Effects of hypocholesterolemia and chronic hormonal stimulation on sterol and steroid metabolism in a Leydig cell tumor

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Abstract The studies presented herein were done to investigate the effects of drug-induced hypocholesterolemia and chronic hormonal stimulation on cholesterol metabolism and steroid biosynthesis in a functional Leydig cell tumor. It was found that 4-aminopyrazolo(3,4-d)-pyrimidine (4-APP)-induced hypocholesterolemia had no effect on (a) the amount of cholesterol present in the tumor, (b) cholesterol biosynthesis, and (c) steroid production. Chronic stimulation with choriogonadotropin also had no effect on the amount of cholesterol present in the tumor, but it increased steroid production and cholesterol biosynthesis. These results suggest that the Leydig tumor cells primarily use intracellular cholesterol for steroid biosynthesis. Other data show that 4-APP treatment reduces gonadotropin binding in the Leydig tumor cells. - Ascoli, M.

Supplementary key words 4-aminopyrazolopyrimidine · human choriogonadotropin · cholesterol

Our understanding of the mechanism by which lipoproteins regulate cholesterol metabolism in cultured cells (1) has led to new experiments on the source of the cholesterol used for steroid biosynthesis in several steroidogenic tissues (2–6).

Thus, when rats or mice are made hypocholesterolemic by injecting them with 4-aminopyrazolopyrimidine (4-APP) or 7α-ethyl estradiol, cholesterol metabolism in the adrenal gland and other extrahepatic tissues is affected: the cholesteryl ester content falls, and the activity of HMG CoA reductase rises (7–9). Likewise, other experiments have shown that lipoproteins regulate cholesterol metabolism and steroid biosynthesis in a clonal strain of malignant mouse adrenocortical cells (Y-1), in cultured normal bovine adrenocortical cells (10, 11), and the human fetal adrenal cortex (12, 13). In these systems, most of the cholesterol used for steroid synthesis is derived from lipoproteins.

A similar phenomenon appears to be operative in luteal cells. Luteal cells isolated from pseudopregnant rats, made hypocholesterolemic with 4-APP, show the alterations of cholesterol metabolism expected for a tissue that has an operative lipoprotein pathway, i.e., reduced levels of cholesteryl esters, and increased de novo synthesis of cholesterol (5, 6). Moreover, these cells show a reduced ability to synthesize progesterone, which can be restored by supplying lipoproteins (5, 6).

In a recent report, Andersen and Dietschy (4) compared the effects of hypocholesterolemia and hormonal stimulation on cholesterol metabolism and steroid production in the three steroidogenic tissues (adrenals, ovaries, and testes) of the rat. Their data suggest that in the absence of hormonal stimulation, cholesterol metabolism in the testes is not sensitive to reduced plasma cholesterol.

The experiments presented herein were done to test the effects of reduced plasma cholesterol and hormonal stimulation on cholesterol metabolism and steroid production in a functional Leydig cell tumor (14, 15). The results presented indicate that the metabolism of cholesterol in the M5480P Leydig cell tumor is not sensitive to the levels of plasma cholesterol.

MATERIALS AND METHODS

Hormones and supplies

HCG (Batch CR-121) was obtained from the National Institute of Child Health and Human Development and iodinated as described elsewhere (13). 4-Aminopyrazolo(3,4-d)-pyrimidine was from Aldrich Chemical Co. 8-Br-adenosine 3',5'Monophosphate and o-phthalaldehyde were from Sigma. Cholesterol, cholesteryl oleate, and progesterone were from Stera-
loids. Sodium [1-14C]acetate (56 mCi/mmol), [4-14C]-
cholesteryl oleate (54 mCi/mmol), [1,2-3H(N)]-chole-
sterol (44 Ci/mmol), and [1,2,6,7-3H(N)]-progesterone
(97 Ci/mmol) were from New England Nuclear. All
other supplies were obtained as described elsewhere
(15–17).

Animal treatment

Male (5–6 wks old) C57Bl/6J mice (Jackson Labs)
were injected subcutaneously with a suspension of
freshly prepared M5480P cells (14, 15), and housed
in a room with a light-dark cycle of 12 hr. All experi-
ments were started 11 days after the injection of tumor
cells, when the tumors were readily visible. 4-APP was
dissolved in 10 mM sodium phosphate buffer, pH 3.0,
and injected intraperitoneally. HCG was dissolved in
10 mM sodium phosphate buffer, pH 4.7, and injected
subcutaneously. The animals were allowed free access
to food and water during the treatment period.

Preparation of tumor cells

Mice were killed by cervical dislocation and blood
(about 0.5 ml) was collected by cardiac puncture in
0.1% EDTA. Individual tumors were dissected and
cells were isolated as described previously (15), with
two modifications: i) the lysis of the red blood cells was
omitted, and ii) the cells were suspended in Way-
mouth MB752/1 medium containing 20 mM Hepes,
1.12 g/ml NaHCO3, 1 mg/ml albumin, and 40 
µg/ml Gentamycin, pH 7.4.

Measurement of cholesterol content

Total plasma cholesterol was measured by the
method of Rudel and Morris (18).

The content of cellular cholesterol was determined as
follows: 2-ml aliquots of the cell suspensions (100–
200 µg DNA) were centrifuged and resuspended in
0.1 ml of isotonic saline. Four ml of chloroform-
methanol 2:1 containing 1 µCi/ml of [1,2-3H]chole-
sterol and [4-14C]cholesteryl oleate were added (to
determine procedural losses). The mixture was agitated
and allowed to stand at room temperature for 30
min. Each tube then received 0.9 ml of water. After
mixing and centrifugation, the bottom phase was re-
moved, evaporated, and redissolved in benzene. Free
and esterified cholesterol were separated on silicic acid–
celite columns as described by Brown, Faust, and Gold-
stein (19). At this stage, the recovery of free cholesterol
was 70–80%. The cholesteryl ester fraction was
 evaporated and hydrolyzed by heating in alcoholic
KOH (3.3% w/v) for 30 min at 70°C. The mixture was
extracted with hexane (18), evaporated, and redissolved
in benzene. At this stage, the recovery of cholesterol
was 60–70%. Double label counting indicated that
there was no cross-contamination between the free
and esterified cholesterol fractions. The content of
cholesterol in each fraction was then determined by
the method of Rudel and Morris (18). All determina-
tions were done in duplicate.

Measurement of plasma progesterone

Duplicate aliquots of plasma (25–50 µl) were
brought up to a total volume of 500 µl with water. After
addition of 1,000 cpm of [3H]progesterone
(used to determine procedural losses), the samples
were extracted with 5 ml of diethyl ether and the
extracts were dried. Progesterone was then separated
from other steroids by chromatography on celite
microcolumns (20) and quantitated by radioimmuno-
assay (21).

Cell incubations

Binding of 125I-labeled hCG was determined during
a 1-hr incubation at 37°C in the presence of 20 ng/ml
125I-labeled hCG (15). Nonspecific binding was deter-
mined in the presence of 2.5 µg/ml hCG and was sub-
tracted from all data. It accounted for 10% of the total
binding at most.

The ability of the cells to respond to steroidogenic
stimuli was measured in duplicate incubations during
a 4-hr incubation (37°C) in the presence of saturating
concentrations (16) of hCG or 8-Br-cAMP. Pro-
gesterone was measured by radioimmunoassay in suit-
able aliquots of the incubation medium.

Incorporation of [1-14C]acetate was measured during
a 4-hr incubation in medium containing 2 µM
[1-14C]acetate (56 mCi/mmol). Preliminary experi-
ments (not shown) indicated that acetate incorpora-
tion was linear during this time period. The incorpo-
ration of radioactivity into cholesterol and cholesteryl
esters was determined as described by Goldstein,
Dana, and Brown (22). All determinations were done
in duplicate. The content of DNA in the cell incuba-
tions varied between 50 and 100 µg.

Other methods

DNA was measured in cell pellets by the method
of Burton (23). Statistical analysis was performed by
analysis of variance (Duncan’s multiple range test).

RESULTS

Effects of 4-APP treatment on plasma
and tumor cholesterol

C57Bl/6J male mice bearing 11-day-old M5480P
Leydig cell tumors had plasma cholesterol levels in the
range of 0.8–1.0 mg/ml. The amounts of free and esterified cholesterol in the tumor were in the range of 0.3–0.5 and 0.1–0.2 μg/μg DNA, respectively.

Treatment of tumor-bearing mice with 4-APP for 3 days resulted in a 70–85% reduction in plasma cholesterol, but had little or no effect on the levels of free and esterified cholesterol in the tumor (Figs. 1 and 2). This treatment also had little or no effect on body weight and on the weight of the tumors. These results agreed well with the demonstrated ability of 4-APP to lower plasma cholesterol (4, 5, 7, 8). It should also be noted that in other steroid-producing tissues, such as the mouse and rat adrenals (2–4, 7–9) and in the luteinized rat ovary (5, 6), a reduction in plasma cholesterol results in a reduction in the levels of esterified cholesterol in the tissue. In the M5480P tumor, 4-APP induced a modest reduction in the levels of esterified cholesterol in only one (Fig. 1) of the three experiments shown (cf. Figure 2 and Table 2).

**Effects of hCG on plasma and tumor cholesterol**

To test the effect of hCG on sterol levels in the plasma and tumors, a single dose of hormone (50 IU, 4 μg/mouse) was injected. This treatment has been previously shown to be effective in stimulating sterol metabolism in the tumors (24, 25).

The data presented in Fig. 3 show that hCG treatment resulted in a 23-fold increase in plasma progesterone, but had little or no effect in the plasma or tumor cholesterol (also see Table 2).

These data indicate that steroid production can be chronically stimulated without major changes in the levels of free and esterified cholesterol in the tumor.

**Effects of combined hCG and 4-APP treatments on plasma and tumor cholesterol**

The data presented in Table 1 show again that while 4-APP reduces plasma cholesterol by 85%, hCG has no effect, and that a combination of hCG and 4-APP treatments was as effective as 4-APP alone in reducing plasma cholesterol. Measurements of plasma progesterone, revealed that while 4-APP treatment produces only a small (2-fold) reduction in the level of plasma progesterone in tumor-bearing mice, it inhibited the stimulatory effect of hCG by 93%. In contrast to normal Leydig cells, the major steroid produced by the M5480P tumor is progesterone (15, 21).
Metabolism of cholesterol in tumor cells isolated from mice treated with 4-APP and/or hCG

The following experiments were done to test the effects of reduced plasma cholesterol, and/or in vivo stimulation of steroid production on the metabolism of cholesterol and steroidogenic properties of the tumor cells. Cells were isolated from the tumors of control animals, or animals that had been injected with 4-APP, hCG, or both, and tested for a) incorporation of [14C]acetate into cholesterol and cholesteryl esters, b) 125I-labeled hCG binding, and c) progesterone production. The results presented in Table 3 show that Leydig tumor cells isolated from hypocholesterolemic mice show the same levels of [14C]acetate incorporation into cholesterol and cholesteryl esters as the control animals. On the other hand, cells isolated from hCG-treated or 4-APP/hCG-treated mice showed a 2 to 4-fold increase in acetate incorporation into cholesterol and cholesteryl esters.

When tested for 125I-labeled hCG binding activity, it was found that the cells isolated from hCG-treated animals bound as much 125I-labeled hCG as the controls (Table 4). In contrast, cells isolated from 4-APP- or 4-APP/hCG-treated mice showed reduced levels of 125I-labeled hCG binding. Note also that the 4-APP/hCG treatment was more effective than 4-APP alone in reducing 125I-labeled hCG binding.

The ability of hCG to stimulate steroid production in freshly isolated Leydig tumor cells was consistent with the levels of 125I-labeled hCG binding activity in these cells (Table 5). Thus, the stimulation of steroid production by hCG in cells isolated from 4-APP- or 4-APP/hCG-treated animals was lower than in control cells. In spite of this, 8-Br-cAMP stimulated steroid production to the same extent in all groups of cells. These data indicate that the reduced ability of hCG to stimulate steroid production in Leydig tumor cells isolated from 4-APP treated mice is due to the loss of 125I-labeled hCG binding activity rather than to alterations in cholesterol metabolism. Moreover, the results obtained with 8-Br-cAMP show that none of the treat-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma Cholesterola</th>
<th>Plasma Progesteroneb</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1003 ± 204</td>
<td>5.8 ± 1.2</td>
</tr>
<tr>
<td>4-APP</td>
<td>152 ± 20</td>
<td>10 ± 0.6</td>
</tr>
<tr>
<td>HCG</td>
<td>854 ± 59</td>
<td>114 ± 20.0</td>
</tr>
<tr>
<td>APP + hCG</td>
<td>149 ± 12</td>
<td>9.9 ± 1.5</td>
</tr>
</tbody>
</table>

a A vs B, P < 0.01; A vs C, not significant (0.01 < P < 0.05); A vs D, P < 0.01; B vs D, not significant (P > 0.05). 

b A vs B, not significant (P > 0.05); A vs C, P < 0.01; A vs D, not significant (P > 0.05); B vs D, not significant (P > 0.05); C vs D, P < 0.01.
The metabolism of cholesterol in steroid-producing tissues (adrenals, ovaries, and testes) may be regulated by the supply of exogenous cholesterol (i.e., in the form of lipoproteins), de novo synthesis, and the demand of cholesterol for steroid biosynthesis (2–4).


discussion

The ability of 4-APP treatment to reduce 125I-labeled hCG binding activity in the Leydig tumor cells is not understood. Two observations (not shown) relevant to this question have been made: i) short term (1–4 hr) incubations of freshly isolated Leydig tumor cells with 4-APP (100–200 µg/ml) had no effect on 125I-labeled hCG binding, or progesterone production in response to hCG or 8-Br-cAMP, and ii) 4-APP treatment (40 mg/kg daily for 3 days) reduced the level of 125I-labeled hCG binding in normal mouse Leydig cells by 30–40%.

**TABLE 3.** Effects of hypocholesterolemia and stimulation of steroid production on cholesterol biosynthesis in Leydig tumor cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>14C</th>
<th>Acetate Incorporated</th>
<th>Cholesterol</th>
<th>Cholesteryl Ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (A)</td>
<td>0.38 ± 0.09</td>
<td>0.25 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-APP (B)</td>
<td>0.34 ± 0.04</td>
<td>0.18 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCG (C)</td>
<td>1.52 ± 0.23</td>
<td>0.55 ± 0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCG + 4-APP (D)</td>
<td>1.18 ± 0.14</td>
<td>0.48 ± 0.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Cells were isolated from the tumors of mice treated as outlined on the column. The incorporation of 14C|Acetate was measured in duplicate incubations as described in Materials and Methods. Each value represents the average (±SEM) of six different tumors.

* Animal treatments were as described in the legend to Table 1. *A vs B, not significant (P > 0.05); A vs C, P < 0.01; A vs D, P < 0.01; C vs D, not significant (P > 0.05).

**DISCUSSION**

Thus, drug-induced hypocholesterolemia leads to a decrease in the cholesteryl ester content, and an increase in cholesterol biosynthesis in the rat and/or mouse adrenals and ovaries (2–9). This treatment, however, is not effective in inducing these changes in the rat testes (4). On the other hand, chronic stimulation of steroid production with the appropriate hormone increases the cholesteryl ester content of the rat adrenals and testes, and decreases that of the ovaries. The same treatment stimulates de novo synthesis of cholesterol in the ovaries and testes, but has no effect on the adrenals (4). Taken all together, these data suggest that the relative importance of the supply and demand of cholesterol in regulating cholesterol metabolism may be different in these steroidogenic tissues. The importance of lipoprotein-derived cholesterol in regulating steroid production in the adrenals and ovaries has been well characterized. Thus, other investigators have shown that adrenocortical (7) or luteal cells (5, 6) isolated from hypocholesterolemic rats have a reduced ability to produce steroids in vitro when exposed to the appropriate hormone or other steroidogenic stimuli. Likewise, cultured adrenocortical cells of murine (Y-1) or bovine origin rely on lipoprotein cholesterol for steroid production (2, 10, 11).

The studies reported herein were designed to obtain information about these processes in the Leydig cells. We have employed the M5480P Leydig cell tumor (14, 15) as a model system for normal Leydig cells because of its ease of experimental manipulation.

**TABLE 4.** Effects of hypocholesterolemia and stimulation of steroid production on 125I-labeled hCG binding in Leydig tumor cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>125I-labeled hCG Bound</th>
<th>pg/µg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (A)</td>
<td>11.7 ± 1.06</td>
<td></td>
</tr>
<tr>
<td>4-APP (B)</td>
<td>4.1 ± 0.49</td>
<td></td>
</tr>
<tr>
<td>HCG (C)</td>
<td>9.8 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>4-APP + hCG (D)</td>
<td>0.62 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

* Cells were isolated from the tumors of mice treated as outlined on the left column (see legend to Table 1 for details). 125I-labeled hCG binding was measured in duplicate samples during a 1-hr incubation in the presence of 20 ng/ml 125I-labeled hCG. Each number represents the average (±SEM) of six different tumors.

* A vs B, P < 0.01; A vs C, not significant (P > 0.05); A vs D, P < 0.01; B vs C, P < 0.01; B vs D, P < 0.01.

**TABLE 5.** Effect of hypocholesterolemia and stimulation of steroid production on the in vitro steroidogenic responses of Leydig tumor cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal</th>
<th>HCG</th>
<th>8-Br-CAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (A)</td>
<td>0.65 ± 0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-APP (B)</td>
<td>0.77 ± 0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCG (C)</td>
<td>2.52 ± 0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-APP + HCG (D)</td>
<td>2.01 ± 0.17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* See legend to Table 4 and Materials and Methods for experimental details. Each number represents the average (±SEM) of six different tumors.

* A vs B, not significant (P > 0.05); A vs C, P < 0.01; A vs D, P < 0.01; B vs C, P < 0.01; B vs D, P < 0.01; C vs D, not significant (P > 0.05).

* A vs B, P < 0.01; A vs C, not significant (P > 0.05); A vs D, P < 0.01; B vs C, P < 0.01; B vs D, not significant (P > 0.05); C vs D, P < 0.01.

* None of these values are different from the control or from each other (P > 0.05).
Andersen and Dietschy (4) on the normal rat testes. The data presented here show that the content of cholesterol, and cholesterol biosynthesis in the Leydig tumor cells is not affected by 4-APP-induced hypocholesterolemia. Chronic in vivo stimulation of steroid production also failed to change the content of cholesterol in the tumor cells of control or hypocholesterolemic mice. On the other hand, this treatment increased [14C]acetate incorporation to the same extent in the cells isolated from control or hypocholesterolemic mice. Chronic hormonal stimulation also increased steroid biosynthesis in control and hypocholesterolemic rat testis (4). In this tissue, however, hCG injections increased the content of tissue cholesterol (free and esterified) in normal rats, and the content of esterified cholesterol in hypocholesterolemic rats (4). The results presented herein show that increased steroid production, or reduced cholesterol supply have no effect on the content of cholesterol in the tumor cells. Moreover, the maximal steroidogenic potential (measured by the in vitro response to 8-Br-cAMP) of cells isolated from hypocholesterolemic, hCG-stimulated controls, or hCG-stimulated hypocholesterolemic mice remained unchanged. Thus, taken all together, these results suggest that, under chronic hormonal stimulation, the Leydig tumor cells may use intracellular cholesterol (obtained either from de novo synthesis or from preexisting pools) rather than extracellular cholesterol to meet the increased demand in steroid production.

In spite of these findings, it appears that under some conditions lipoproteins may play a role in the regulation of testicular cholesterol metabolism. Thus, Andersen and Dietschy (4) showed that high density lipoprotein infusion in 4-APP/hCG-treated rats decreases testicular cholesterol biosynthesis and increases the tissue cholesterol content. Moreover, high or low density lipoprotein receptors have been demonstrated in normal rat Leydig cells, bovine testis, and porcine Leydig cells, respectively (2, 3, 26, 27). We have recently established several clonal strains of Leydig tumor cells in culture (28). Studies in this system should help our understanding of the role of extracellular cholesterol in testicular steroidogenesis, and the effects of 4-APP on 125I-labeled hCG binding.

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