Human Chorionic Gonadotropin and 8-Bromo Cyclic Adenosine Monophosphate Promote an Acute Increase in Cytochrome P450scc and Adrenodoxin Messenger RNAs in Cultured Human Granulosa Cells by a Cycloheximide-insensitive Mechanism

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Abstract

Treatment of human granulosa cells with human chorionic gonadotropin (hCG) or an analogue of its second messenger, cyclic AMP (cAMP), promotes a rapid accumulation of the messenger RNAs (mRNAs) for cytochrome P450 side-chain cleavage (scc) and adrenodoxin. A twofold increase in the cellular content of these mRNAs was observed within 4 h of exposure to 8-bromo-cAMP, and was maintained for up to 48 h. Inhibition of protein synthesis by cycloheximide did not prevent the hCG- or 8-bromo-cAMP-stimulated accumulation of either cytochrome P450scc or adrenodoxin mRNAs. We conclude that human granulosa cells respond rapidly to hCG and cAMP analogues with a coordinate increase in levels of the mRNAs encoding two key proteins of the steroidogenic machinery, and that this stimulation does not require synthesis of a protein intermediate.

Introduction

The conversion of cholesterol to pregnenolone is the rate-limiting step and principal site of hormonal regulation of steroidogenesis. This reaction is catalyzed by the cholesterol side-chain cleavage (scc)[1] complex, consisting of a specific cytochrome P450, termed P450scc, and two electron transport proteins, adrenodoxin and adrenodoxin reductase (1).

Gonadotropic hormones can augment the capacity of gonadal cells to synthesize steroid hormones by increasing the cellular contents of the cholesterol scc system by a specific tropic effect on the rate of synthesis of these proteins (2–4). In bovine adrenal cortex cells, activation of gene transcription is the principal mechanism by which ACTH increases the synthesis of steroidogenic enzymes, such as P450scc and adrenodoxin (5). A "labile" protein intermediate has been suggested to be required for the action of ACTH and cAMP on gene expression in this system, since cycloheximide, an inhibitor of protein synthesis, blocks the increase in accumulation of messenger RNAs (mRNAs) encoding these proteins (5).

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 ovarian cells to synthesize progesterone. In these situations increased synthesis of steroidogenic enzymes is probably an important event. We have shown that human chorionic gonadotropin (hCG) and its second messenger, cyclic AMP (cAMP), stimulate human granulosa cells to synthesize adrenodoxin and P450scc by increasing the levels of their respective mRNAs in a coordinate fashion. These actions of hCG and cAMP analogues appear to be independent of ongoing protein synthesis. This finding is in direct contrast with reports of an obligatory labile protein factor in the hormonal induction of steroidogenesis in bovine adrenocortical cells.

Methods

Cell culture and RNA isolation. Granulosa cells were aspirated from preovulatory follicles of women undergoing ovum retrieval for in vitro fertilization, and were cultured as previously described (6). Granulosa cells were separated from erythrocytes by centrifugation through lymphocyte separation medium (Litton Bionetics, Kensington, MD), plated in Dulbecco's minimum essential medium containing 25 mM glucose, 4 mM L-glutamine, 50 μg/ml gentamicin, 25 mM Hepes, and 20% vol/vol human male serum, and cultured at 37°C in humidified air. After 48 h, serum-supplemented medium was replaced with serum-free medium for an additional 48 h. Subsequently, this culture medium was replaced with media containing the treatments described below. After the prescribed treatment interval for a given experiment, cells were harvested with a plastic spatula, collected by centrifugation at 4°C, and redispersed in 200 μl of a solution containing 4 M guanidine isothiocyanate, 0.1 M 2-mercaptoethanol, 0.5% *N*-laurylsarkosine, and 5 mM sodium citrate, and frozen at −70°C. Cells from two to three experiments were usually pooled to obtain RNA for blot hybridizations. Total cellular RNA was obtained by centrifugation through 5.7 M CsCl (7).

The treatments selected for these experiments were based on our previous studies of human granulosa cells (6, 8). hCG (500 mIU/ml) or 8-bromo-cAMP (1.5 mM) were used at concentrations that produce maximal stimulation of steroidogenesis and low density lipoprotein (LDL) receptor synthesis. Cycloheximide (20 μg/ml) was used at a concentration that decreased protein synthesis by 97% within 30 min

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1. *Abbreviations used in this paper:* hCG, human chorionic gonadotropin; scc, side-chain cleavage; 1× SSC, 150 mM sodium chloride, 15 mM sodium citrate.

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of treatment, as assessed by incorporation of [^{35}S]methionine into trichloroacetic acid–precipitable radioactivity.

RNA electrophoresis and blot hybridizations. RNA was denatured in morpholinopropanesulfonic acid–formaldehyde, electrophoresed in 1% agarose gels, and transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, NH) by standard procedures (7). A Hind III digest of bacteriophage lambda DNA was also electrophoresed in these gels as nucleic acid size markers and transferred to Nytran along with the RNA samples.

Hybridizations with nick-translated complementary DNA (cDNA) probe were conducted according to Berent et al. (9). Dried Nytran filters were first moistened in 6× SSC (1× SCC is 150 mM sodium chloride, 15 mM sodium citrate), then placed in plastic bags with 5–8 ml prehybridization solution (50% formamide, 5× SSC, 0.1% each of Ficoll, polyvinylpyrollidone, bovine serum albumin, sodium dodecyl sulfate (SDS), and 250 μg/ml denatured salmon sperm DNA) and incubated in 42°C for 4 h. This solution was replaced with fresh solution containing nick-translated cDNA (2–5 × 10^6 cpm/ml) for P450scc or adrenodoxin and nick-translated lambda DNA (1–5 × 10^6 cpm per hybridization). Hybridizations were for 18–22 h; filters were then washed twice for 30 min at room temperature in 2× SCC, 0.1% SDS, followed by two 45-min washes at 65°C in 1× SSC, 0.1% SDS. After washing, the filters were placed with x-ray film (Kodak X-Omat) for autoradiography at −20°C for 2–96 h. Autoradiograms of mRNA bands were scanned with a densitometer (Kontes/Martin, Vineland, NJ) coupled with a 3390A integrator (Hewlett-Packard Co., Palo Alto, CA). The areas under densitometric tracings were normalized relative to control bands, which were set to a value of 1.0.

Statistical analysis. Analysis of variance was conducted utilizing a randomized complete block design on data obtained from densitometric tracings of autoradiograms. Significant treatment effects were further partitioned by orthogonal contrasts (10).

Cytochrome P450scc and adrenodoxin cDNAs. Plasmid pUC71 containing a 1,687-basepair cDNA insert for the human cytochrome P450scc mRNA has been previously described (11). Plasmid phadx-6 contains a full length human adrenodoxin cDNA clone prepared from our human adrenal cDNA library (12) and is described elsewhere (13). The cDNA inserts were removed from vectors with Eco RI and isolated from low-melting-point agarose gels for nick-translation. cDNAs were labeled to a specific activity of 1.2–3 × 10^8 cpm/μg with a nick-translation kit from Bethesda Research Laboratories (Gaithersburg, MD).

Reagents and supplies. Ficoll, polyvinylpyrollidone, bovine serum albumin, cycloheximide, *N*-lauryl sarkosine, and 8-bromo–cAMP were purchased from Sigma Chemical Co., (St. Louis, MO); [^{32}P]dCTP (specific activity > 3,000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). All other reagents for mRNA analyses were purchased from Bethesda Research Laboratories. hCG (CR-121) was provided by the National Pituitary Agency.

Results

Fig. 1 shows representative autoradiograms of granulosa cell RNA hybridized with ^{32}P-labeled cDNA for P450scc (Fig. 1 *A*) and adrenodoxin (Fig. 1 *B*). The cytochrome P450scc probe hybridized to a single RNA species of ~ 2.2 kilobase (kb), consistent with previous findings (11, 12). In this experiment, treatment of cells with hCG or 8-bromo–cAMP for 6 h increased levels of this mRNA by threefold. The human adrenodoxin cDNA hybridized with a predominant mRNA band of 1.6 kb and minor RNA bands of 1.4, and 1.1 kb (Fig. 1, arrows). This hybridization pattern is consistent with that obtained when bovine or human adrenal RNAs are probed with bovine or human adrenodoxin cDNAs, respectively (13, 14). hCG and 8-bromo–cAMP treatment for 6 h also increased granulosa cell adrenodoxin mRNA contents by approximately

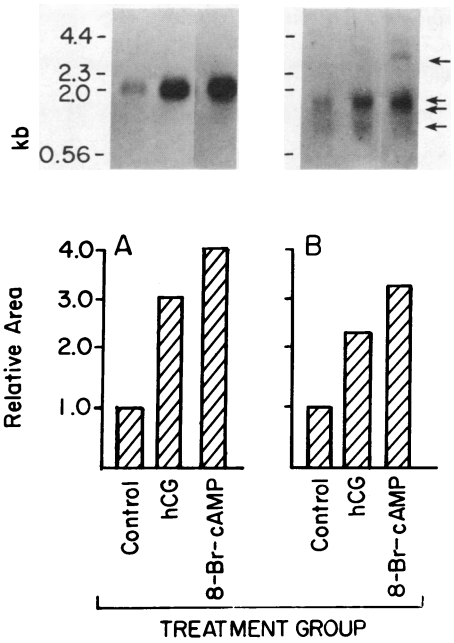

Figure 1. Autoradiograms of hybridization with P450scc (*A*) or adrenodoxin (*B*) cDNAs to RNA from cultured human granulosa cells. Cells were exposed for 6 h to control medium, 500 mIU/ml hCG, or 1.5 mM 8-bromo–cAMP. After treatment, cells were scraped from dishes and total cell RNA was prepared. Denatured RNA (5 μg) from each treatment group was electrophoresed in 1% agarose gels and transferred to nitrocellulose paper. Filters were hybridized with ^{32}P-labeled human adrenodoxin or P450scc cDNAs. 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.

twofold. The 1.6- and 1.4-kb mRNA bands are not distinguishable from one another in the autoradiogram shown. An apparent precursor band of ~ 3 kb, which may represent an

Table I. Time Course of Adrenodoxin and P450scc mRNA Abundances after Treatment of Human Granulosa Cells with hCG

Time of exposure to hCG	Relative mRNA abundance	
	Cytochrome P450scc	Adrenodoxin
h		
Control	1.0	1.0
1	1.2±0.1	1.1
2	1.7±0.3	1.6
4	2.1±0.1	3.1
6	2.8±0.3	8.1

Human granulosa cells were cultured as described in Fig. 1 and exposed to control medium, or medium containing hCG (500 mIU/ml) for 1, 2, 4, or 6 h. 5 μg of total RNA was analyzed for adrenodoxin and cytochrome P-450scc mRNA content as described earlier. The results are presented as area of the densitometric scan of the adrenodoxin and P450scc RNA bands relative to controls from untreated cells harvested at the same time as treated cells. Values presented are means±SE of three separate experiments for P450scc mRNA and the mean of two experiments for adrenodoxin mRNA.

Table II. Effect of Prolonged Exposure of Human Granulosa Cells to hCG or 8-Bromo-cAMP on P450scc and Adrenodoxin mRNA Abundances

Treatment group	Time of exposure	Relative mRNA abundances	
		P450scc	Adrenodoxin
	h		
Control	6	1.0	1.0
8-bromo-cAMP	6	2.0	2.2
Control	24	1.0	1.0
hCG	24	2.7	1.7
8-bromo-cAMP	24	2.2	2.8
Control	48	1.0	1.0
hCG	48	2.6	1.9
8-bromo-cAMP	48	2.5	2.9

Human granulosa cells were cultured as described in Fig. 1 and exposed to control medium or medium containing hCG (500 mIU/ml) for 24–48 h, or 8-bromo-cAMP (1.5 mM) for 6, 24, or 48 h. RNA samples were prepared and 5 μg of total RNA was analyzed as described earlier. The results are presented as the relative area of the densitometric scan of the P450scc and adrenodoxin mRNA bands relative to the control bands at each time point from a single experiment.

mRNA precursor species, as others have reported (5, 14), is also apparent in this autoradiogram.

The effects of hCG and 8-bromo-cAMP on accumulation of mRNAs for both P450scc and adrenodoxin are relatively rapid in onset: the earliest appreciable increases were seen within 2 h of exposure to hCG (Table I). Similar time courses were observed with 8-bromo-cAMP stimulation. The elevated levels of these mRNAs were maintained during 48 h of treatment (Table II). Hybridization of the granulosa cell RNA samples to a cDNA for actin demonstrated that hCG and 8-bromo-cAMP do not affect actin mRNA levels during these times of exposure. Thus, the effects of hCG and 8-bromo-cAMP are specific for P450scc and adrenodoxin mRNAs, and do not reflect a general increase in cellular mRNA content.

Recent reports have suggested that a cycloheximide-sensitive factor is required for the ACTH- or cAMP-stimulated increase in transcription of steroid hydroxylase genes, including cytochrome P450scc and adrenodoxin, in bovine adrenal cells (5). To determine if a cycloheximide-sensitive mechanism is also operative in the hCG- and 8-bromo-cAMP–induced increase in these mRNAs in human granulosa cells, some cultures were pretreated for 30 min with 20 μg/ml cycloheximide and then exposed to control medium, hCG, or 8-bromo-cAMP for 6 h. The cellular content of P450scc and adrenodoxin mRNAs were determined by Northern blot analysis. Under these conditions cycloheximide reduced protein synthesis by < 97% within 30 min, as assessed by monitoring incorporation of [^{35}S]methionine into trichloroacetic acid–precipitable radioactivity. Treatment with hCG or 8-bromo-cAMP significantly increased cytochrome P450scc and adrenodoxin mRNAs from 2.0- to 3.6-fold during the 6-h exposure period (Table III). Treatment with cycloheximide for this period did not change the amount of P450scc mRNA. Furthermore, in contradistinction to the inhibition suggested by the bovine adrenal experiments, cycloheximide tended to increase the amount of adrenodoxin mRNA, although the increase was not statistically significant. Additionally, cycloheximide did not prevent the stimulatory effects of either hCG or 8-bromo-cAMP on cytochrome P450scc mRNA. The combination of the protein synthesis inhibitor and hCG or 8-bromo-cAMP resulted in increases in adrenodoxin mRNA that were not significantly different from those for tropic agent alone.

Discussion

Previous studies showed that prolonged (8–48 h) tropic stimulation increases P450scc mRNA in granulosa cells that had been cultured 8–12 d (15). By contrast, the present study used cells cultured for only 4 d before tropic stimulation. Since the hormonal treatments of the donor women were the same in both studies, this difference in culture conditions probably accounts for the different kinetics of the P450scc mRNA response in the two studies. Thus, cells preincubated for 4 d responded maximally within 4 h of tropic stimulation, while cells preincubated for 8–12 d responded more slowly, reaching maximal values of P450scc mRNA after 36 h of tropic stimulation (15). Adrenodoxin mRNA was not studied in the earlier report (15), but under the culture conditions used here the kinetics of its response to tropic stimuli were similar to the P450scc mRNA, indicating coordinate regulation. hCG and 8-bromo-cAMP also rapidly increase LDL receptor mRNA levels in the cultured granulosa cells (14). However, neither 8-bromo-cAMP nor hCG affected the abundance of actin mRNA. Thus, the changes in mRNA are specific to the gene transcripts encoding proteins involved in steroidogenesis.

Table III. Effects of Cycloheximide on Basal and hCG- or 8-bromo-cAMP–Stimulated Adrenodoxin and P450scc mRNA in Cultured Human Granulosa Cells

Treatment group	Relative mRNA abundances					
	Control	CHX	hCG	hCG + CHX	8-br-cAMP	8-br-cAMP + CHX
Adrenodoxin	1.0*	1.7±0.2*‡	2.1±0.1§	3.7±1.2§	3.6±0.8§	3.6±0.4§
P450scc	1.0‖	1.0±0.1‖	2.0±0.5¶	2.5±0.2¶	2.2±0.2¶	2.5±0.7¶

Ganulosa cells were cultured as described in Fig. 1. Cells were then exposed to 20 μg/ml cycloheximide (CHX) for 30 min where indicated, after which hCG or 8-bromo(br)-cAMP was added to some cultures. Incubations were continued for 6 h, after which cells were harvested and RNA was prepared. 5 μg of total cell RNA was analyzed. The means±SE are presented from five experiments measuring adrenodoxin mRNA, and three experiments measuring P450scc mRNA. Values without a common superscript are significantly different within each mRNA. *§ $P < 0.01$. *‡§ $P < 0.05$. ‖¶ $P < 0.025$.

Nuclear run-off assays indicate that the ACTH-mediated increases in P450scc and adrenodoxin mRNAs in bovine adrenocortical cells are principally due to increased gene transcription (5). Since both ACTH and gonadotropins stimulate cholesterol scc by using cAMP as a second messenger, it is likely that the increases in P450scc and adrenodoxin mRNAs in granulosa cells reported here are also due to increased transcription of those genes. However, an effect on mRNA half-life has not been ruled out.

The studies in bovine adrenal cells have also revealed that cycloheximide, an inhibitor of protein synthesis, blocks this increase in transcription (5, 16). This suggested that a labile protein mediates the action of cAMP on the genome. In contrast to these findings, we have found that cycloheximide treatment does not attenuate the increase in adrenodoxin or cytochrome P450scc mRNA induced by hCG or cAMP. Moreover, cycloheximide accentuates the stimulatory effects of hCG and 8-bromo-cAMP on granulosa cell LDL receptor mRNA levels (14). These findings argue strongly against an obligatory role for a labile protein mediating the mRNA response to tropic stimulation of human granulosa cells cultured under our conditions. Further studies are needed to determine if these differences are species specific (cattle vs. man), cell specific (adrenal vs. granulosa), or are due to subtle changes induced by varying culture conditions. Nevertheless, our findings suggest that labile protein intermediates are not a general feature in the intracellular regulation of genes encoding the steroidogenic machinery.

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