8-Bromoadenosine Cyclic 3′,5′-Phosphate Rapidly Increases 3-Hydoxy-3-methylglutaryl Coenzyme A Reductase mRNA in Human Granulosa Cells: Role of Cellular Sterol Balance in Controlling the Response to Tropic Stimulation†

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ABSTRACT: Exposure of cultured human granulosa cells to 8-bromoadenosine cyclic 3′,5′-phosphate (8-bromo-cAMP) resulted in a rapid increase in the content of the mRNA for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in the de novo synthesis of cholesterol. HMG-CoA reductase mRNA levels increased within 2 h of stimulation and remained elevated for at least 6 h. Treatment of granulosa cells with 25-hydroxycholesterol, a soluble cholesterol analogue, in combination with aminoglutethimide to block conversion of cellular sterols to pregnenolone, resulted in suppression of HMG-CoA reductase mRNA. When cells were stimulated with 8-bromo-cAMP in the presence of 25-hydroxycholesterol and aminoglutethimide, the increase in HMG-CoA reductase mRNA provoked by the tropic agent was markedly attenuated. This indicates that 8-bromo-cAMP raises HMG-CoA reductase mRNA levels indirectly by accelerating steroidogenesis and depleting cellular sterol pools, thus relieving sterol-mediated negative feedback of HMG-CoA reductase gene expression. 25-Hydroxycholesterol in the presence of aminoglutethimide suppressed low-density lipoprotein (LDL) receptor mRNA, but 8-bromo-cAMP effected a significant stimulation of LDL receptor mRNA levels when added with hydroxysterol and aminoglutethimide. These findings reveal differential regulation of HMG-CoA reductase and LDL receptor mRNAs in the presence of sterol negative feedback.

The formation of the corpus luteum from the preovulatory ovarian follicle following ovulation is characterized by a marked increase in the capacity for steroidogenesis in the luteinizing cells. This is the result of the ovulatory surge of LH[1] which stimulates increased granulosa cell synthesis of components of the cholesterol side chain cleavage complex (Rodgers et al., 1986, 1987b; Waterman & Simpson, 1985) and the low-density lipoprotein (LDL) receptor (Golos et al., 1986; Gwynne & Strauss, 1982). The increase in LDL receptor facilitates acquisition of lipoprotein cholesterol as steroidogenic substrate. Another mechanism by which luteinizing cells can obtain cholesterol for synthesis of steroids is through an increased ability to synthesize cholesterol from acetyl coenzyme A (Kovanen et al., 1978; Schuler et al., 1981; Savion et al., 1982). The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the rate-limiting step in the de novo synthesis of cholesterol. In most cells, regulation of cholesterol levels is mediated by the negative feedback of a cellular sterol pool on the expression of the HMG-CoA reductase and LDL receptor genes (Brown & Goldstein, 1984). Thus, when cellular sterol pools are replete, transcription of these genes is suppressed. Depletion of cellular sterol leads to an increase in transcription of these genes (Sudhof et al., 1987; Osborne et al., 1985).

We have previously demonstrated that the expression of the LDL receptor gene in cultured human granulosa cells can be regulated by human chorionic gonadotropin (hCG), via the intermediacy of cAMP, by two apparently distinct mechanisms: relief of sterol negative feedback and a cAMP-mediated mechanism which can overide sterol feedback to some extent such that LDL receptors are increased even in the presence of a potent suppressive sterol, 25-hydroxycholesterol (Golos & Strauss, 1985, 1987). The regulation of HMG-CoA reductase activity and synthesis in rat ovaries and cultured bovine granulosa and adrenocortical cells by tropic hormones has been reported to be mediated by alterations in cellular sterol balance subsequent to tropic stimulation (Schuler et al., 1981; Rainey et al., 1986; Rodgers et al., 1987a). We wished to learn if HMG-CoA reductase is controlled in a similar fashion in cells of the human ovary and whether the control mechanisms operate to regulate the level of the enzyme's mRNA. Moreover, we wanted to determine if there is coordinate regulation of HMG-CoA reductase and LDL receptor mRNA expression in the cultured human granulosa cells.

Experimental Procedures

Materials

8-Bromo-cAMP was purchased from Sigma (St. Louis, MO); [^{32}P]dCTP (sp act. >3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). 25-Hydroxycholesterol was obtained from Steraloids (Wilton, NH).

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[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoproteins; hCG, human chorionic gonadotropin; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AG, aminoglutethimide; 25-OHC, 25-hydroxycholesterol or 5-cholestene-3β,25-diol; 1× SCC, 0.15 M sodium chloride–0.015 M sodium citrate; LH, luteinizing hormone.

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Aminoglutethimide (Cytadren) was a gift from Ciba-Geigy Corp. (Summit, NJ).

Methods

Culture and Treatment of Human Granulosa Cells. Granulosa cells were aspirated from preovulatory follicles of women undergoing ovum retrieval for in vitro fertilization and embryo transfer and separated from red blood cells in the follicular aspirate by centrifugation through lymphocyte separation medium (Organon Teknika, Irving, TX). Cells were plated in 35-mm dishes and cultured for 48 h at 37 °C in Dulbecco's minimum essential medium containing 25 mM glucose, 4 mM L-glutamine, 50 μg/mL gentamicin, 25 mM Hepes, and 20% (v/v) human male serum. After the initial period of 48 h, serum-supplemented medium was replaced with serum-free medium for an additional 48-h period. This culture medium was then replaced with medium containing the experimental treatments described below. After the prescribed treatment interval for a given experiment, cells were harvested with a plastic spatula, and total cellular RNA was isolated as previously described (Golos & Strauss, 1987). Cells from two to three experiments were sometimes pooled to obtain RNA for blot hybridizations. It should be recognized that there is considerable variability in the function of granulosa cells harvested from individual patients. Thus, the relative responsiveness of the cells in each experiment to tropic stimulation differs due to the biological variation in the ovarian response to the regimen employed for follicular recruitment.

The treatments employed in these experiments were selected on the basis of previous experience in the study of LDL receptor expression in cultured human granulosa cells (Golos & Strauss, 1985, 1987). 8-Bromo-cAMP was added at a concentration of 1.5 mM. This concentration produced maximal stimulation of LDL receptor synthesis in our previous studies. To suppress HMG-CoA reductase expression, 25-OHC (10 μg/mL, added in 5 μL of ethanol) was added in the presence of aminoglutethimide (100 μg/mL, added in 5 μL of dimethyl sulfoxide). Aminoglutethimide, an inhibitor of cholesterol side chain cleavage, was added in order to prevent metabolism of hydroxysterol and endogenous cholesterol to pregnenolone. These concentrations of hydroxysterol and aminoglutethimide were previously shown to reduce LDL receptor synthesis and mRNA levels (Golos & Strauss, 1985, 1987).

RNA Electrophoresis and Blot Hybridizations. RNA was quantitated by absorbance at 260 nm, and equal amounts for each treatment group in a given experiment were electrophoresed in 1% agarose gels and transferred to Nytran filters (Schleicher & Schuell, Keene, NH) for RNA blot hybridization as previously described (Golos et al., 1987). A plasmid containing a full-length cDNA for human HMG-CoA reductase (pHRED-2) (Luskey & Stevens, 1985) was obtained from American Type Culture Collection (Rockville, MD). The human LDL receptor cDNA (pHH1-LDLR1) (Yamamoto et al., 1984) was provided by Drs. David Russell, Michael Brown, and Joseph Goldstein of the University of Texas Health Science Center, Dallas, TX. A γ-actin cDNA (Gunning et al., 1983) was provided by Dr. Peter Gunning of the University of California, Berkeley, CA. All cDNAs were nick-translated with kits obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Hybridizations with nick-translated cDNA probe [(2–5) × 10^6 cpm/mL] were conducted as previously described (Golos & Strauss, 1987). Hybridizations were conducted at 42 °C for 18–22 h. Following hybridization, filters were washed twice for 10 min each at room temperature in 2× SSC containing 0.1% SDS, followed by two 45-min washes at 65 °C in 1× SCC containing 0.1% SDS. Following washes, filters were briefly blotted and wrapped in plastic and placed with X-ray film (Kodak X-Omat, Rochester, NY) for autoradiography at −20 °C for 3–96 h. Blots were exposed for various periods of time to obtain autoradiograms with appropriate band intensities for quantitative analysis. The autoradiograms were analyzed with a Technology Resources Inc. (Nashville, TN) gel analysis system. Control bands were assigned a value of 1.0, and treatment effects are reported relative to this value.

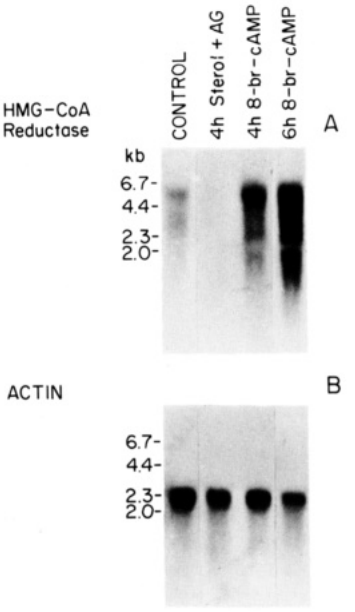

FIGURE 1: Effects of 25-hydroxycholesterol (25-OHC) plus aminoglutethimide and 8-bromo-cAMP on HMG-CoA reductase (panel A) and actin (panel B) mRNA. Granulosa cells were aspirated from preovulatory follicles of women undergoing ovum retrieval for in vitro fertilization and collected and cultured as described in the text. Following 48 h of serum-free culture, cells were treated for 4 or 6 h with 25-OHC (10 μg/mL) plus aminoglutethimide (100 μg/mL) or 1.5 mM 8-bromo-cAMP. Equal amounts (10 μg) of total RNA from control or treated cells were electrophoresed in 1% agarose gels and transferred to Nytran. The migration of fragments from a *Hin*dIII digest of λ DNA appears to the left of the autoradiogram. The filter was hybridized with nick-translated HMG-CoA reductase cDNA (panel A) or actin cDNA (panel B).

Results

We first wished to learn if a sterol negative-feedback regulatory pathway of HMG-CoA reductase mRNA expression is operative in cultured human granulosa cells. We investigated the time course of suppression of HMG-CoA reductase mRNA levels following treatment with 25-hydroxycholesterol (25-OHC) and aminoglutethimide (AG) (Figure 1 and Table I). Table I presents the results of three experiments where granulosa cells were exposed to 25-OHC and AG for up to 6 h. Total cell RNA was prepared and electrophoresed and transferred to Nytran membranes and probed with a ^{32}P-labeled cDNA for human HMG-CoA reductase. Autoradiograms revealed a major mRNA band of approximately 5.8 kb (Figure 1, panel A). This is similar to the expected size of the HMG-CoA reductase mRNA (Luskey & Stevens, 1985). The results demonstrate (Table I) that there is suppression of HMG-CoA reductase mRNA within 2 h of exposure to 25-OHC and AG and that mRNA levels are further reduced to approximately one-fifth that of control levels after 6 h of treatment.

We next examined the effect of 8-bromo-cAMP on HMG-CoA reductase mRNA levels (Figure 1, panel A). Granulosa

Table I: Time Course of Sterol Suppression of HMG-CoA Reductase mRNA in Human Granulosa Cells[a]

time of exposure (h)	relative mRNA abundance
0	1.0
1	0.90 ± 0.30
2	0.52 ± 0.15
4	0.51 ± 0.17
6	0.20 ± 0.02

[a] Human granulosa cells were cultured as described in Figure 1 and exposed to 25-OHC plus AG for 1–6 h. RNA samples were prepared, and 10 μg of total cellular RNA from each time point was analyzed as described in the legend to Figure 1. Autoradiograms were densitometrically scanned and integrated, and the areas under the tracings were expressed relative to control bands, which were set to 1.0. The means ± SE of three experiments are presented.

Table II: Time Course of Response of HMG-CoA Reductase mRNA in Human Granulosa Cells following Exposure to 8-Bromo-cAMP[a]

time of exposure (h)	relative mRNA abundance
0	1.0
1	1.8 ± 0.49
2	2.3 ± 0.60
4	4.2 ± 0.15
6	4.4 ± 1.82

[a] Human granulosa cells were cultured as described in Figure 1 and exposed to 8–bromo–cAMP (1.5 mM) for 1–6 h. RNA samples were prepared and 8–10 μg of total cellular RNA from each time point was analyzed as described earlier. Autoradiograms were densitometrically scanned, and the areas under the tracings were expressed relative to control bands, which were set to 1.0. The results presented are the means ± SE of data from three separate experiments.

cells exposed to 8-bromo-cAMP for 6 h had a more than 2.5-fold increase in HMG-CoA reductase mRNA compared to untreated cultures. The response of HMG-CoA reductase mRNA to 8-bromo-cAMP was relatively rapid; a significant increase of approximately 2-fold was seen within 2 h of treatment (Table II), and this increase was sustained for 6 h. The RNA filters were also probed with an actin cDNA (Figure 1, panel B). 25-OHC plus AG and 8-bromo-cAMP did not cause significant consistent changes in actin mRNA levels, demonstrating that the effects of sterol and cyclic nucleotide analogue on HMG-CoA reductase mRNA are specific and not due to changes in the levels of all cellular mRNAs.

We conducted studies to determine if the effects of 8-bromo-cAMP on HMG-CoA mRNA levels were dependent on cellular sterol balance. To this end, granulosa cells were treated with a combination of 25-OHC and AG in the absence or presence of 8-bromo-cAMP. The results of a representative experiment are shown in Figure 2, panel A. Treatment with 25-OHC and AG alone resulted in an appreciable decrease in the cellular content of the HMG-CoA reductase mRNA within 6 h. While 8-bromo-cAMP stimulated HMG-CoA reductase mRNA by severalfold, 25-OHC and AG markedly reduced the ability of the cyclic nucleotide to increase mRNA levels. When the RNA filter from this experiment was probed with the LDL receptor cDNA (panel B of Figure 2), it was found that 25-OHC and AG also substantially suppressed LDL receptor mRNA but that treatment with 8-bromo-cAMP plus 25-OHC and AG resulted in a more than 10-fold increase of this mRNA over the levels seen with sterol and aminoglutethimide alone. Table III summarizes the results of the experiment shown in Figure 2 with two additional experiments conducted in the same fashion. A consistent finding in these experiments was marked inhibition of the 8-bromo-cAMP-induced increase in HMG-CoA reductase mRNA in the presence of 25-OHC and AG. Although the 8-bromo-cAMP-induced rise in LDL receptor mRNA was also blunted

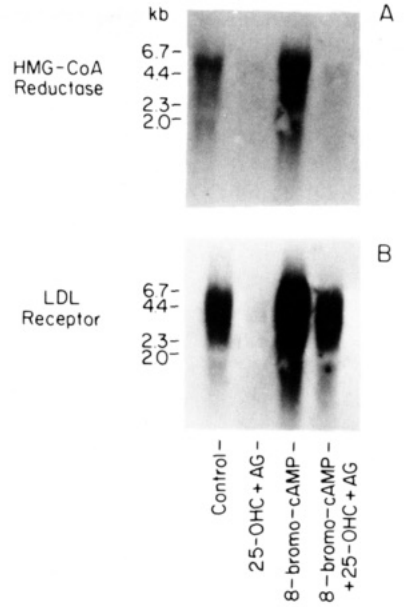

FIGURE 2: Effect of 8-bromo-cAMP and 25-hydroxycholesterol (25-OHC) plus aminoglutethimide (AG) on HMG-CoA reductase (panel A) and LDL receptor (panel B) mRNAs. Following 48 h of serum-free culture, cells were exposed for 6 h to control medium, 1.5 mM 8-bromo-cAMP, 10 μg/mL 25-OHC plus 100 μg/mL AG, or a combination of 8-bromo-cAMP and 25-OHC plus AG. The filter was hybridized with nick-translated HMG-CoA reductase cDNA (panel A), or LDL receptor cDNA (panel B).

Table III: Effect of 8-Bromo-cAMP and 25-Hydroxycholesterol and Aminoglutethimide (25-OHC + AG) on HMG-CoA Reductase and LDL Receptor mRNA Levels in Cultured Human Granulosa Cells[a]

	relative mRNA abundance			
	control	25-OHC + AG	8-bromo-cAMP	8-bromo-cAMP + 25-OHC + AG
HMG-CoA reductase	1.0	0.24 ± 0.1	2.6 ± 0.1	0.65 ± 0.3
LDL receptor	1.0	0.22 ± 0.1	5.0 ± 0.7	2.2 ± 0.8

[a] Human granulosa cells were cultured and treated as described in Figure 1. Cells were exposed for 6 h to control medium, 1.5 mM 8-bromo-cAMP, 10 μg/mL 25-hydroxycholesterol (25-OHC), and 100 μg/mL aminoglutethimide (AG) or a combination of 8-bromo-cAMP and 25-OHC plus AG. After the 6-h treatment interval, cells were harvested, RNA was prepared, and 8–10 μg of total cellular RNA from each treatment group was analyzed as described in Figure 1. Autoradiograms were densitometrically scanned, and the areas under the tracings were expressed relative to control bands, which were set to 1.0. The values shown represent the means ± SE of three experiments.

in the presence of 25-OHC and AG, LDL receptor mRNA rose a mean of (11.3 ± 2.4)-fold above levels in cells treated with 25-OHC and AG alone, whereas HMG-CoA reductase mRNA increased only (2.75 ± 0.2)-fold: fold increase in LDL receptor mRNA vs fold increase in HMG-CoA reductase mRNA ($p < 0.025$ by Student's t test).

Discussion

We have demonstrated that 8-bromo-cAMP rapidly increases cellular mRNA levels for HMG-CoA reductase, the rate-limiting enzyme in the de novo synthesis of cholesterol. The effect of the cyclic AMP analogue is essentially abolished in the presence of 25-OHC and AG. These findings suggest that the action of 8-bromo-cAMP with respect to HMG-CoA reductase expression is due primarily to the removal of sterol-mediated negative feedback of the HMG-CoA reductase gene subsequent to the acute increase in steroidogenesis. The 5′-flanking region of the HMG-CoA reductase gene has been

found to contain both positive and negative regulatory elements, as has the human LDL receptor gene (Osborne et al., 1985; Sudhof et al., 1987), and it has been postulated that these genes may be negatively regulated by a coordinate end-product inhibition mechanism (Chin & Chang, 1981) via cholesterol or another sterol metabolite (Gupta et al., 1986; Saucier et al., 1985). Although our studies have not uncovered the molecular mechanisms by which HMG-CoA reductase mRNA levels are controlled in cultured human granulosa cells, they do suggest that upon removal of sterol negative feedback HMG-CoA reductase mRNA expression is rapidly increased.

In contrast to HMG-CoA reductase, the mechanisms regulating the increase in LDL receptor mRNA levels promoted by 8-bromo-cAMP appear to be more complex since 8-bromo-cAMP raises LDL receptor message levels under conditions in which increases in HMG-CoA reductase mRNA are minimal. These results provide new information on the mechanisms mediating increases in de novo cholesterol synthesis which result from tropic hormone stimulation of steroidogenic tissues (Schuler et al., 1981; Simpson & Waterman, 1983; Azhar et al., 1984). They also underscore the importance of the LDL pathway in supporting maximal rates of steroidogenesis, in that upregulation of LDL receptors can occur even when cellular sterol stores are replete. The mechanism of this override of sterol negative feedback in terms of LDL receptor expression is not yet known. However, the 5′-flanking region of the LDL receptor gene contains sequences which are similar to the recently described consensus sequence (TGACGTCA) found in many genes transcriptionally regulated by cyclic AMP (Silver et al., 1987). Alternatively, cyclic AMP could affect LDL receptor mRNA at a posttranscriptional level (e.g., message stabilization). Thus, cyclic AMP could play a role in controlling the receptor gene or the mRNA in addition to the putative coordinate cholesterol-mediated regulation of both HMG-CoA reductase and LDL receptor expression by a common factor (Chin & Chang, 1981).

We have previously demonstrated that exogenous LDL is a primary source of cholesterol for steroidogenesis in cultured human granulosa cells (Soto et al., 1984; Golos et al., 1985). These data, along with those derived from in vivo and in vitro models (Gwynne & Strauss, 1982), have fostered the concept that in vivo, circulating lipoproteins are a major source of cholesterol for luteal progestin synthesis in many species. However, the granulosa cells of the preovulatory follicle are deprived of LDL by the anatomical nature of their separation from the circulation by a basement membrane (Simpson et al., 1980; Carr et al., 1982). Hence, an important consideration for steroidogenesis by the preovulatory granulosa cell may be the amount of HMG-CoA reductase activity. Moreover, mevalonate, the product of the HMG-CoA reductase reaction, is also used in the synthesis of dolichol, ubiquinone, and isopentenyl-RNA. These substances are likely to be essential for luteinizing granulosa cells, and it is appropriate that tropic hormones would rapidly upregulate the enzyme essential for their synthesis.

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Registry No. cAMP, 60-92-4; HMG-CoA reductase, 9028-35-7; cholesterol, 57-88-5.

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