Lipid composition and gonadotropin-mediated lipid metabolism of the M5480 murine Leydig cell tumor[1]

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Abstract The effects of human choriogonadotropin (HCG) stimulation on lipid composition in the murine Leydig cell tumor M5480 grown subcutaneously were determined. The main lipids of the Leydig cell tumor were found to be largely triacylglycerols and phospholipids. Daily in vivo administration of human choriogonadotropin to tumor-bearing mice for 3 days increased the phospholipid content and altered the phospholipid composition of the tumors. There was no demonstrable change in the levels of triacylglycerols, cholesterol, and cholesteryl esters. HCG had no major effect on the fatty acid patterns of the major lipid fractions with the exception of cholesteryl esters, which had a decreased amount of arachidonic acid following HCG-treatment. Results of in vitro incubations of tumor cells prelabeled with [1-^{14}C]arachidonate showed that the label was lost more rapidly from cholesteryl esters of HCG-treated cells than from control cells during (the 12-hour) incubation. Moreover, less [1-^{14}C]acetate was incorporated into the cholesteryl ester fraction of hormone-treated cells than in control cells. HCG stimulated the activity of cholesteryl ester hydrolase in dispersed cells within 3 hours. These results demonstrate that an acute effect of HCG on tumor Leydig cell metabolism is to increase the metabolism of cholesteryl esters, probably by stimulating cholesteryl ester hydrolase activity. The long term effect is an accumulation of phospholipids which may be utilized for membrane synthesis.—**Albert, D. H., M. Ascoli, D. Puett, and J. G. Coniglio.** Lipid composition and gonadotropin-mediated lipid metabolism of the M5480 murine Leydig cell tumor. *J. Lipid Res.* 1980. **21:** 862–867.

Supplementary key words human choriogonadotropin · cholesteryl ester hydrolase · phospholids · cholesteryl esters

It is well established that Leydig cells respond to luteinizing hormone and human choriogonadotropin (HCG), via increased androgen production (1). However, the mechanism of action of the glycoprotein hormones has not been established. Of particular interest is the possible role that lipids may play in the steroidogenic process, not only as direct precursors to steroids (e.g., cholesterol), but also as major constituents of membranes, the biogenesis of which may be important to steroid secretion (2). Evidence has accumulated indicating that the metabolism of cholesteryl esters is under hormonal control in the adrenal (3), corpus luteum (4), and testis (5). It has also been demonstrated that gonadotropins stimulate phospholipid biosynthesis in the testis (6, 7); however, it is not clear if this response is due to the germinal cells or to the nongerminal cells (i.e., Sertoli and Leydig cells).

In an attempt to define the lipid composition of Leydig cells and to clarify the role of lipid metabolism in the steroidogenic response of these cells to gonadotropins, an investigation of a mouse Leydig cell tumor M5480 was undertaken. Previous biochemical studies have demonstrated that the tumor and variants are ideally suited for detailed mechanism studies (8–11). The morphology of the tumor has been reported (12), and the tumors provide sufficient Leydig cells free of germinal cells for quantitative analysis. In the present investigation the effects of gonadotropin stimulation on the composition and metabolism of lipids and on cholesteryl ester hydrolase activity were determined.

MATERIALS AND METHODS

Hormones and supplies

HCG was purified as previously described (13). Sodium [1-^{14}C]acetate, [1-^{14}C]arachidonic acid, and cholesteryl [1-^{14}C]linoleate were purchased from Amersham Searle (Arlington Heights, IL). Medium 199 and RPMI-1640 were obtained from the Grand

Abbreviation: HCG, human choriogonadotropin.

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TABLE 1. Lipid composition of Leydig cell tumors before and after prolonged HCG administration

Class	Treatment[a]	
	Control (n = 4)	HCG-Treated (n = 3)
Phospholipids	13.7 (2.8)	26.3 (6.6)[b]
Cholesterol	1.8 (1.0)	2.2 (1.0)
Cholesteryl esters	0.9 (0.7)	1.4 (0.6)
Triacylglycerols	15.0 (4.4)	18.6 (6.1)

[a] The data are in μmol/g and standard deviations are given in parentheses. Animals were injected with 4 μg HCG daily for 3 days and the tumors were analyzed 2 hr after the last injection.
[b] Significantly different from control ($P < 0.05$).

TABLE 2. Phospholipid composition of Leydig cell tumors before and after prolonged HCG administration

Class	Treatment[a]	
	Control (n = 5)	HCG-Treated (n = 3)
Sphingomyelin	9.6 (3.4)	8.5 (1.5)
Lysophosphatidylcholine	6.0 (4.2)	2.7 (0.3)
Phosphatidylcholine	36.1 (2.7)	44.5 (2.2)[b]
Phosphatidylinositol	6.3 (1.1)	7.1 (1.5)
Phosphatidylserine	3.2 (3.1)	3.4 (2.7)
Phosphatidylethanolamine	27.6 (3.4)	28.2 (1.4)
Solvent front	11.1 (7.1)	4.8 (2.5)

[a] The data are expressed as % of total lipid phosphorus, and standard deviations are given in parentheses. In vivo studies as described in Table 1.
[b] Significantly different from control ($P < 0.05$).

Island Biological Company (Grand Island, NY). All other supplies were obtained as described previously (8, 9).

Leydig cell tumors and cell preparation

Conditions for the isolation and in vitro incubations of tumor cells are given elsewhere (8). In some experiments, tumor-bearing mice were given daily subcutaneous injections of 4 μg of HCG (50 IU) for 3 days. The mice were killed 2 hr after the last injection and the tumors were removed for analysis (see below).

Other methods

Lipids were extracted from the Leydig cell tumors, dispersed tumor cells (after the medium had been removed by centrifugation), and normal mouse testes by the method of Folch, Lees, and Sloane Stanley (14). Neutral lipids were separated by thin-layer chromatography on silica gel using hexane–diethyl ether–acetic acid 80:20:1 (by volume). Phospholipids were separated using chloroform–methanol–acetic acid–water 50:25:8:4 (by volume). The lipids were visualized by brief exposure to iodine vapors and identified by comparison with authentic standards. After the iodine sublimed, the lipids were scraped into vials or tubes for the determination of either radioactivity or phosphorus.

Phosphorus was determined by the method of Bartlett (15). The silica gel was removed by centrifugation prior to measuring the absorbancy. For other measurements, the different lipids were eluted from the gel with chloroform–methanol 2:1 (by volume). Cholesterol and cholesteryl esters were measured by the method of Zlatkis and Zak (16) and triacylglycerols by the method of Sardesai and Manning (17). Methyl esters were obtained from isolated lipid classes by transesterification with boron trifluoride-methanol and analyzed by gas–liquid chromatography as previously described (18). Cholesteryl ester hydrolase activity was determined in cell homogenates by the method of Takatori, Phillips, and Privett (19), except that cholesteryl [1-^{14}C]linoleate (200 μg, 0.5 μCi) was used as substrate.

The Student's *t* test was used for statistical evaluation.

RESULTS

The lipid composition of the Leydig cell tumor is given in **Table 1.** Most of the lipids were phospholipids and triacylglycerols, but nonesterified and esterified cholesterol were also present. The in vivo administration of HCG led to a significant increase in the phospholipid content of the tumor; however, there was no significant alteration in the levels of cholesterol, cholesteryl esters, and triacylglycerols under these conditions (Table 1). As shown in **Table 2,** phosphatidylcholine and phosphatidylethanolamine are the major phospholipids in this testicular tumor, and pretreatment of tumor-bearing animals with HCG led to an increase only in phosphatidylcholine.

The total fatty acid composition of the Leydig cell tumor before and after in vivo administration of HCG is given in **Table 3.** These results, based on limited data, suggest an increase in 18:1[5] following gonadotropin treatment. This increase was noted in all individual lipid classes but was statistically significant only in cholesteryl esters. Following HCG treatment there was also a significant decrease in 18:2, 20:4, and 22:6 of cholesteryl esters (**Table 4**).

In order to obtain more detailed information on the effects of HCG on cholesteryl ester metabolism, in vitro studies were designed using cell suspensions incubated with [^{14}C]acetate. Radioactivity incorporated into the lipid classes of the dispersed cells was determined at various times, either in the absence or

[5] Number of carbons:number of double bonds.

TABLE 3. Total fatty acid composition of Leydig cell tumors and of tumors after prolonged HCG administration

Fatty Acid	Treatment[a]	
	Control	HCG-Treated
16:0	21.0 (17.9, 24.1)	22.5 (22.9, 22.1)
18:0	13.8 (15.3, 12.3)	12.0 (12.7, 11.2)
18:1	24.1 (22.5, 25.7)	31.8 (30.1, 32.8)
18:2	11.3 (7.6, 14.9)	13.8 (11.6, 16.0)
18:3	1.9 (1.8, 1.9)	1.7 (2.1, 1.3)
20:3 (n-6)	2.3 (3.1, 1.5)	1.7 (2.1, 1.3)
20:4	8.0 (8.4, 7.6)	6.3 (6.8, 5.8)
22:4	2.1 (2.6, 1.6)	1.4 (1.4, 1.5)
22:5 (n-6)	1.7 (2.9, 0.5)	trace
22:5 (n-3)	2.7 (3.1, 2.3)	1.5 (1.8, 1.2)
22:6	3.1 (3.2, 3.0)	2.3 (2.5, 2.0)

[a] Data presented as weight % represent the mean of two separate analyses. Individual values are given in parentheses. In vivo studies as described in Table 1.

presence of HCG. Acetate incorporation into the unesterified fatty acid fraction represented less than 2% of the total and was the same for the treated and untreated cells at each of the time intervals (data not shown). Incorporation into the major lipid classes of the HCG treated cells, with the exception of the cholesteryl ester fraction, was also the same or higher as that found in the control cells (**Fig. 1**). However, the cholesteryl ester fraction of the treated cells exhibited a substantially lower specific activity compared to the control within 4 hr and remained significantly lower throughout the incubation period (Fig. 1). These results suggest that the hormone either increased the hydrolysis or decreased the synthesis of cholesteryl esters but not of cholesterol. A reduction in the rate of acetate incorporation into the fatty acid moiety of cholesteryl esters of the treated cells was not ruled out. However, this was considered unlikely since the specific activities of the other lipid classes containing acyl groups were at least as high as the control values (Fig. 1).

As demonstrated in **Table 5,** HCG stimulated the activity of cholesterol ester hydrolase, a further indication that increased hydrolysis may have been responsible for the reduction in specific activity of the cholesteryl ester fraction after HCG stimulation. Although hydrolase activity was measured using cholesteryl linoleate as substrate, it is probable that the observed increase in enzymatic activity also occurred in cholesteryl esters containing other polyunsaturated fatty acids including arachidonate. Support for this is found in the compositional data in which a decrease in arachidonate was observed in the cholesteryl esters isolated from HCG-treated tumors (Table 4).

Confirmatory evidence was obtained in an experiment in which the tumors were pre-labeled with [^{14}C]arachidonate in vivo, after which the cells were exposed to HCG in vitro. As shown in **Fig. 2,** the gonadotropin treatment did not substantially alter, relative to control, the percentage of label retained in the triacylglycerol, phosphatidylcholine, and phosphatidylinositol fractions, but did cause a relative retention of label in the phosphatidylethanolamine fraction. In contrast, the cholesteryl esters from HCG-treated cells exhibited a loss, relative to control, of labeled arachidonate for about 10 hr.

TABLE 4. Fatty acid composition of the major lipid fractions of Leydig cell tumors and of tumors after prolonged HCG administration

Fatty Acid	Lipid Class[a]					
	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-inositol	Triacyl-glycerols	Cholesteryl Esters	
	Cont	*Cont*	*Cont*	*Cont*	*Cont (n = 4)*	*HCG (n = 3)*
16:0	24.1 (5.5)	15.6 (2.1)	8.0 (0.1)	18.2 (6.5)	14.1 (7.2)	21.9 (7.9)
18:0	18.9 (1.9)	17.0 (5.0)	37.1 (4.2)	9.5 (3.4)	8.2 (0.8)	12.0 (0.5)
18:1	24.2 (3.0)	15.3 (6.2)	11.2 (3.2)	32.3 (2.0)	26.9 (0.6)	35.4 (2.7)[b]
18:2	12.5 (3.1)	8.4 (5.8)	3.5 (1.6)	14.0 (4.7)	17.4 (4.2)	7.4 (1.6)[b]
18:3	1.0 (0.2)	1.4 (0.2)	0.4 (0.3)	3.8 (0.8)	3.6 (0.3)	4.9 (1.2)
20:3 (n-6)	3.2 (1.1)	6.3 (2.7)	5.1 (1.7)	2.4 (1.1)	trace	0.3 (0.3)
20:4	7.9 (2.2)	15.9 (2.3)	22.8 (2.6)	5.4 (2.5)	14.7 (4.5)	6.4 (4.6)[b]
22:4	1.0 (0.7)	3.0 (0.9)	1.6 (2.0)	2.6 (2.1)	3.9 (1.4)	2.7 (2.7)
22:5 (n-6)	trace	0.7 (0.1)	0.5 (0.4)	trace	trace	trace
22:5 (n-3)	trace	2.5 (0.5)	1.8 (2.3)	2.5 (0.8)	trace	1.8 (0.3)
22:6	2.5 (0.5)	6.5 (0.1)	6.0 (4.2)	2.9 (0.1)	6.0 (0.9)	2.2 (1.4)[b]

[a] The data are given as weight %, and standard deviations are in parentheses. In vivo studies as described in Table 1. Cont, control; HCG, hormone-treated animals. With the exception of the cholesteryl esters, the data are presented for the controls only since the values did not change significantly following HCG treatment (n = 2 for controls and n = 3 for HCG treated animals).

[b] Significantly different from control ($P < 0.05$).

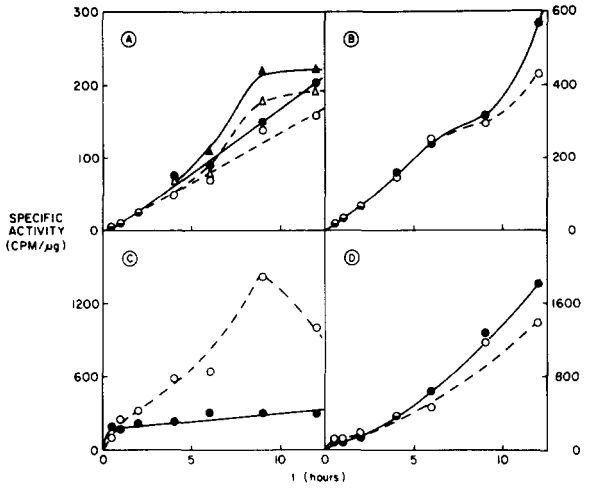

Fig. 1. Effect of HCG on the incorporation of [^{14}C]acetate into lipids of dispersed Leydig tumor cells. Cells were incubated with 1 μCi of [^{14}C]acetate in either non-supplemented media (open symbols) or media supplemented with 200 ng HCG (closed symbols) at 37°C with shaking under 95% O_2–5% CO_2. Each point represents the mean of four measurements, and standard errors were within ±10% of the means in all cases. A. triacylglycerol (triangles) and phosphatidylethanolamine (circles); B. phosphatidylcholine; C. cholesteryl ester; and D. cholesterol. Values for cholesteryl ester from cells in supplemented media were significantly different from control ($P < 0.05$) at 4, 6, 8, and 12 hr.

DISCUSSION

The results reported herein demonstrate that lipid metabolism of the M5480 Leydig cell tumor was affected by gonadotropins. Stimulation of the tumors with HCG led to the accumulation of phospholipids, principally phosphatidylcholine, and to altered metabolism of cholesteryl esters. The hormone-dependent increase in phospholipids is similar to the response previously observed in whole testis (6, 7) and parallels a proliferation of endoplasmic reticulum in Leydig cells (2). Thus, the accumulation of testicular phospholipid in response to gonadotropins may be a reflection of increased membrane biogenesis in the Leydig cells associated with steroid production.

The HCG-dependent effects on the metabolism of cholesteryl esters in the M5480 Leydig cell tumor may result from the 2- to 3-fold stimulation of cholesteryl ester hydrolase activity observed in this study. An analogous hormone-sensitive cholesteryl ester hydrolase activity has been shown to exist in ovarian tissue (20). Adrenal cortex also responds to hormones in a manner similar to Leydig cells and ovarian tissue by increasing mobilization of cholesteryl esters (3). Interestingly, cholesteryl esters containing arachidonate and other polyunsaturated fatty acids are

TABLE 5. Effect of HCG on cholesteryl ester hydrolase activity of dispersed Leydig tumor cells

Experiment[a]	Control[b]	HCG-Treated[b]	Fold-Stimulation[c]
1	0.35	1.01	2.9
2	0.30	1.15	3.8
3	0.13	0.28	2.2
4	0.13	0.22	1.7

[a] Dispersed cells were maintained in medium alone (control) or in medium supplemented with 200 ng HCG for 3 hr (HCG-Treated).
[b] Enzymic activity of homogenates in nmoles/hr/mg protein.
[c] Ratio of enzymic activity of HCG-treated cells to that of control cells.

preferentially utilized in response to hormone stimulation in Leydig cells (cf. Fig. 2) and in ovaries (21). Thus, in the steroidogenic tissues, cholesteryl esters may serve as precursors for prostaglandins as well as steroids. Preliminary investigations have shown that prostaglandins are present in the Leydig cell tumor[6], but it remains to be established whether or not HCG stimulates prostaglandin synthesis as a result of increased availability of arachidonate derived from cholesteryl esters.

Other polyunsaturated fatty acids such as 22:5 and 22:6 are only found in small amounts in the Leydig

[6] Albert, D. H., and A. R Whorton. Unpublished observations.

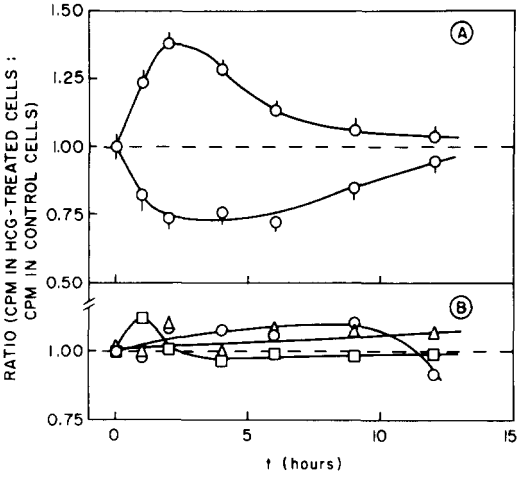

Fig. 2. Comparison of the retention of radioactivity from [^{14}C]-arachidonate in lipids of dispersed Leydig tumor cells with and without HCG present in the media. Cells, pre-labeled in vivo with [^{14}C]arachidonate, were incubated as described in the legend to Fig. 1. The values represent the mean of three determinations and are expressed as the ratio of the total counts in the HCG-treated cells to those present in the control. Standard errors were always within ±10% of the mean. A. phosphatidylethanolamine (○) and cholesteryl ester (♀). B. triacylglycerol (□); phosphatidylinositol (○); and phosphatidylcholine (△). Values for cholesteryl ester from treated cells were significantly different from control ($P < 0.05$) at 1–6 hr.

cell tumors, although they are present in high concentrations in whole testis (22). This is in agreement with recent studies which have established that these polyunsaturated fatty acids, which may be important to testicular function (23), are located primarily in the germinal or Sertoli cells of the testis (24).

In view of the known precursor role of cholesterol and cholesteryl esters for steroids, it is somewhat surprising that no decrease was observed in these fractions following HCG administration for 3 days. Our in vitro labeling studies showed that cholesteryl esters were metabolized more rapidly shortly after HCG stimulation. Others have demonstrated a reduction in the cholesteryl ester content of the Leydig cell tumor within 5 hr after an injection of HCG (25). Taken together, these results indicate that in vivo the tumor cells recover their cholesteryl ester content after an initial gonadotropin-induced depletion. This interpretation is consistent with observations in ovarian metabolism (26, 27). In light of recent studies which demonstrate that circulating high density lipoproteins are a significant source of cholesterol for the adrenal gland, ovary, and testes of the rat (28), the recovery of cholesteryl ester content of the tumor cells may reflect the role of plasma lipoproteins in maintaining total cholesterol levels in endocrine cells in vivo. Lastly, it must be pointed out that prolonged administration of HCG will, by virtue of receptor down-regulation (8, 29, 30), lead to temporary non-responsiveness of the Leydig cell tumors. Thus, it is likely that less than maximal steroid production is occurring on the second and third days of HCG injection.

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