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## 22. Enzymes in Steroid Synthesis

# REGULATION OF LOW DENSITY LIPOPROTEIN RECEPTOR AND CYTOCHROME P-450scc mRNA LEVELS IN HUMAN GRANULOSA CELLS

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**Summary**—hCG and 8-bromo-cAMP increase the levels of the mRNAs encoding the LDL receptor and cytochrome P-450scc in human granulosa cells. The increment in these mRNAs occurs rapidly after stimulation (within hours). Actin mRNA levels are not changed by hCG and 8-bromo-cAMP treatment. The tropic hormone effects on LDL receptor mRNA are observed even in the presence of 25-hydroxycholesterol and aminoglutethimide, which by themselves suppress LDL receptor mRNA. Actinomycin D blocks the hCG and 8-bromo-cAMP promoted augmentation of LDL receptor mRNA, suggesting that the tropic factors act to increase transcription of this gene. Treatment of cells with cycloheximide increases LDL receptor mRNA levels, and the stimulatory response to hCG and 8-bromo-cAMP is enhanced in the presence of cycloheximide. This indicates that tropic hormones act to increase LDL receptor mRNA levels without requiring synthesis of intermediary proteins and suggests that some short-lived protein may actually be reducing LDL receptors mRNA levels in these cells. We conclude that gonadotropins increase steroidogenesis in human granulosa cells, in part, by increasing expression of mRNAs encoding specific proteins involved in cholesterol metabolism.

## INTRODUCTION

Tropic hormones acutely regulate hormone synthesis by steroidogenic cells, as well as increase the long-term capacity for steroidogenesis [1-3]. In the short term, hormones acting via the intermediacy of cyclic AMP stimulate steroid synthesis by increasing delivery of cholesterol to the inner mitochondrial membrane where cleavage of the sterol side-chain is catalyzed by cytochrome P-450scc. The rapid movement of cholesterol to the inner mitochondrial membrane seems to require the action of a short-lived or "labile" protein whose synthesis or activity is increased by cyclic AMP [2]. Synthesis of enzymes involved in steroidogenesis is apparently not required for the acute response.

In the long term, tropic hormones augment cellular steroidogenic capacity at least in part by increasing the amounts of the components of the steroidogenic machinery, including lipoprotein receptors and the cholesterol side-chain cleavage system [2, 4]. Cyclic AMP also serves as the second messenger in this circumstance. The increased content of proteins involved in steroidogenesis arises from a specific tropic effect on the rate of synthesis of these proteins [2, 5]. In bovine adrenal cortex cells, activation of gene transcription has recently been shown to be one mechanism by which ACTH increases the synthesis of steroid hydroxylases, including cytochrome P-450scc [6]. The action of a "labile" protein, related or unrelated to the factor involved in the acute response, seems also to be required for the action of ACTH and cAMP on steroidogenic enzyme gene expression in the latter system.

The luteinization of granulosa cells during corpus luteum formation, as well as the conversion of the corpus luteum of the cycle into the corpus luteum of pregnancy, is characterized by a marked increase in the capacity of the ovarian cells to synthesize progesterone. In these situations the increase in synthesis of key proteins of the steroidogenic pathway is likely to be an important event in the mechanism of regulation of ovarian steroidogenesis. We have thus investigated whether hCG and its second messenger, cyclic AMP, stimulate human granulosa cells to synthesize two of these components, the LDL receptor and cytochrome P-450scc, by increasing the levels of their mRNAs. We also examined whether such increases are brought about in a co-ordinated fashion. The granulosa cells used in these studies were obtained from women participating in an *in vitro* fertilization/embryo transfer program. These women had been treated with human menopausal gonadotropins to induce follicular development and had received hCG (5000 IU) approximately 34 h prior to follicular aspiration. The experiments were conducted with cells cultured under serum-free conditions as we have previously reported [5, 7, 8]. We present in this report a summary of our findings.

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*Effects of hCG and 8-bromo-cAMP on LDL receptor and cytochrome P-450scc mRNA*

Nick-translated $^{32}$P-labeled cDNA specific for the human LDL receptor hybridized with a single human granulosa cell RNA species of approximately 5.3 kb, the size of the human LDL receptor mRNA [9]. "Northern" blot analysis revealed that treatment of granulosa cells with hCG at a concentration of 500 mIU/ml, a dose of hormone that maximally stimulates LDL receptor content and synthesis rates [5, 7, 8], produced a significant increase (2–4-fold) in LDL receptor mRNA within 6 h (Fig. 1). Treatment with the cAMP analog 8-bromo-cAMP (1.5 mM) also promoted a similar increase in the LDL receptor mRNA content (Fig. 1).

Granulosa cell RNA was also probed with a specific cDNA for human cytochrome P-450scc [10]. After 6 h of treatment with hCG or 8-bromo-cAMP a notable increase (3-fold) was seen in an RNA band of approximately 2.2 kb, the expected size of the P-450scc mRNA [10, 11] (Fig. 2). These findings confirm previously published studies demonstrating that tropic hormones which increase human granulosa cell progesterone secretion increase cytochrome P-450scc mRNA [11].

The effects of hCG and 8-bromo-cAMP on LDL receptor mRNA accumulation are relatively rapid in onset: appreciable increases in mRNA are seen after several hours, with elevated mRNA levels maintained for up to 24 h of exposure to hCG (Table 1). The time course of P-450scc mRNA induction appeared to be generally similar to that of the LDL receptor. An essentially similar time course was observed when 8-bromo-cAMP was used to stimulate the cells (unpublished observations). It is important to note that treatment with hCG or 8-bromo-cAMP did not affect expression of actin mRNA in our human granulosa cell system. Thus, the effects are specific for the LDL receptor and cytochrome P-450scc mRNAs and do not reflect an increase in total cellular mRNA content.

*Effects of 25-hydroxycholesterol and aminoglutethimide on LDL receptor mRNA levels and the response to hCG and 8-bromo-cAMP*

In both steroidogenic and nonsteroidogenic cells, cholesterol modulates LDL receptor expression through a negative feedback mechanism either directly or via a sterol precursor or metabolite [12]. Thus, when fibroblasts are cultured in the presence of 25-hydroxycholesterol, which readily enters cultured cells, LDL receptor mRNA levels are suppressed and LDL receptor synthesis dramatically declines [9]. When we cultured granulosa cells in the presence of 25-hydroxycholesterol and aminoglutethimide (an inhibitor of cholesterol side-chain cleavage which prevents metabolism of endogenous as well as exogenous sterols to steroid hormones [13]), within 6 h a decline in LDL receptor mRNA was detectable by hybridization analysis (Table 2). These findings would indicate that the sterol negative feedback system of LDL receptor regulation is operative in human granulosa cells.

In contrast to the effects of sterol alone, either hCG or 8-bromo-cAMP stimulated LDL receptor mRNA levels despite the presence of 25-hydroxycholesterol and aminoglutethimide (Table 2). Thus, a cAMP-mediated mechanism can stimulate LDL receptor mRNA expression in the presence of a sterol negative-feedback signal. These data rule out the possibility that tropic hormones increase LDL receptor expression primarily as a result of relief of sterol negative feedback subsequent to depletion of cellular sterol stores via accelerated steroidogenesis.

*Effects of actinomycin D on hCG stimulation of LDL receptor mRNA*

An inhibitor of RNA synthesis, actinomycin D, completely blocked the effects of hCG on LDL receptor mRNA (Fig. 3). These findings demonstrate that on-going RNA synthesis is required for tropic hormones to increase the cellular contents of LDL receptor mRNA, as has been reported for cytochrome P450scc mRNA [6]. While these

Table 1. Time course of the increase in LDL receptor and cytochrome P-450scc mRNA levels following treatment of human granulosa cells with hCG

| Time of exposure to hCG (h) | Relative mRNA level | |
|---|---|---|
| | LDL receptor | Cytochrome P-450scc |
| Control | 1.0 | 1.0 |
| 2 | 2.3 | 2.3 |
| 4 | 2.1 | 2.0 |
| 6 | 2.6 | 3.0 |
| 24 | 2.7 | 4.7 |

Human granulosa cells were cultured as described in Fig. 1 and exposed to control medium, or medium containing hCG (500 mIU/ml) for 2, 4, 6, or 24 h. Total cellular RNA (5 μg) was analyzed for LDL receptor and cytochrome P-450scc mRNA content as described earlier. The results are presented as area of the densitometric scan of the LDL receptor and cytochrome P-450scc RNA bands relative to controls from untreated cells harvested at the same time as treated cells.

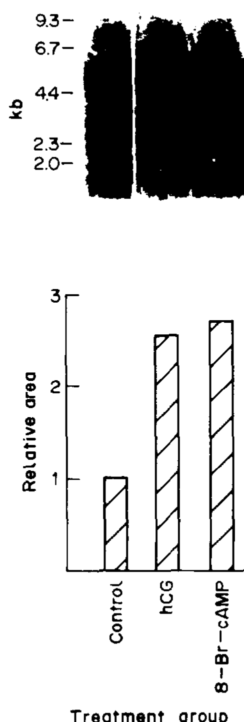


Fig. 1. Effect of hCG and 8-bromo-cAMP on LDL receptor mRNA levels in cultured human granulosa cells. Granulosa cells were aspirated from preovulatory follicles of women undergoing ovum retrieval for *in vitro* fertilization, separated from red blood cells by centrifugation through a Ficoll gradient, and were plated in 35-mm culture dishes as previously described [8]. Cells were cultured for 48 h in Dulbecco's modified Eagle medium containing 20% human male serum, followed by 48 h in serum-free medium. Experiments were begun on the 5th day of culture. Cells were exposed for 6 h to control medium, 500 mIU hCG, or 1.5 mM 8-bromo-cAMP. After the 6-h treatment interval cells were scraped from dishes and total cell RNA was obtained by the guanidine isothiocyanate–cesium chloride method [18]. Cells from 2–3 experiments were pooled for RNA blot hybridizations. RNA was denatured in 1 M glyoxal, 0.01 M $NaH_2PO_4$, and equal amounts (5 μg) from each treatment group were electrophoresed in 1% agarose gels and transferred to nitrocellulose paper. A *Hind* III digest of lambda DNA was also electrophoresed in these gels for nucleic acid size markers. A 1919 bp cDNA insert for the human LDL receptor mRNA was isolated from a plasmid (pHHI-LDLR1) generously provided by Dr David Russell of the University of Texas Health Science Center, Dallas, TX. Filters were hybridized with $^{32}$P-nick-translated LDL receptor cDNA (sp. act. $0.5\text{–}3 \times 10^8$ cpm/μg) according to Berent *et al.* [19]. Following hybridization, filters were washed and briefly blotted, wrapped in plastic and placed with X-ray film for 16 h. Autoradiograms were densitometrically scanned and integrated, and the areas under the tracings were expressed relative to control bands, which were set to 1.0. These data appear as histogram bars under the corresponding autoradiographic band.

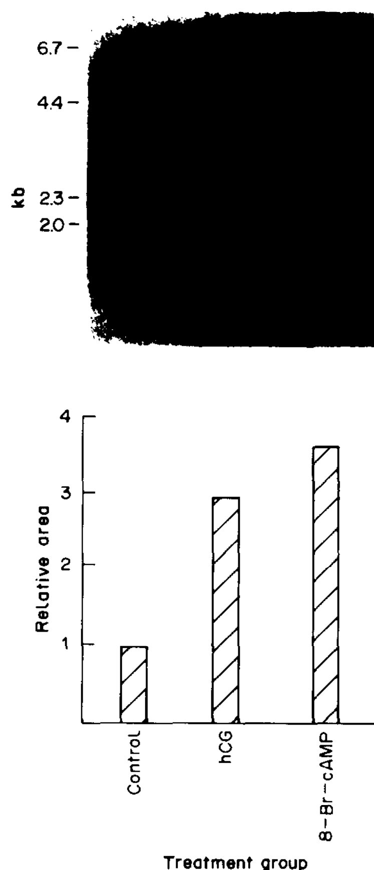


Fig. 2. Effect of hCG and 8-bromo-cAMP on cytochrome P-450scc mRNA levels in cultured human granulosa cells. Experiments were conducted and total cellular RNA was prepared as described in Fig. 1. Granulosa cell RNA (5 μg) from each treatment group was electrophoresed, transferred to nitrocellulose filters, and hybridized with $^{32}$P-labeled cytochrome P-450scc cDNA (pUC-71) [11]. The relative areas of the densitometric scans of the cytochrome P-450scc mRNA band appear below each band.

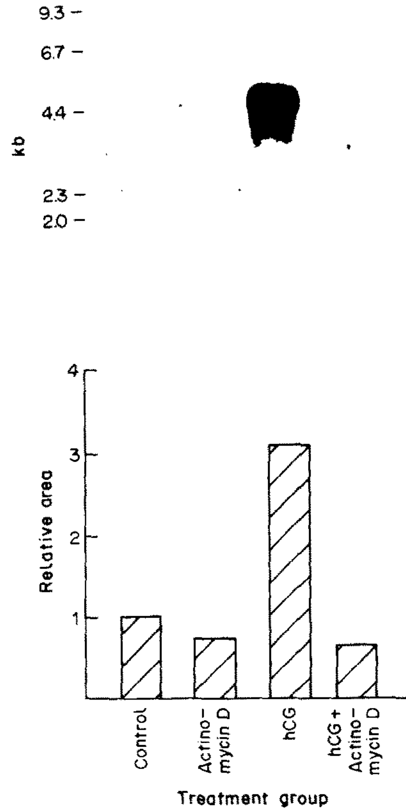


Fig. 3. Effect of actinomycin D on hCG-stimulated increases in LDL receptor mRNA levels in human granulosa cells. Cultured cells were exposed to 500 mIU/ml hCG, 2 μg/ml actinomycin D, or a combination of both for 4 h. Total cellular RNA was prepared and analyzed for LDL receptor mRNA as in Fig. 1. The relative areas of the densitometric scans are presented with the control band equal to 1.0.

Table 2. Effects of 25-hydroxycholesterol and aminoglutethimide (25-OHC + AG) on basal and hCG- or 8-bromo-cAMP-stimulated LDL receptor mRNA levels in cultured human granulosa cells

| Treatment group | Control | 25-OHC + AG | hCG | hCG + 25-OHC + AG | 8-Br-cAMP | 8-Br-cAMP + 25-OHC + AG |
|---|---|---|---|---|---|---|
| Relative area | 1.0 | 0.6 | 3.6 | 2.7 | 2.9 | 2.5 |

Human granulosa cells were cultured as described in Fig. 1. Cells were exposed for 6 h to control medium, 500 mIU/ml hCG, 10 μg/ml 25-hydroxycholesterol (25-OHC) and 100 μg/ml aminoglutethimide (AG) or a combination of hCG and 25-OHC plus AG. After the 6-h treatment interval cells were harvested and RNA was prepared and analyzed as described in Figs 1 and 2. Autoradiograms were densitometrically scanned and the areas under the tracings were expressed relative to control bands, which were set to 1.0.

observations do not prove conclusively that the increase in LDL receptor mRNA is a consequence of increased gene transcription, they are fully consistent with this notion. Moreover, they argue against the possibility that the tropic hormones act to increase mRNA levels by stabilizing pre-formed mRNA.

*Effects of cycloheximide on hCG stimulation of LDL receptor mRNA*

A cycloheximide-sensitive factor ("labile" protein) is believed to mediate the cyclic AMP-induced acute increase in steroidogenesis [1]. Moreover, there is recent evidence to indicate that a cycloheximide-sensitive factor is required for hormones which activate adenylate cyclase to increase transcription of steroid hydroxylase genes in bovine adrenal cells [6]. To determine if a cycloheximide-sensitive factor is required for the hCG-induced increase in LDL receptor mRNA in human granulosa cells, cells were pretreated for 30 min with 20 μg/ml cycloheximide and then exposed to control medium or hCG for 4 h. LDL receptor mRNA levels were determined by Northern blot analysis. The cycloheximide treatment reduced protein synthesis by >97% within 30 min, as assessed by monitoring incorporation of [$^{35}$S]methionine into trichloroacetic acid-precipitable radioactivity. In these experiments, we observed a 2-fold increase in LDL receptor mRNA in cycloheximide-treated cells compared to controls, and an augmentation of the hCG- and 8-bromo-cAMP-induced increase in LDL receptor mRNA levels (Table 3). These observations would suggest that some factor dependent on protein synthesis may act to reduce cellular LDL receptor mRNA content, and that the acute effects of tropic stimulation on LDL receptor gene expression are not mediated by a "labile" protein. Indeed, the response to tropic stimulation was augmented when protein synthesis is inhibited. This is in marked contrast to the reported findings on ACTH induction of steroid hydroxylases in bovine adrenal cells [6].

## DISCUSSION

The present studies were conducted to gain a fuller understanding of the molecular mechanisms of regulation of steroidogenesis in human granulosa cells. The observations reviewed in this report demonstrate that tropic hormones can promote rapid increases in the cellular contents of mRNA encoding two key proteins of the steroidogenic machinery, the LDL receptor and cytochrome P-450scc. The number of LDL receptors expressed by the granulosa cells will determine their capacity to acquire LDL-carried cholesterol, which is a primary substrate for steroidogenesis [14, 15]. The cytochrome P-450scc catalyzes the first committed reaction in steroid biosynthesis and the amount of this enzyme will be a major determinant of the steroidogenic capacity of the granulosa cells [2, 13]. Our previous studies have demonstrated that hCG and 8-bromo-cAMP increase progesterone secretion by human granulosa cells at least in part by increasing the synthesis and thus the content of LDL receptors [5, 7, 8]. Similar increases in cytochrome P-450scc synthesis and enzyme content have also been found (unpublished observations). Our earlier studies on LDL receptor

Table 3. Effects of cycloheximide on hCG-stimulated LDL receptor mRNA levels in cultured human granulosa cells

| Treatment group | Control | CHX | hCG | hCG + CHX |
|---|---|---|---|---|
| Relative area | 1.0 | 2.6 | 2.3 | 6.8 |

Human granulosa cells were cultured as described in Fig. 1 and exposed to control medium, medium containing 500 mIU/ml hCG, 20 μg/ml cycloheximide (CHX) or hCG plus CHX. CHX was added before other treatments were initiated. RNA samples were prepared and 5 μg of total cellular RNA were analyzed for LDL receptor mRNA content as described earlier. The results are presented as area of the densitometric scan relative to control RNA from untreated cells harvested at the same time as treated cells.

synthesis revealed that hCG and 8-bromo-cAMP promote an increase in rates of receptor synthesis within 2.5 h, and that a maximal increase in synthesis rates is found after 5–7 h of tropic hormone treatment. Measurement of LDL receptor content by immunoblotting and LDL receptor activity as assessed by binding, internalization and degradation of [$^{125}$I]-LDL have shown increases detectable by 6 h, with progressive accumulation of receptor over the subsequent 18 h. The finding that both hCG and 8-bromo-cAMP stimulate an increase in LDL receptor mRNA by 2 h would suggest that increased LDL receptor synthesis is primarily the consequence of increased receptor mRNA levels.

The stimulation of cytochrome P-450scc mRNA accumulation in response to hCG and 8-bromo-cAMP seems to take place with a relatively similar time course. That the increase in both LDL receptor and cytochrome P-450scc mRNAs was specific was demonstrated by the fact that tropic stimulation did not affect actin mRNA levels. The increase in LDL receptor mRNA levels requires on-going RNA synthesis, as actinomycin D completely blocked the tropic hormone effect. Thus, a control mechanism presumably at a transcriptional level seems to be responsible for the increase in expression of LDL receptor mRNA. Recent studies employing nuclear run-off assays have shown that ACTH-mediated increases in steroid hydroxylase levels, including cytochrome P-450scc, involves increased gene transcription [6]. Therefore, transcriptional activation may be a common aspect of hormonal regulation of the expression of genes involved in steroidogenesis.

The above-mentioned studies of ACTH regulation of steroid hydroxylases in bovine adrenal cells have also revealed that the protein synthesis inhibitor cycloheximide blocks the increase in transcription [6]. This has led to the postulation that a "labile" protein mediates the action of tropic hormones and their cAMP second messenger on the genome. In contrast to these findings, we have observed that cycloheximide treatment alone increases LDL receptor mRNA levels, and augments the response to hCG and 8-bromo-cAMP. These findings would seem to rule out a role for a "labile" factor in the mRNA response to tropic stimulation. Indeed, these findings suggest that some rapidly-turning-over factor acts to reduce LDL receptor mRNA expression in human granulosa cells. Further studies are required to determine if this is a finding unique to control of the LDL receptor gene and not extended to cytochrome P-450sdc expression.

If we conclude from our available results that tropic hormones enhance LDL receptor expression via a cyclic AMP-mediated increase in LDL receptor gene transcription, we would expect to find regulatory elements in the LDL receptor gene which are sensitive to cAMP or a cAMP-associated factor. Inspection of the 5′ flanking region of the LDL receptor gene reveals the presence of sequences at bases −101 to −94 and −178 to −171, which are similar to a sequence (TGACOTCA) in the 5′ flank (−123 to −116) of the human alpha gonadotropin subunit gene that is known to be part of the 5′ flanking sequence which is required for cAMP responsiveness [16, 17]. Future studies will be directed at defining such regulatory regions of the LDL receptor gene and determining if other genes which encode proteins required for steroidogenesis also contain such sequences. The existence of common regulatory sequences could explain how tropic hormones promote co-ordinated expression of several genes.

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