table II

Effects of hCG and LDL on the activity of HMG-CoA reductase

On day 2 the medium was replaced with 4 ml of lipoprotein-deficient medium. After a 24-h incubation, the medium was replaced again, and LDL and hCG added as indicated. After a 4- or 12-h incubation at 37°C, the cells were harvested for the determination of HMG-CoA reductase activity. Each number represents the average ± range of two independent experiments (duplicate dishes in each experiment). The LDL concentration is expressed in terms of cholesterol content.

<table>
<thead>
<tr>
<th>Additions to medium</th>
<th>HMG-CoA reductase activity</th>
<th>4 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG</td>
<td></td>
<td>40 ng/ml</td>
<td>120 ng/ml</td>
</tr>
<tr>
<td>40 ng/ml</td>
<td>PMol/mg protein × min</td>
<td>457 ± 95</td>
<td>532 ± 31</td>
</tr>
<tr>
<td>LDL (50 μg/ml)</td>
<td></td>
<td>956 ± 136</td>
<td>539 ± 45</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>388 ± 15</td>
<td>273 ± 49</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>900 ± 18</td>
<td>519 ± 54</td>
</tr>
</tbody>
</table>

* Significant difference from time-matched control (i.e. no LDL or hCG) at p < 0.01

Table III

Effects of hCG and LDL on the conversion of [14C]oleate to cholesteryl[14C]oleate

On day 2 the medium was replaced with 4 ml of lipoprotein-deficient medium. After a 24-h incubation at 37°C, the medium was replaced again, and LDL and hCG added as indicated. The cells were then incubated for 4- or 12-h (at 37°C) as indicated, and pulse-labeled for 1 h with 0.1 μM [14C]oleate albumin. Each number represents the average ± range of two independent experiments. In each experiment each point was run in duplicate. The LDL concentration is expressed in terms of its cholesterol content.

<table>
<thead>
<tr>
<th>Additions to medium</th>
<th>4 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG</td>
<td>(Pmol/μg DNA × h)</td>
<td>40 ng/ml</td>
</tr>
<tr>
<td>40 ng/ml</td>
<td>19 ± 2</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>+</td>
<td>4 ± 1</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>LDL (50 μg/ml)</td>
<td>37 ± 1</td>
<td>50 ± 12</td>
</tr>
<tr>
<td>+</td>
<td>5 ± 1</td>
<td>17 ± 5</td>
</tr>
</tbody>
</table>

* Significant difference from time-matched control (i.e. no LDL or hCG) at p < 0.01

Table IV

Effects of compstatin and LDL on hCG-stimulated progesterone production

On day 2 the medium was replaced with 4 ml of lipoprotein-deficient medium. After 24 h at 37°C, the medium was replaced with 2 ml of lipoprotein-deficient medium, and LDL was added as indicated (concentration is expressed in terms of cholesterol content). All the dishes received 40 ng/ml of hCG, and Compstatin (5 μM) was added as indicated. After 4 h at 37°C, half of the dishes were collected and the medium saved for the determination of progesterone. The rest of the dishes were washed five times with warm medium containing 1 mg/ml albumin. Each dish then received 2 ml of the appropriate medium containing hCG (40 ng/ml) with or without Compstatin (5 μM). The medium was collected after a further 4-h incubation. The values shown are the results of a representative experiment. Each value represents the average (± S.E.) of quadruplicate dishes. The numbers in parentheses represent percentages relative to the appropriate controls. In several experiments, the average amount of steroid produced in the presence of Compstatin (expressed as % of control) was 85% (0-4 h) and 3% (4-8 h); and 100% (0-4 h) and 58% (4-8 h) for the cells incubated in the presence or absence of LDL, respectively.

<table>
<thead>
<tr>
<th>Additions to medium</th>
<th>Compstatin*</th>
<th>Progesterone production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-4 h</td>
<td>4-8 h</td>
</tr>
<tr>
<td>LDL</td>
<td>0</td>
<td>30.8 ± 1.9 (100)</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>22.6 ± 1.4 (73)</td>
</tr>
<tr>
<td>LDL (50 μg/ml)</td>
<td>0</td>
<td>33.0 ± 1.5 (100)</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>35.6 ± 1.6 (108)</td>
</tr>
</tbody>
</table>

* The concentration of Compstatin used blocked [14C]acetate incorporation into total cellular cholesterol by 95-98% (data not shown).

results presented, one can calculate the relative contribution of the intracellular cholesterol stores, de novo synthesis, and LDL cholesterol to progesterone biosynthesis as follows: 1) the amount of progesterone derived from intracellular stores was calculated from the amount of progesterone synthesized by the cells incubated with Compstatin in lipoprotein-deficient medium, and assumed to be identical in both groups; 2) the amount of progesterone derived from de novo synthesis was calculated from the data obtained when each group of cells was incubated with or without Compstatin; and 3) the amount of progesterone derived directly from LDL cholesterol was calculated by subtracting the amounts calculated in steps 1 and 2, and subtracting this from the amount of progesterone produced in the absence of Compstatin. Thus, it can be calculated that during the first 4 h of stimulation in lipoprotein-deficient medium, the cells derive 73% (22.6 ng) and 27% (8.2 ng) of the synthesized progesterone from intracellular stores and de novo synthesis, respectively. When LDL is present, 69% (22.6 ng), 0% (0 ng), and 32% (10.4 ng) of the progesterone is derived from intracellular stores, de novo synthesis, and LDL, respectively.

During the next 4 h of stimulation in lipoprotein-deficient medium, the cells derive 4% (0.3 ng) and 96% (8.1 ng) from intracellular stores and de novo synthesis, respectively. When LDL is present, it provides 65% (13.7 ng) of the cholesterol; intracellular stores, and de novo synthesis contributes 1% (0.3 ng) and 34% (7.1 ng), respectively.

These results suggest that LDL enhances the hCG-stimulated progesterone production entirely by providing a direct source of substrate, presumably via the classic LDL pathway (19-21). In fact, during long-term stimulation, the percentage of steroid derived from LDL cholesterol is similar in the MA-10 cells (65%) and adrenocortical cells (75%) (4, 5).

DISCUSSION

The studies presented in this paper were designed to characterize the sources of cholesterol that Leydig tumor cells use for steroid biosynthesis under hCG stimulation.

Our data shown that under acute hCG stimulation (0-4 h),
60-70% of the steroid produced is derived from the intracellular stores of free and esterified cholesterol. The remaining steroid is derived from either the de novo synthesis of cholesterol or from lipoproteins. During prolonged stimulation (t > 4 h), when the intracellular pools are partially depleted, the rate of steroid production falls, and the cells derive 96% of the steroid produced from the de novo synthesis of cholesterol if LDL is not present. If LDL is present, it directly provides 65% of the cholesterol substrate, the contribution of de novo synthesis falls to 35%, and the rate of steroid production remains elevated. In this respect, the MA-10 cells are similar to cultured adrenocortical cells which, under prolonged hormonal stimulation, derive 75% of their steroids from LDL cholesterol (4, 5).

The general aspects of the utilization of LDL-derived cholesterol are similar in the MA-10 cells, and adrenocortical and ovarian cells (1-5, 7-11, 21, 50). Recent studies show that the MA-10 cells have LDL receptors that mediate the internalization and degradation of LDL. The results presented here show that LDL induces the expected changes in cholesterol metabolism; it decreases HMG-CoA reductase activity and increases cholesterol esterification (Table I). These changes are observed only in the absence of hCG stimulation, presumably because when the cells are stimulated with hCG most of the LDL-derived cholesterol is quickly converted to steroids.

Inasmuch as LDL appears to be more active than HDL in enhancing hCG-stimulated progesterone biosynthesis, we presume that LDL is the preferred source of exogenous cholesterol for the MA-10 cells. It is important to note that the routine use of horse serum in our cultures results in a relative deficiency in LDL, because the predominant cholesterol carrier in horse serum is HDL (51). Thus, the finding (Table I) that a 24-h incubation in lipoprotein-deficient horse serum does not result in an increase in HMG-CoA reductase activity and a decrease in the intracellular levels of cholesterol (i.e. the changes expected from LDL removal, see Refs. 19-21) does not seem surprising, since the cells were already in a state of "LDL deficiency" even before they were placed in lipoprotein-deficient serum. The increase in esterified cholesterol and cholesterol esterification (Table I), however, cannot be explained on this basis.

Our experiments on the effects of lipoprotein-deficient serum on cholesterol metabolism in the MA-10 cells are consistent with previous data on the effects of drug-induced hypocholesterolemia on the metabolism of cholesterol in normal rat testes (13, 17, 44), or in the Leydig cell tumor grown in mice (16). Those studies have shown that a reduction in the levels of circulating cholesterol does not result in an increase in HMG-CoA reductase activity (or [14C]acetate incorporation) or a reduction in the cholesterol content of the tissue. In contrast, the same treatment results (in mice or rats) in an increase in adrenal and ovarian HMG-CoA reductase activity and a decrease in the cholesterol content of these tissues (1, 3, 8, 9, 13, 17). Thus, one must still consider the possibility that there are some differences among these three steroidogenic tissues.

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REFERENCES
Cholesterol Metabolism in Leydig Tumor Cells


