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LH/hCG-Stimulated Androgen Production and Selective HDL-Cholesterol Transport Are Inhibited by a Dominant-Negative CREB Construct in Primary Cultures of Rat Theca-Interstitial Cells

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Theca-interstitial (T-I) cells synthesize androgens that are converted to estrogen by the granulosa cells. In rat ovary, T-I cells primarily utilize HDL-derived cholesteryl esters (CE) as a precursor for androgen synthesis. The HDL-CE is delivered to steroidogenic cells by a process termed "selective" uptake in which CE is internalized without the simultaneous uptake of apolipoprotein(s). This process is mediated by an HDL receptor, scavenger receptor class B, type I (SR-BI) and is stimulated by trophic hormone (LH/hCG), which also activates the cAMP cascade. In this study, we tested whether the adenoviral (Ad)-mediated introduction of a dominant-negative analog of cyclic AMP response element binding protein (A-CREB) inhibits the stimulatory effect of LH/hCG on the selective uptake of high-density lipoprotein (HDL)-cholesterol and androgen production in primary cultures of rat T-I cells. Androstenedione production by cultured T-I cells was stimulated by hCG and by the adenoviral overexpression of wtCREB. Additionally, the stimulatory effect observed with hCG was amplified in the presence of HDL. Androgen synthesis was increased 17-fold in the presence of HDL and hCG but the stimulatory effect of hCG was inhibited by Ad A-CREB by approx 70%. In the selective uptake studies, cell-surface association of the labeled HDL was significantly enhanced by hCG treatment, and this effect was inhibited by Ad A-CREB. The selective uptake of HDL-cholesterol was also enhanced by hCG but exposure to Ad A-CREB also abrogated this effect. It is concluded that CREB plays an intermediary role in the stimulatory action of LH/hCG on androgen synthesis and the selective uptake of HDL-cholesterol in T-I cells.

Key Words: LH; steroidogenesis; CREB; theca-interstitial cells; cholesterol; selective cholesterol uptake.

Introduction

Steroidogenesis by T-I cells is critical for the development and maintenance of ovarian follicle function *(1–6)*. Specifically, the T-I cells, by converting cholesterol into androgens, provide a vital precursor needed for estrogen production by ovarian granulosa cells. However, excessive androgen production by T-I cells has been associated with various pathophysiological conditions in humans such as polycystic ovarian syndrome, hyperthecosis, hirsutism and anovulation *(7–12)*.

Although T-I cells can synthesize cholesterol *de novo*, a major source of cholesterol for steroid production is provided by plasma-derived low-density lipoproteins (LDL) and high-density lipoproteins (HDL). In the rat, HDL is the major cholesterol-carrying lipoprotein and provides cholesterol precursor for steroid hormone synthesis *(13–15)*, although LDL cholesterol can also serve as a cholesterol donor *(13,15–17)*.

The process by which internalization of HDL-derived cholesteryl esters (CEs) occurs is known as "selective" cholesterol uptake *(17,18)* and differs from the classic endocytic, LDL receptor pathway *(19)* in that circulating lipoproteins contribute their CEs to cells without internalization of intact lipoprotein particle *(16,17,20–22)*. Thus, in the selective cholesterol uptake process, lipoprotein cholesterol enters cells unaccompanied by apolipoproteins. Scavenger receptor class B, type I (SR-BI), a bona fide HDL receptor, binds HDL (and other lipoprotein types) in steroidogenic tissues *(16,17,22–24)* and mediates selective CE uptake *(18,25–27)*. Recently, our laboratory has conclusively demonstrated that exposure of rat theca-interstitial cells LH/hCG stimulates the selective delivery of HDL-CE for androgen production, and that the addition of a highly specific anti-rat SR-BI peptide antibody attenuates this stimulatory effect *(26)*.

Considerable evidence now indicates that the conversion of HDL-derived cholesterol into androgens is subject to hormonal (LH/hCG) regulation through the intermediary role of cyclic AMP *(28,29)*. The increased cAMP production and activation of the cAMP/PKA signaling cascade leads to phosphorylation of the cAMP response element binding protein

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(CREB) and the formation of CREB homodimers that bind to the cyclic AMP response element (CRE), the CREB recognition site in the DNA *(30–32)*. Although LH-stimulated steroidogenesis is believed to occur mainly through the activation of the cAMP/PKA/CREB signaling cascade, the regulation of steroid production has also been shown to occur through CREB-independent mechanisms *(28,33,34)*.

The present study examined the potential involvement of CREB signaling cascade in hCG-stimulated selective uptake of cholesterol and its subsequent conversion into androgen by the ovarian T-I cells. Because CREB is expressed ubiquitously in mammalian systems, we have employed an adenoviral dominant-negative CREB construct, A-CREB, to interfere with the CREB function in primary cultures of rat T-I cells. A-CREB contains an acidic extension fused onto the N-terminus of the CREB leucine zipper domain *(30)*. This acidic extension interacts with the basic region of CREB and forms a stable A-CREB:native CREB heterodimer that prevents binding of native CREB to the CRE sequence, thus blocking the biological activity of CREB *(30)*. Employing this strategy we have examined whether adenoviral-mediated expression of A-CREB interferes with the stimulatory actions of LH/hCG on the selective uptake of HDL cholesterol and androgen biosynthesis in rat T-I cells.

Results

Effect of Adenoviral Infection Titers on Androstenedione Production by Rat Theca Interstitial Cells

Initial studies were conducted to determine the appropriate, nontoxic maximal viral load and exposure time of the T-I cells to the adenoviral constructs. We established that exposure of up to 3×10^{12} viral particles per mL (ppmL) of adenoviral constructs for 48 h did not elicit any signs of toxicity such as detachment of cells or pH changes in incubation media, compared to control uninfected incubations. Subsequently, using an adenovirus containing β-gal (Ad β-gal), we determined that exposure of the cells to concentrations of 3×10^{12} ppmL for up to 48 h did not exert a negative effect on basal androgen production [855 ± 64 vs 1034 ± 39 pg androstenedione/100 μg DNA ($n = 4$) for uninfected and Ad β-gal control incubations, respectively], showing that the adenovirus was not toxic to the cultured T-I cells.

Assessment of Adenoviral Uptake in T-I Cells

In order to evaluate infection efficiency of the adenoviral constructs in cultured T-I cells, we conducted experiments assessing the *in situ* staining of β-gal, using an Ad β-gal construct. It was observed that an adenoviral concentration equal to 6×10^{11} ppmL and a time of exposure of 24 h was necessary to achieve high levels of infection. The top image in Fig. 1 shows that T-I cells incubated in the absence of the adenovirus (control) lacked endogenous β-gal, while the lower image shows a robust β-gal staining when cells were incubated with the Ad β-gal construct. On average, over 85% cell staining was achieved, as assessed by light microscopy.

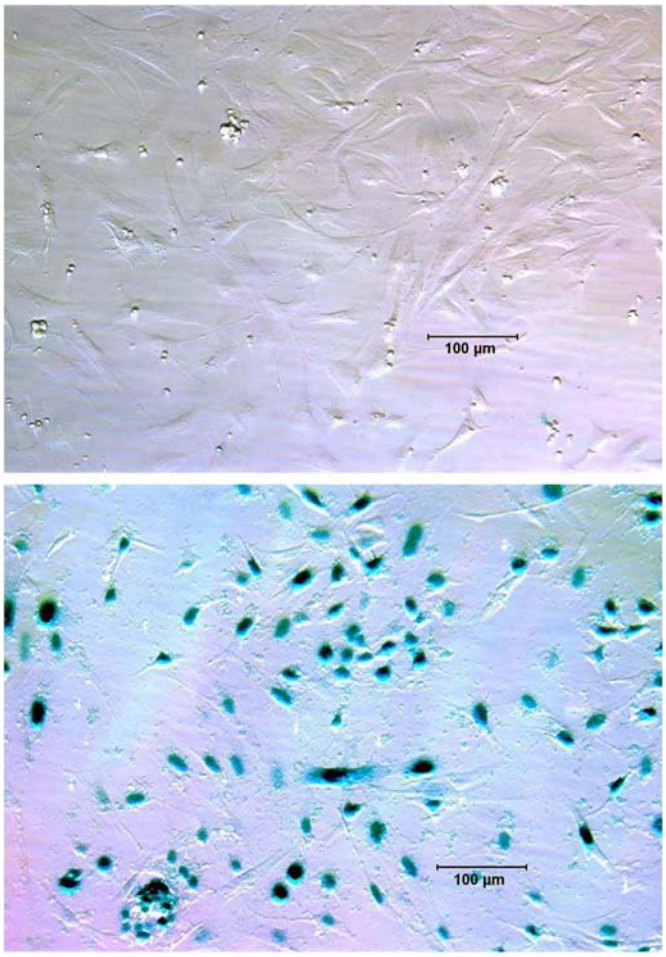

Fig. 1. Determination of adenoviral infectivity in rat theca-interstitial cells by β-gal staining. Rat theca-interstitial cells were harvested, plated, and allowed to attach for 24 h. The cells were then incubated in fresh media in the absence and presence of 6×10^{11} ppmL adenovirus containing the β-gal. After 24 of incubation at 37°C, the media were removed and the cells fixed in 0.5% glutaraldehyde and processed for the histochemical detection of β-gal. The top panel shows a representative area of control theca-interstitial cell incubations without adenovirus and the bottom panel depicts a representative area of cells stained for β-gal after the 24 h exposure to the adenovirus.

Effect of Ad wtCREB on Androstenedione Production by Rat T-I Cells

The specific effect of native CREB on the stimulation of androgen production by T-I cells was then examined. For these studies, the cells were incubated in the absence and presence of 3×10^{12} ppmL Ad β-gal and Ad wtCREB (native CREB construct) for 48 h. The incubations were carried out in the absence of added hormone (hCG), to more directly assess the contribution of wtCREB construct on basal androgen production. The representative data of such studies are

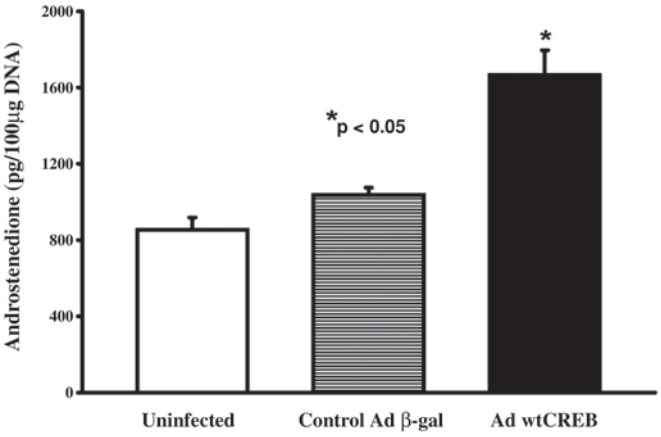

Fig. 2. Stimulation of androstenedione production by Ad wtCREB in rat T-I cells. The cells were isolated, plated, and allowed to attach overnight. The media were then replaced with fresh media and the cells were incubated for 48 h in the absence or presence of 3×10^{12} ppmL Ad wt CREB or Ad β-gal. After this period, the media and T-I cells were harvested and the androstenedione was extracted and measured by RIA. Values depicted are the mean ± SEM of four independent determinations. The asterisk represents a statistically significant ($p < 0.05$) difference over uninfected and Ad β-gal incubations.

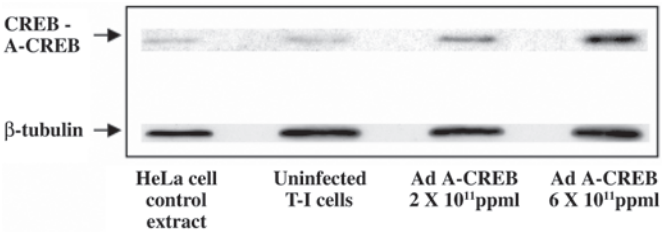

Fig. 3. Immunoblot detection of adenoviral-mediated overexpression of A-CREB in rat T-I cells. The cells were isolated, plated, and incubated for 24 h at 37°C in the absence and presence of 2×10^{11} ppmL (low Ad A-CREB) and 6×10^{11} ppmL (high Ad A-CREB) for 24 h. After this exposure, the media were removed and the cells were incubated for another 24 h to resemble the conditions in the experiments with hormone treatment. After this 24 period, the cells were collected, lysed, and homogenized. The detection of CREB and A-CREB was conducted by SDS-PAGE and Western blot assay. The bands were identified by molecular weight size and by running a control HeLa cell extract alongside the samples. The detection of β-tubulin was performed to ascertain gel loading and transfer accuracy.

depicted in Fig. 2, and show that Ad wtCREB increased T-I cell androstenedione production by approx 60% ($p < 0.05$) as compared to uninfected and Ad β-gal–infected control cells. These studies thus clearly demonstrate a stimulatory role of CREB in androgen production.

Assessment of Adenoviral-Mediated Expression of A-CREB in Rat T-I Cells

The expression of A-CREB was verified in cells infected with the adenoviral construct, using the same experimental time-frame that was utilized for the subsequent experiments with hCG treatment. In these studies, the cells were incubated in the absence and presence of 2×10^{11} (low concentration) and 6×10^{11} ppmL (high concentration) Ad A-CREB for 24 h and then the media were replaced with fresh media and the incubations were continued for another 24 h. Following this period, the cells were collected, cell homogenates were prepared, and the proteins were resolved by SDS-PAGE followed by Western blot detection of A-CREB with a polyclonal antibody known to recognize both A-CREB and native CREB *(35)*. The results of these experiments are depicted in Fig. 3 and show that there was an increase in the signal for CREB/A-CREB in the cells that were incubated in the presence of the adenovirus construct. The increases in the density of the bands were adenoviral concentration–related and at the high adenoviral concentration, the densitometric scanning of the bands indicated a threefold increase compared to uninfected controls. These results demonstrate that infection with A-CREB constructs yielded A-CREB expression in cultured T-I cells.

Effects of Ad A-CREB on hCG-Stimulated Androstenedione Production by Rat T-I Cells

Studies were then conducted to determine whether the adenoviral-mediated introduction of a dominant-negative A-CREB could inhibit hCG-stimulated androstenedione production. For these studies, the exposure time to the adenovirus was kept to 24 h, to preserve the responsiveness of the cells to hCG during the subsequent 24 h incubation, as responsiveness to hCG declines considerably 48 h post-attachment *(36)*. Cultured T-I cells were incubated in the absence and presence of 6×10^{11} ppmL of adenovirus containing either β-gal or A-CREB. Following the initial 24 h adenoviral incubation, the culture media were replaced by fresh media in the absence and presence of 100 ng/mL hCG. The results presented in Fig. 4 show that, as expected, hCG markedly increased androstenedione production in cells exposed to β-gal–containing adenovirus. In contrast, the stimulatory effect of hCG on androgen production, in cells infected with the Ad-A-CREB construct, was significantly ($p < 0.05$) decreased (approx 70% inhibition) compared to Ad β-gal controls treated with hCG. Confirmatory experiments were also conducted on the inhibition of hCG-stimulated androstenedione production by the biochemical inhibition of the cAMP-PKA cascade (data not shown). In these studies, addition of the PKA inhibitor H89 blunted the stimulatory effect of hCG to a degree similar to that observed with Ad A-CREB. In the adenoviral studies, basal androstenedione concentrations in the adenoviral-infected cultures were statistically similar to those found in uninfected cells, confirming the lack of adenoviral toxicity.

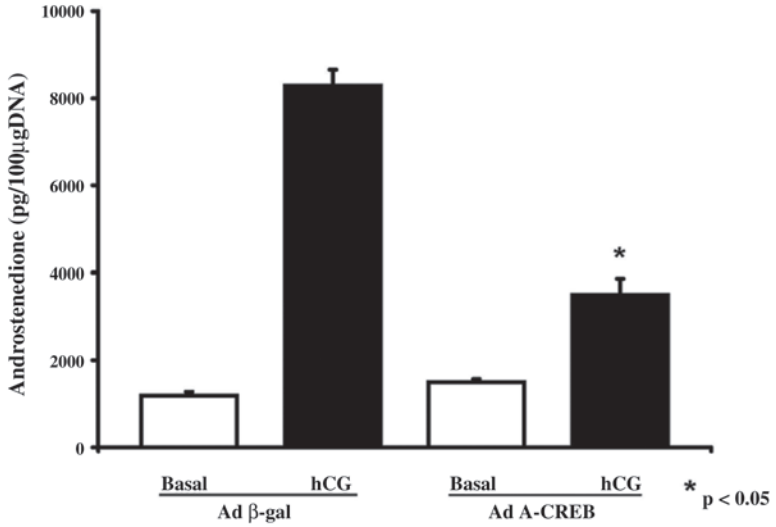

Fig. 4. Ad A-CREB inhibition of hCG-stimulated androstenedione production by rat theca-interstitial cells. The cells were isolated, plated, and incubated for 24 h at 37°C, in the absence and presence of 6×10^{11} ppmL of the adenoviral constructs containing the control β-gal or A-CREB cDNA sequences. After adenoviral exposure, the media were replaced with fresh media and the cells were incubated for another 24 h in the absence or presence of 100 ng/mL hCG. Cells and media were then harvested and androstenedione was extracted and measured by RIA. Values represent the mean ± SEM of three independent determinations. The asterisk over bar indicates a statistically significant difference ($p < 0.05$) of the hCG stimulation in the Ad A-CREB incubations compared to the hCG effect in Ad β-gal–treated controls.

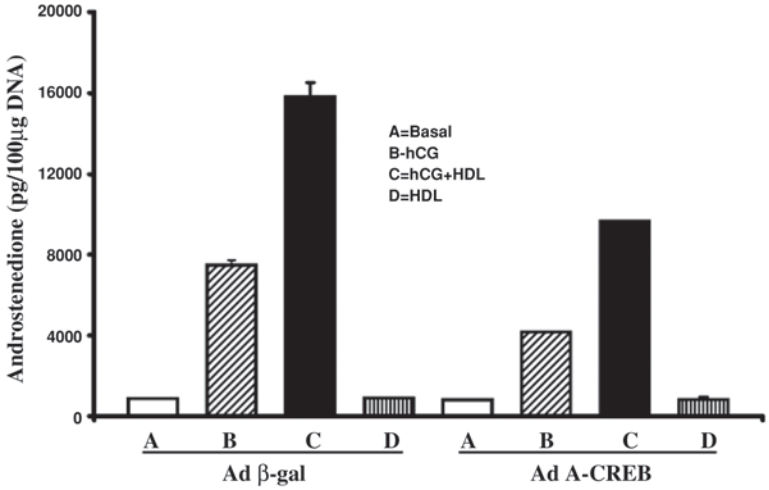

Fig. 5. Ad A-CREB inhibition of hCG-stimulated androstenedione production by rat theca-interstitial cells incubated with HDL. The theca-interstitial cells were incubated in media containing 6×10^{11} ppmL Ad β-gal or A-CREB for 24 h at 37°C. The media were then replaced and the incubations continued in the absence or presence of 100 ng/mL hCG, 50 μg/mL HDL cholesterol, or a combination of 100 ng/mL hCG plus 50 μg/mL HDL cholesterol. The incubations continued for 24 h and then the cells and media were collected and the androstenedione was extracted and measured by radioimmunoassay. The figure is a representative experiment of three separate experiments with similar inhibitory results of Ad A-CREB.

Effects of Ad A-CREB Treatment on the hCG-Stimulated and HDL-Supported Androstenedione Production in T-I Cells

The above studies (Fig. 4) clearly indicate the conversion of cholesterol to androgens requires the participation of the cAMP–PKA/CREB signaling cascade. Given that previous studies from our laboratory have shown *(26)* that hCG also stimulates HDL-supported androstenedione production under identical conditions, studies were conducted to determine if the stimulatory effect of hCG on HDL-supported steroid production could also be abrogated by the adenoviral-mediated expression of A-CREB in the cells. Figure 5 depicts representative data from three identical experiments demonstrating that Ad A-CREB inhibits hCG-stimulated and HDL-supported androstenedione production. The hCG stimulation of HDL-supported androstenedione production was markedly reduced from a 17-fold stimulation over basal in the Ad β-gal control, to a 10-fold stimulation

Table 1
Effect of Ad A-CREB on hCG-Stimulated Cell Surface Association of HDL in Rat T-I Cells

Treatment	(*n*)	Cell surface association (ng/100 μg protein ± SE)	Percentage change
Ad β-Gal basal	(4)	29.5 ± 0.37	—
Ad β-Gal + hCG	(4)	37.3 ± 0.92*	26.4
Ad A-CREB basal	(4)	43.0 ± 1.20	—
Ad A-CREB + hCG	(4)	40.5 ± 1.34	−5.8

The data presented are the means ± SEM of four independent measurements as indicated (*n*). *Mean is different from basal ($p < 0.05$).

Table 2
Effect of Ad A-CREB on hCG-Stimulated Selective Uptake of HDL Cholesterol in Rat T-I Cells

Treatment	(*n*)	Cholesterol uptake (ng/100 μg protein ± SE)	Percentage change
Ad β-Gal basal	(4)	266.5 ± 18.8	—
Ad β-Gal + hCG	(4)	365.1 ± 21.5*	37.0
Ad A-CREB basal	(4)	392.7 ± 20.4	—
Ad A-CREB + hCG	(4)	414.0 ± 7.4	5.4

The data presented are the means ± SEM of four independent measurements as indicated (*n*).

*Mean is different from basal ($p < 0.05$).

over basal in the Ad A-CREB infected cells. Interestingly, the basal levels of androstenedione production remained unchanged between the two groups. The data also show that the incubations conducted in the presence of HDL alone produced androstenedione that was comparable to that elicited by cells incubated under basal conditions, further establishing that hCG causes a specific increase in the selective uptake of lipoprotein cholesterol. These observations raised the possibility that A-CREB might also exert an inhibitory effect on the selective transport of HDL-derived CE into the T-I cells.

Effect of Ad A-CREB on hCG-Mediated Cell-Surface HDL Binding and Selective HDL-CE Uptake

The uptake of cholesterol from HDL involves the binding of the HDL to its receptor, SR-BI, on the cell surface followed by selective uptake of cholesterol from HDL. To determine whether exposure of T-I cells to A-CREB inhibits either cell-surface association of HDL to the HDL receptor or the selective uptake of cholesterol from HDL, primary cultures of T-I cells were infected with Ad A-CREB or control Ad β-gal for 24 h as previously described and then incubated with fresh media containing $^{125}I/^{3}H$-labeled hHDL (50 μg protein/mL), in the absence and presence of 100 ng/mL hCG for12 h. The results presented in Table 1 show that after 12 h of hCG treatment cell-surface association of doubly labeled HDL was significantly increased in cells exposed to the control β-gal adenovirus but that this stimulatory effect was blunted in cells preincubated with Ad A-CREB. The hCG-stimulated selective uptake of HDL cholesterol following 12 h hormone exposure is shown in Table 2. These data show that the selective uptake of HDL cholesterol was significantly stimulated by hCG in cells that had been exposed to the control adenoviral vector, but that this stimulatory effect was greatly reduced in the Ad A-CREB–treated group. The results further demonstrate that the inhibition of CREB action leads to attenuation of the hCG-mediated increase in HDL-cholesterol uptake.

Discussion

Previous studies from this laboratory *(26,36)* have clearly established that hCG stimulates the binding and the selective uptake of HDL-derived cholesterol in rat ovarian T-I cells. In the present study, these findings were extended to determine whether LH/hCG-stimulated selective uptake of HDL- cholesterol for androgen synthesis is mediated by signaling pathways culminating in CREB-mediated transcriptional activation. The specific involvement of CREB in androgen production by T-I cells was substantiated by the results from the incubations with Ad wtCREB, which demonstrated increased production of androstenedione. The involvement of CREB in the actions of LH/hCG on steroidogenesis and on the selective uptake pathway was examined by employing A-CREB, a dominant-negative mutant form of CREB that blocks the ability of CREB to homodimerize and bind to CRE sequences required for transcriptional activation. We used an adenoviral construct of A-CREB to infect primary cultures of T-I cells, because it has been difficult to achieve consistent transfection of primary cultures with plasmid vectors due to their low levels of transfection efficiency. The results with the immunoblot detection of adenoviral-directed expression of A-CREB demonstrate the viability and usefulness of this approach.

Our results show that hCG, as expected, markedly increased androstenedione production in the adenoviral-free (data not shown) and in the control Ad β-gal treatments and that this stimulatory effect of hCG was further increased by the addition of HDL to the incubations. The results with Ad A-CREB show that this dominant negative form of CREB decreased the stimulation of androstenedione production by hCG both in the absence and presence of HDL. The marked inhibition of the stimulatory effect of hCG by A-CREB indicates that CREB is a major component in the transduction of the LH/hCG signal for androgen production by T-I cells. However, the inhibition was not total and this may have been due to several possibilities. One possibility is that, although

the assessment of adenoviral infectivity showed that most (85%) of the cells were infected and that there was active expression of A-CREB in the cells, not all of the cells incorporated the adenovirus and therefore it is likely that Ad A-CREB could not completely block the effect of endogenous CREB. Additionally, it has been shown in a variety of steroidogenic models that the modulation of steroidogenesis also involves CREB-independent pathways *(28,33,34)*. For instance, recent studies using adenoviral-directed *(31)* or metabolic inhibition *(37–40)* of the cAMP–PKA cascade have shown to produce partial inhibition, suggesting that LH/hCG signaling may also involve additional CREB-independent mechanisms. Therefore, the lack of total inhibition by A-CREB on CREB action could be attributed to one or both of these possibilities.

The results also show that hCG had a moderate but significant stimulatory effect on the selective uptake of HDL cholesterol and this stimulatory effect of hCG was blocked by Ad A-CREB. The first step, cell-surface association of the HDL particle, was significantly increased by hCG in the Ad β-gal incubations, and this effect was blocked by Ad A-CREB. The second step, the selective uptake of labeled HDL-cholesterol into T-I cells, shows that hCG again elicited a moderate but significant stimulatory effect on the selective pathway and that A-CREB abrogated this effect. The HDL preparation used contained cholesteryl ether tags that accumulate in the T-I cell *(41,42)* that permitted the measurement of the uptake of HDL-associated cholesterol. The results from the experiments on the selective uptake of HDL cholesterol indicate that the enhancement of the selective uptake of HDL-cholesterol by hCG is dependent on the action of CREB. The moderate stimulatory effects observed may have been due to high basal rates of selective uptake in the T-I cells that could only be moderately increased by hCG. The elevation of basal rates of steroidogenic enzyme activity and hormone production during inhibition of the cAMP–PKA cascade is not an unusual finding *(37,38)*. A relevant additional point is that the partial inhibition by Ad A-CREB of hCG action on the stimulation of androgen production in the presence of HDL and the block of hCG-stimulated selective uptake of HDL-cholesterol should not be interpreted to mean that selective uptake is only a minor contributor to hCG-modulated androgen production. The results also need to take into consideration the known stimulatory effects of hCG on the stimulation of *de novo* cholesterol synthesis and the utilization of preexisting intracellular cholesterol for androgen synthesis.

At present the pressing question of how CREB regulates SR-BI expression and selective CE uptake remains unanswered. A close sequence analysis of human, rat, and mouse SR-BI promoters indicates that none of them contains CRE binding motifs *(43–46)*, but all three promoters contain several binding sites for steroidogenic factor-1 (SF-1, or Ad4BP), an orphan nuclear receptor (NR5A1) of the Fushi Tarazu factor-1 (Ftz-F1) transcription factor family, which controls the expression of genes involved in steroid hormone biosynthesis *(47,48)*. Previous studies have shown that treatment of model steroidogenic cells with cAMP agonists increases dramatically SR-BI mRNA levels, and this stimulatory action is due to PKA-dependent phosphorylation of SF-1 *(49)*. Another study, however, suggested that gonadotropin-mediated increased SR-BI transcription is caused by PKA phosphorylation of SP-1 and its increased binding to DNA in MA-10 mouse Leydig tumor cells *(45)*. The SP-1 and SP-3 transcription factors with three zinc fingers bind to proximal GC-rich sequences in the SR-BI promoter *(45)*, and are essential for constitutive or inducible expression of many genes in response to hormones *(50)*. It is possible that CREB functions as a functional partner of SF-1 and/or SP-1. This role may be analogous to that of SF-1 and CREB co-regulation of steroidogenic enzyme genes *(51–56)*. Likewise, SP-1 in cooperation with CREB either directly or indirectly, is known to regulate the expression of steroidogenic enzymes *(57,58)*. Together the data in the present studies provide evidence of the involvement of the cAMP–PKA–CREB cascade in hCG-mediated stimulation of the binding of HDL and the selective uptake of cholesterol for androgen synthesis by T-I cells.

Materials and Methods

Reagents

Peroxidase, cholesterol oxidase, and cholesterol esterase were obtained from Calbiochem (La Jolla, CA). Human chorionic gonadotropin (hCG CR-125) was kindly provided by the Center for Population Research, NICHD, National Institutes of Health. Medium 199, L-glutamine, and McCoy's 5A medium were purchased from Life Technologies, Inc. (Gaithersburg, MD). Collagenases (CLS I) were obtained from Worthington Biochemical Corp. (Freehold, NJ). Reagents for androstenedione RIA (DSL-4200) were obtained from Diagnostic Systems Laboratories, Inc. (Webster, TX). The β-gal *in situ* staining set was purchased from Roche Diagnostics Corporation (Indianapolis, IN). All other chemicals and reagents used were of analytical grade.

Adenoviral Constructs

The replication-deficient, adenovirus type 5 (variant dl309) constructs containing either the β-gal, wild-type (wt) CREB or A-CREB sequences under the control of the cytomegalovirus (CMV) promoter, were generated and kindly provided by Dr. Marc Montminy (The Salk Institute for Biological Studies, San Diego, CA). The detailed procedures for adenoviral construct generation have been previously published and described in detail *(31,32,59)*. The propagation of the adenoviral constructs was performed following previously published procedures *(31,59)* and adenoviral titers were determined as viral particles per mL (ppmL) by measuring the absorbance at 260 nm of propagated adenoviral dilutions following published standard procedures

(31). The assessment of infectivity of the adenoviral constructs was performed by microscopic evaluation of the percentage of plated T-I cells showing β-gal staining, after incubation with Ad β-gal, using a β-gal staining kit (Roche Diagnostics; Indianapolis, IN).

Animals

Sprague Dawley female rats (25 d old) were purchased from Harlan, Inc. (Indianapolis, IN) and were used without further treatment. The animals were killed by CO_2 asphyxiation. The ovaries were removed under sterile conditions and were processed immediately for the isolation of theca-interstitial cells.

Isolation and Culture of Theca-Interstitial Cells

The theca-interstitial cells were isolated, dispersed, and cultured following a procedure previously published *(26)*. Briefly, freshly removed ovaries were placed in warm (37°C) medium 199 containing 25 m*M* HEPES (pH 7.4), 2 m*M* L-glutamine, 1 mg/mL BSA, 100 U/mL penicillin, and 100 μg/mL streptomycin. The ovaries were subsequently cleaned of adhering fat, punctured actively and repeatedly with a 27G needle under a dissecting microscope to release granulosa cells. The ovaries were then washed three times with medium to remove remaining adhering granulosa cells. The tissue was then minced thoroughly and incubated for 45 min at 37°C in the same medium, supplemented with 1.5 mg/mL collagenase type 1 and 10 μg/mL deoxyribonuclease. The dispersion was aided mechanically by pipetting the preparation with a 10 mL pipette. The theca-interstitial cells thus released were centrifuged at 250*g* for 5 min and washed in collagenase-free medium two times to eliminate collagenase contamination. Subsequently, the dispersed cells were resuspended in McCoy's 5A medium containing 2 m*M* L-glutamine, 1 mg/mL BSA, 100 U/mL penicillin, and 100 μg/mL streptomycin and subjected to a single 5-min unit gravity purification to sediment and eliminate small clusters of undispersed ovarian tissue. Cell viability was checked by trypan blue exclusion and was above 90%. The dispersed cells were either seeded in 12-well plates to determine steroid production (8×10^5 viable cells/well) or in 35-mm dishes (2×10^6 viable cells/dish) to assay for lipoprotein-derived cholesteryl ester (CE) transport. The plated cells were maintained overnight in McCoy's 5A medium containing 2 m*M* L-glutamine, 1 mg/mL BSA, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere of 95% air–5% CO_2 at 37°C. Theca-interstitial cell purity, averaging 95%, was determined by histochemical staining *(60)*. Additionally, in control experiments, theca-interstitial cells were prepared by a Percoll gradient procedure *(15)* and yielded similar responses in hCG-stimulated androstenedione production.

Western Blot Assay

T-I cells were harvested, plated, and allowed to attach overnight. The media was then replaced with fresh media and the cells were incubated in the absence and presence of 2×10^{11} and 6×10^{11} ppmL Ad A-CREB for 24 h. After this period, the media were replaced with fresh media and the cells were incubated for another 24 h to resemble the schedule and conditions followed in the experiments with hCG treatments. After this period, the media were removed and the cells were rinsed twice with ice-cold PBS and lysed in RIPA lysis buffer. The cells were homogenized by further sonication and 30 μg protein per lane were resolved by SDS-PAGE. CREB and A-CREB was detected by Western blot analysis with a rabbit, polyclonal anti-CREB antibody (Upstate biotechnology, NY) that has been shown to detect both forms of CREB *(35)*. Gel loading and transfer accuracy were monitored by re-probing the blots for β-tubulin with a specific antibody (Santa Cruz Biotechnology, Inc., CA). Detection of the appropriate bands was conducted with an enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech). The density of the bands was assessed using an Arcus II scanner (Agfa, Wilmington, MA) and the NIH Image 1.61 program (Bethesda, MD).

Measurement of Androstenedione Production

For the determination of the effect of Ad wtCREB on androstenedione production, T-I cells were isolated, plated, and incubated with fresh media in the absence or presence of 3×10^{12} ppmL of Ad wtCREB or Ad β-gal for 48 h. Ad β-gal was used to provide an adenoviral-infected control counterpart to Ad wtCREB, as well as to assess, by *in situ* staining, the infection efficiency in the T-I cell cultures. Additionally, an adenovirus-free treatment group was carried through the same sequence of procedures. After this period, the cells and media were collected from each dish, kept in a water bath at 75°C for 20 min to inactivate the adenoviral content and lyse the cells, and stored at –20°C until assayed for androstenedione by radioimmunoassay (RIA). To examine the effect of dominant-negative forms of CREB on androgen production, the cultured theca-interstitial cells were preincubated in the absence and presence of Ad A-CREB (6×10^{11} ppmL) or the control adenovirus containing β-gal (Ad β-gal) (6×10^{11} ppmL). The cells were exposed to the adenoviral constructs for 24 h. After the adenoviral preincubation, the media in the wells were replaced with fresh adenovirus-free media and the cells were maintained for an additional 24 h in the presence or absence of hCG (100 ng/mL), human HDL_3 (HDL) (50 μg/mL cholesterol), or hHDL plus hCG. Following this period, the cells and media were collected as described above and androstenedione was measured by RIA.

Uptake and Internalization of Lipoprotein-Derived CEs

In these experiments, theca-interstitial cells were cultured in 35-mm dishes. After overnight attachment, the cells were exposed to β-gal or A-CREB adenovirus (both at 6×10^{11} ppmL) and incubated at 37°C for 24 h. Subsequently, the media were replaced with fresh, adenoviral-free medium

containing doubly (^{125}I/^{3}H)-labeled hHDL3 particles with [^{3}H]labeled, nonreleasable CE tags that accumulate within the cells even when degraded and apolipoproteins labeled with ^{125}I *(41,42)*. The incubations were conducted with the labeled HDL (^{125}I-labeled dilactitol tyramine (DLT)-[^{3}H] COE-hHDL$_3$) (50 μg protein/mL), in the absence or presence of hCG (100 ng/mL) for 12 h at 37°C. At the end of incubation, the dishes were rinsed with PBS and the cells were solubilized with 0.1 *N* sodium hydroxide. Duplicate sets of aliquots were taken. A set was used to determine soluble and insoluble ^{125}I radioactivity before and after addition of 20% trichloroacetic acid (TCA), and the second set was extracted with organic solvents to assay ^{3}H-radioactivity. The endocytic uptake of lipoprotein particles was determined from TCA-soluble ^{125}I label alone as described previously *(41,42)*. Briefly, the difference between total and TCA-soluble radioactivity is considered to be cell surface–associated ^{125}I-radioactivity. Since both ^{125}I and ^{3}H labels are on the same particle, the surface-bound ^{3}H is also equal to the surface-bound ^{125}I. Therefore, total ^{3}H minus surface-bound ^{3}H equals the amount of internalized ^{3}H. The selective uptake of CE was then calculated by subtracting the soluble ^{125}I radioactivity from soluble ^{3}H radioactivity.

Miscellaneous Techniques

Human apolipoprotein E-free HDL$_3$ was isolated and characterized as described previously *(13)*. Cellular DNA content was assayed colorimetrically *(61)*. Cellular protein concentration was determined by the bicinchoninic acid procedure of Smith et al. *(62)*. Cholesterol content of the lipoproteins was determined following the method of Deacon and Dawson *(63)*.

Statistical Analysis

The statistical analysis was carried out using ANOVA followed by Tukey's test for pairwise comparisons. Each experiment was repeated at least three times, with comparable results. Significance was accepted as $p < 0.05$.

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