BIOLOGY OF REPRODUCTION **81,** 284–292 (2009)
Published online before print 18 March 2009.
DOI 10.1095/biolreprod.108.074351

Regulation of Sterol Regulatory Element-Binding Transcription Factor 1a by Human Chorionic Gonadotropin and Insulin in Cultured Rat Theca-Interstitial Cells[1]

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ABSTRACT

Theca-interstitial (T-I) cells of the ovary synthesize androgens in response to luteinizing hormone (LH). In pathological conditions such as polycystic ovarian syndrome, T-I cells are hyperactive in androgen production in response to LH and insulin. Because cholesterol is an essential substrate for androgen production, we examined the effect of human chorionic gonadotropin (hCG) and insulin on signaling pathways that are known to increase cholesterol accumulation in steroidogenic cells. Specifically, the effect of hCG and insulin on sterol regulatory element-binding transcription factor 1a (SREBF1a) required for cholesterol biosynthesis and uptake was examined. Primary cultures of T-I cells isolated from 25-day-old rat ovaries responded to hCG and insulin to increase the active/processed form of SREBF1a. The hCG and insulin significantly reduced insulin-induced gene 1 (INSIG1) protein, a negative regulator of SREBF processing. Furthermore, an increase in the expression of selected SREBF target genes, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*) and mevalonate kinase (*Mvk*), was also observed. Protein kinase A (PRKA) inhibitor completely abolished the hCG-induced increase in SREBF1a, while increasing INSIG1. Although the hCG-induced depletion of total and free cholesterol was abolished by aminoglutethimide, the stimulatory effect on SREBF1a was not totally suppressed. Treatment with 25-hydroxycholesterol abrogated the effect of hCG on SREBF1a. Inhibition of the phosphatidylinositol 3-kinase pathway did not block the insulin-induced increase in SREBF1a, whereas mitogen-activated protein kinase inhibition reduced the insulin response. These results suggest that the increased androgen biosynthesis by T-I cells in response to hCG and insulin is regulated, at least in part, by increasing the expression of sterol response element-responsive genes by increasing SREBF1a.

INSIG1, insulin, LH, luteinizing hormone, Mvk, *PCOS, SREBF1, theca cells, theca-interstitial cells*

INTRODUCTION

Although androgen synthesis by theca-interstitial (T-I) cells is necessary for estrogen biosynthesis by the growing follicles, excess androgen production has been associated with anovulatory conditions such as polycystic ovarian syndrome (PCOS) [1–4]. The stimulatory effect of luteinizing hormone (LH) and insulin on androgen production by T-I cells has been well established [5–14]. Because cholesterol is the precursor of androgens, its availability in T-I cells, either by uptake from plasma-derived cholesterol or through de novo synthesis, has been shown to have an important role in androgen synthesis [11, 15]. The synthesis and uptake of cholesterol are regulated by a family of membrane-bound transcription factors designated as sterol regulatory element-binding transcription factors (SREBFs) [16–18]. Three major SREBF isoforms, SREBF1a, SREBF1c, and SREBF2, have been identified, and they are encoded by two different genes [16, 19–21]. Both SREBF1a and SREBF1c originate from a single gene. These two isoforms differ in size and transcriptional activity but are formed from a common precursor [18, 22]. While SREBF1a is a potent activator of both cholesterol and fatty acid biosynthetic pathways, SREBF1c is primarily involved in fatty acid synthesis [23]. SREBFs regulate the transcription of genes that possess a sterol response element (SRE) in the promoter sites. This includes key enzymes involved in cholesterol synthesis and uptake by the steroidogenic cells [16, 24, 25]. SREBFs are synthesized as large precursor proteins that are inserted into the endoplasmic reticulum (ER) membranes, where they exist in an inactive form. In the ER, the carboxyl terminus of SREBF interacts with SREBF chaperone, previously known as SREBF-cleavage activating protein (SCAP) [26]. The SREBF-SCAP complex is anchored to ER membranes through another protein designated as insulin-induced gene 1 (INSIG1) [17, 27, 28].

In sterol-depleted cells, the SCAP-SREBF complex dissociates from INSIG1. SCAP then escorts SREBF from the ER to the Golgi, where it is processed by two membrane-associated proteases—membrane-bound transcription factor peptidase, site 1 (MBTPS1, formerly S1P) and membrane-bound transcription factor peptidase, site 2 (MBTPS1, formerly S2P)—which release the active form of SREBF [23, 29]. These transcriptionally active fragments of SREBFs are translocated to the nucleus, where they bind to the promoters of SREBF target genes, resulting in increased transcription of the genes encoding proteins involved in the synthesis and uptake of cholesterol [17, 18]. In contrast, when the cellular cholesterol level is high, the SCAP-SREBF complex remains attached to INSIG1 in the ER [30, 31], which prevents the SCAP-SREBF complex from being incorporated into coat protein complex II vesicles for transport to the Golgi. As a result, SREBF precursor remains trapped in the ER, leading to a decline in the nuclear content of SREBF and a reduction in the synthesis and uptake of cholesterol.

Because androgen synthesis in T-I cells is dependent on the availability of cholesterol, we sought to determine the regulation of SREBF1a by human chorionic gonadotropin (hCG) and insulin, two hormones that have been shown to increase the production of androgens by these cells. Our results show that hCG and insulin stimulate the formation of

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Received: 21 October 2008.
First decision: 24 November 2008.
Accepted: 10 March 2009.

eISSN: 1259-7268 http://www.biolreprod.org
ISSN: 0006-3363

SREBF1a, the active transcription factor required for the induction of genes involved in cholesterol synthesis and uptake.

MATERIALS AND METHODS

Medium 199, McCoy 5A medium, L-glutamine, and Hepes buffer were purchased from Invitrogen/GIBCO (Carlsbad, CA). Penicillin-streptomycin was purchased from Roche Diagnostics (Indianapolis, IN). Collagenase (CLS I) and deoxyribonuclease I were obtained from Worthington Biochemical Corp. (Freehold, NJ). Bovine serum albumin (BSA), insulin, wortmannin, 25-hydroxycholesterol (OHC), aminoglutethimide (AGM), and TUBB (β-tubulin) antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Purified hCG was purchased from Dr. A.F. Parlow (Torrance, CA). SREBF1a mouse monoclonal antibody (IgG-2A4) was raised against amino acids 301–407 of human SREBF1 (ATCC, Manassas, VA). Antibodies for INSIG1, CYP17A1, MAPK1, and anti-goat IgG horseradish peroxidase conjugates were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody against phosphorylated AKT (Ser 473) was purchased from Cell Signaling Technology (Beverly, MA). Texas red-conjugated donkey anti-goat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Mitogen-activated protein (MAP) kinase (MAP2K) inhibitor U0126 was obtained from Promega (Madison, WI). The cholesterol/cholesteryl ester quantitation kits and protein kinase A (PRKA) (H89) inhibitor were purchased from Calbiochem (La Jolla, CA). Anti-mouse anti-rabbit IgG horseradish peroxidase conjugates and enhanced chemiluminescence using the Femto Supersignal Substrate System were obtained from Pierce (Rockford, IL). ^{3}H androstenedione was purchased from New England Nuclear (Boston, MA). Reagents for androstenedione radioimmunoassay (RIA) were obtained from Diagnostic Systems Laboratories (DSL-4200; Webster, TX). All other reagents used were conventional commercial products.

Animals

Sprague-Dawley female rats (25 days old) were purchased from Charles River Laboratories (Wilmington, MA). All the experimental protocols used in this study were approved by the University Committee on the Use and Care of Animals. Animals were housed in a temperature-controlled room with proper D:L cycles as per the guidelines provided by the University Committee on the Use and Care of Animals. The animals were euthanized by CO_2 asphyxiation. The ovaries were removed under sterile conditions and were processed immediately for the isolation of T-I cells.

Isolation and Culture of T-I Cells

The T-I cells were isolated, dispersed, and cultured using a protocol previously published from our laboratory [11, 32]. Briefly, freshly collected ovaries were placed in 100-mm plates in medium 199 containing 25 mM Hepes (pH 7.4), 2 mM L-glutamine, 1 mg/ml BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin. The ovaries were then freed from adhering fat and actively punctured with a 27-gauge needle under a dissecting microscope to release the granulosa cells. The remaining ovarian tissue was then washed three times with medium to release any remaining granulosa cells. The tissue was then minced and incubated for 30 min at 37°C in the same medium, supplemented with 0.65 mg/ml collagenase type 1 plus 10 μg/ml deoxyribonuclease. The dispersion was encouraged by mechanically pipetting the ovarian tissue suspension with a 10-ml pipette. The T-I cells released by this digestion were centrifuged at 250 $\times g$ for 5 min and washed in medium two times to eliminate remaining collagenase. The dispersed cells were then resuspended in McCoy 5A medium containing 2 mM L-glutamine, 1 mg/ml BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin and subjected to unit gravity sedimentation for 5 min to eliminate small fragments of undispersed ovarian tissue. Cell viability was assessed by trypan blue exclusion and was always above 90%. The dispersed cells were seeded in 60-mm plates (3×10^6 viable cells). The plated cells were maintained overnight in McCoy 5A medium containing 2 mM L-glutamine, 0.1% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air-5% CO_2 at 37°C. After allowing cells to attach overnight, they were treated with hCG or insulin for 4 h. Other reagents used are indicated in the figure legends. The T-I cell purity was determined by immunofluorescence staining of CYP17A1 (cytochrome P450, family 17, subfamily a, polypeptide 1, also known as 17α-hydroxylase/17,20-lyase).

Immunofluorescence of CYP17A1

Immunofluorescence analysis of CYP17A1 was performed using cultured T-I cells. In brief, cells were cultured on coverslips for 24 h. After attachment, cells were fixed with 2% formaldehyde solution in PBS for 15 min at room temperature, washed three times with wash solution (PBS containing 0.3% Triton X-100), and treated with 5% normal goat serum for 1 h at room temperature. The cells were washed three times as already described using the wash solution and then incubated with primary CYP17A1 goat polyclonal antibody at 4°C overnight. After washing three times with wash solution, the cells were incubated with Texas red-conjugated secondary anti-goat antibody for 1 h at room temperature in the dark. Finally, the cells were washed and mounted, and the images were captured using a fluorescent microscope (Leica DMR, Wetzlar, Germany).

Real-Time PCR

Aliquots of total RNA (50 ng) extracted from T-I cells were reverse transcribed in a reaction volume of 20 μl using 2.5 μM random hexamer, 500 μM deoxyribonucleotide triphosphates, 5.5 mM $MgCl_2$, 8 U ribonuclease inhibitor, and 25 U Multiscribe reverse transcriptase (Applied Biosystems Inc., Foster City, CA). The reactions were carried out in a PTC-100 (MJ Research, Watertown, MA) thermal controller (25°C for 10 min, 48°C for 30 min, and 95°C for 5 min). The resulting cDNAs were diluted with water. The real-time PCR quantification was then performed using 5 μl diluted cDNAs in triplicate with predesigned primers and probes for rat 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*) and mevalonate kinase (*Mvk*) (TaqMan assay-on-demand gene expression products; Applied Biosystems Inc.). Reactions were carried out in a final volume of 25 μl using an Applied Biosystems Inc. 7300 real-time PCR system (95°C for 15 sec and 60°C for 1 min) after initial incubation for 10 min at 95°C. The fold changes in *Hmgcr* and *Mvk* expression were calculated using the standard curve method with *18S* rRNA as the internal control.

Western Blot Analysis

To examine the effect of hCG and insulin on SREBF1a expression, cultured T-I cells were exposed to hCG (0, 25, and 50 ng/ml) or insulin (0, 0.5, and 1 μg/ml) for 4 h. In experiments with inhibitor H89 (10 μM), to block the PRKA pathway the cells were pretreated with inhibitor for 1 h, followed by hCG treatment for 4 h. In experiments with AGM (5 μg/ml), to inhibit CYP11A1 (cytochrome P450scc) activity the cells were pretreated with AGM for 1 h, followed by hCG treatment for 4 h. In experiments with 25-OHC, cells were preincubated with 25-OHC (10 μg/ml) for 1 h, followed by hCG or insulin treatment for 4 h. In experiments with the MAP2K inhibitor U0126 (10 μM), cells were preincubated for 1 h, whereas the preincubation period was 30 min for the phosphatidylinositol 3-kinase (PIK3) inhibitor wortmannin (100 nM). These preincubations were followed by insulin treatment for 4 h. For phosphorylated AKT measurements, cells were pretreated with wortmannin (100 nM) for 30 min, followed by the addition of insulin for 30 min. Reactions were terminated by removing the media, and the cells were solubilized using radioimmunoprecipitation assay buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). Cell lysates were then sonicated and centrifuged for 10 min at 13 000 $\times g$. The protein concentrations of the supernatants were determined using bicinchoninic acid reagent (Pierce). Proteins (50 μg/lane) were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad) before immunoblot analysis. Membranes were blocked in 5% fat-free milk in 20 mM Tris base [pH 7.45], 137 mM NaCl, and 0.1% Tween 20 (TBST) for 1 h at room temperature and then incubated overnight at 4°C with primary antibody in 5% fat-free milk/TBST. After three 5-min washes with TBST, membranes were incubated in appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After three 5-min washes with TBST, membrane-bound antibodies were detected using the Femto Supersignal Substrate System Western blotting detection kit (Pierce). Protein loading was monitored by reprobing the same blots with β-tubulin antibody.

RIA of Androstenedione

For evaluation of the inhibition of androstenedione production by AGM, T-I cells were grown in 60-mm plates with McCoy 5A medium containing 0.1% BSA. After attachment, cells were pretreated with AGM for 1 h, followed by hCG for 4 h. At the end of treatments, the media were collected, trace amounts of ^{3}H androstenedione were added to monitor recovery, and extracted for the steroid using diethyl ether. The extraction recovery was greater than 85%. Androstenedione production was determined using RIA kits (Diagnostic Systems Laboratories) according to the manufacturer's instructions. The intraassay and interassay coefficients of variation for androstenedione were 2.7%–5.9% and 4.8%–7.0%, respectively.

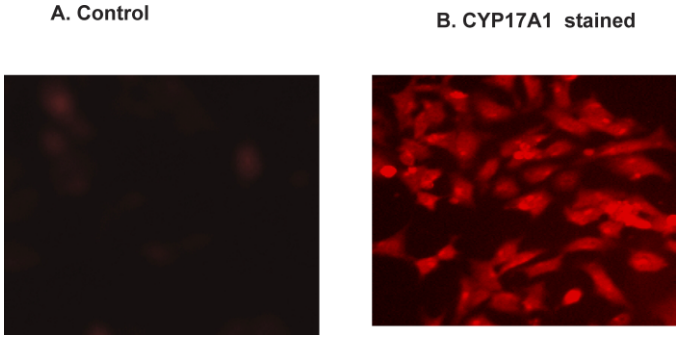

FIG. 1. Immunofluorescence staining of CYP17A1 in T-I cells. Cells were harvested, plated, and allowed to attach for 24 h. Cells were then washed, fixed, and immunostained using a CYP17A1 primary antibody and a Texas red-conjugated secondary antibody. Cells were examined by fluorescence microscopy. **A**) Representative area of control T-I cells incubated without CYP17A1 antibody (original magnification ×20). **B**) Representative area of cells stained with CYP17A1 antibody (original magnification ×20).

Cholesterol Analysis in T-I Cells

The cultured T-I cells (3×10^6) were pretreated with AGM (5 μg/ml) for 1 h, followed by hCG (50 ng/ml) for 4 h. Cells were lysed with 1% Triton X-100 in choloroform and centrifuged for 10 min at 14 000 revolutions per minute. The organic phase was transferred to a clean tube and dried using nitrogen gas. Then the samples were vacuum dried for 30 min to remove residual choloroform. The dried lipids were dissolved in 300 μl cholesterol reaction buffer by vortexing extensively for 5 min. The assay of total and free cholesterol used 25 μl each sample in duplicates. The total and free cholesterol contents were determined using cholesterol/cholesteryl ester quantitation kits according to the manufacturer's (Calbiochem) instructions.

Statistical Analysis

Statistical analysis was carried out using one-way ANOVA, followed by Tukey multiple comparisons test using Prism software (GraphPad Prism, version 3.0; GraphPad Inc., San Diego, CA). Unpaired *t* test was used for the relative mRNA expression. Values were considered statistically significant at $P < 0.05$. Each experiment was repeated at least three times, with similar results. Blots are representative of one experiment, and graphs represent the mean ± SE of three replicates.

RESULTS

Immunofluorescence Detection of CYP17A1

The purity of T-I cells was examined by immunofluorescence staining of CYP17A1, a marker enzyme, as described in *Materials and Methods*. The results in Figure 1 show that greater than 90% of the cells stained positive for CYP17A1, identifying the cells as being T-I cells. The control cells without exposure to CYP17A1 antibody did not show appreciable fluorescence. The cells also showed an absence of aromatase mRNA expression in response to follicle-stimulating hormone, suggesting that T-I cells were free of granulosa cells (data not shown).

Effect of hCG on SREBF1a and INSIG1 Protein Expression

The T-I cells were treated with hCG at different concentrations (0, 25, and 50 ng/ml) for 4 h, and Western blot analysis

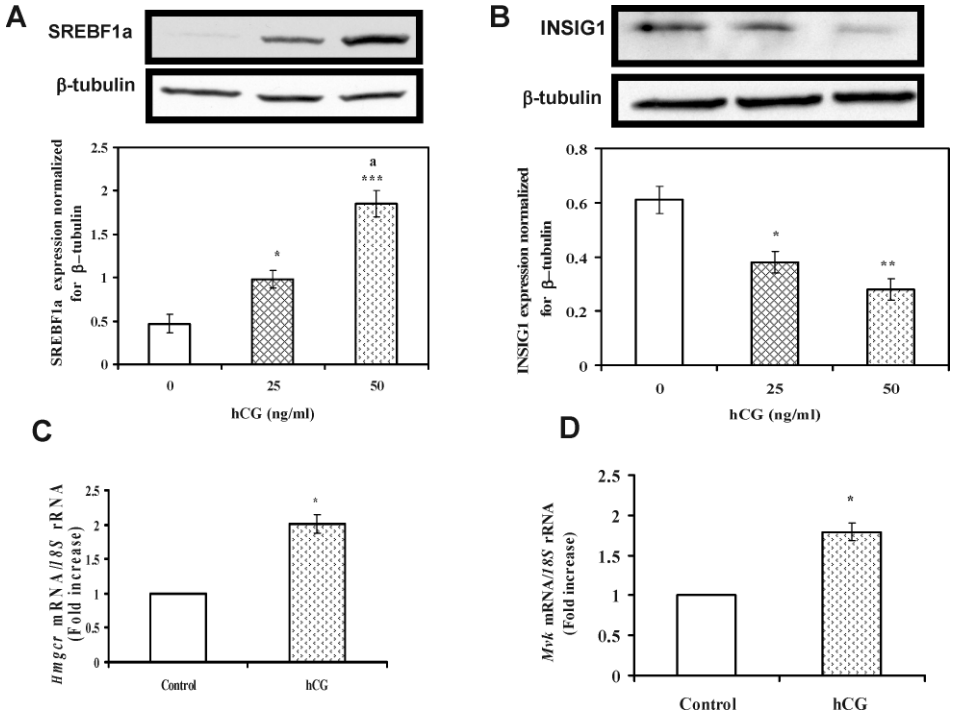

FIG. 2. The hCG-mediated regulation of SREBF1a and its target genes. Ovaries were collected from 25-day-old Sprague-Dawley rats. The T-I cells were isolated by collagenase digestion, and 3×10^6 cells were plated with McCoy 5A medium. After 24-h attachment, cells were treated with different concentrations of hCG (0, 25, and 50 ng/ml) for 4 h. Whole-cell lysates were then analyzed for the expression of the active form of SREBF1a (**A**) and INSIG1 (**B**) by Western blot (top panels). The graphs of the densitometric scans are shown in the bottom panels of **A** and **B**. Protein loading was monitored by stripping and reprobing the same blot with antibody for β-tubulin. Blots are representative of one experiment, and the graphs represent the mean of three experiments. Error bars represent the mean ± SE. **C** and **D**) Cells were treated with hCG (50 ng/ml) for 4 h. Total RNA was reverse transcribed, and the resulting cDNA was subjected to real-time PCR using predesigned primers and probes for rat *Hmgcr* and *Mvk* as described in *Materials and Methods*. **C**) The graph shows the change in *Hmgcr* mRNA expression normalized for *18S* rRNA. **D**) The graph shows the change in *Mvk* mRNA expression normalized for *18S* rRNA. Error bars represent the mean ± SE of two independent experiments, triplicate determinations in each, n = 6. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control. a, Represents significant differences ($P < 0.01$) compared with hCG 25 ng/ml.

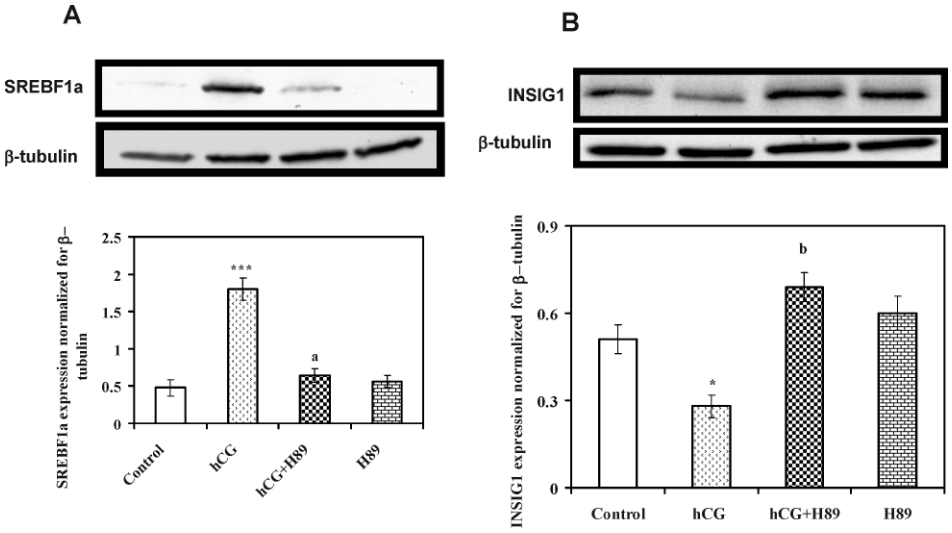

FIG. 3. Effect of hCG and PKA inhibitor on SREBF1a and INSIG1 proteins in T-I cells. Cells were pretreated with the PRKA inhibitor H89 (10 μM) for 1 h, followed by hCG (50 ng/ml) for 4 h. Whole-cell lysates were analyzed for the expression of the active form of SREBF1a (**A**) and INSIG1 (**B**) by Western blot analysis (top panels). The graphs of the densitometric scans are shown in the bottom panels. Protein loading was monitored by stripping and reprobing the same blot with antibody for β-tubulin. Blots are representative of one experiment, and the graphs represent the mean of three experiments. Error bars represent the mean ± SE. * $P < 0.05$ and *** $P < 0.001$ vs. control. a, Represents significant differences ($P < 0.001$) compared with hCG. b, Represents significant differences ($P < 0.01$) compared with hCG.

was performed using an SREBF1a antibody specific for the active form. The results in Figure 2A show that hCG treatment caused a significant increase in the active form of SREBF1a protein.

As stated in the *Introduction*, INSIGs are proteins associated with the ER that block proteolytic activation of SREBFs. To determine whether hCG negatively regulates INSIG1 protein, cultured cells were treated with hCG at different concentrations (0, 25, and 50 ng/ml) for 4 h, and INSIG1 levels were analyzed by Western blot. The results show that hCG caused a decrease in the INSIG1 level at all doses tested (Fig. 2B). This indicates that the increase in SREBF1a in response to hCG treatment is accompanied by a significant reduction in the INSIG1 level, suggesting that the hCG-mediated decrease in INSIG1 allows the increase in the active form of SREBF1a.

Analysis of Transcriptional Target of SREBF

We next sought to determine if the increase in the active form of SREBF was accompanied by a corresponding increase in the mRNA levels of SREBF transcriptional targets. Following 4 h of hCG treatment (50 ng/ml), total RNA was extracted, and the mRNA levels of two representative target genes, *Hmgcr* and *Mvk*, were determined by real-time PCR. The results in Figure 2, C and D, show a significant increase in mRNA levels of SREBF1a target genes *Hmgcr* and *Mvk* in response to hCG.

Effect of PRKA Inhibitor on hCG-Stimulated SREBF1a and INSIG1 Protein Expression

The contribution of the PRKA pathway in the hCG-mediated increase in SREBF1a protein level was then

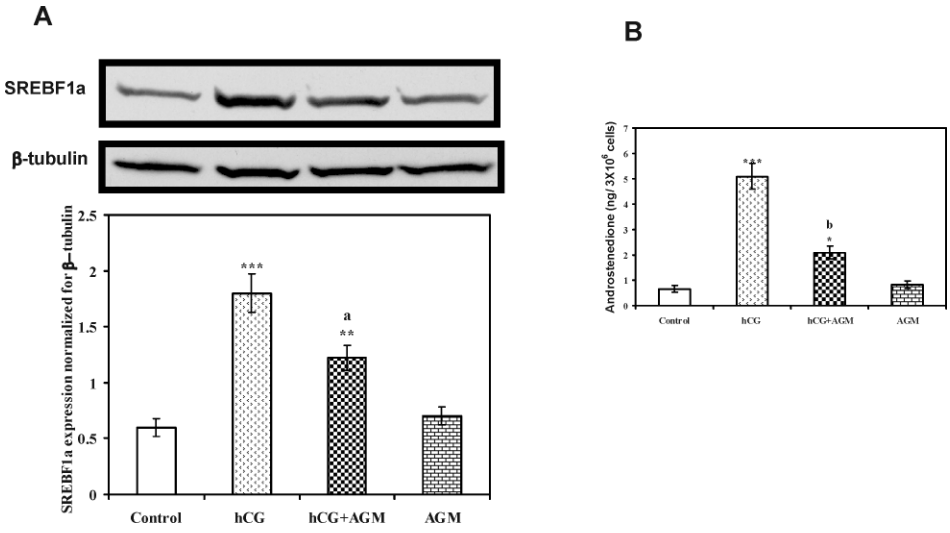

FIG. 4. Effect of hCG and AGM on SREBF1a protein expression and androstenedione production in T-I cells. Cells were treated with AGM (5 μg/ml) for 1 h, followed by hCG (50 ng/ml) for 4 h. **A**) Whole-cell lysates were analyzed for the active form of SREBF1a by Western blot analysis (top panel). The graph of the densitometric scans is shown in the bottom panel. Protein loading was monitored by stripping and reprobing the same blot with antibody for β-tubulin. **B**) The media were collected, and androstenedione was extracted and measured by RIA as described in *Materials and Methods*. The blot in **A** is representative of one experiment, and both graphs represent the mean of three experiments. Error bars represent the mean ± SE. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control. a, Represents significant differences ($P < 0.01$) compared with hCG. b, Represents significant differences ($P < 0.001$) compared with hCG.

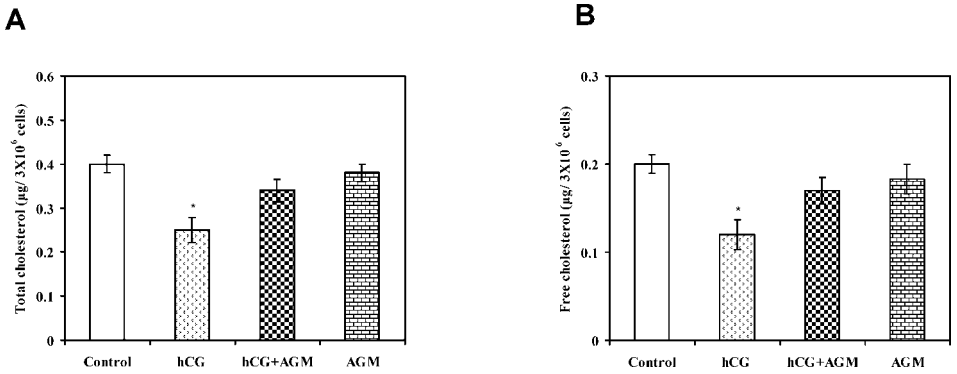

FIG. 5. Effect of hCG and AGM on cellular total (**A**) and free (**B**) cholesterol levels in T-I cells. Cells were treated with AGM (5 µg/ml) for 1 h, followed by hCG (50 ng/ml) for 4 h, and lysed with 1% Triton X-100 in choloroform. Total and free cholesterol contents of the cell lysates were determined using the cholesterol/cholesteryl ester quantitation kit as described in *Materials and Methods*. The graph represents the mean of three experiments. Data are expressed as the mean ± SE. * $P < 0.05$ vs. control.

examined. To test this, cultured cells were preincubated with a PRKA-specific inhibitor (H89 [10 µM]) for 1 h, followed by stimulation with hCG (50 ng/ml) for 4 h. The active form of SREBF1a was then analyzed by Western blot (Fig. 3A). The results show that, while hCG treatment alone caused a three-fold increase in the expression of the active form of SREBF1a, the addition of PRKA inhibitor completely abolished this increase, suggesting that hCG-stimulated SREBF1a activation proceeds through a PRKA-mediated pathway.

Because INSIGs are negative regulators of SREBF1 processing, the effect of PRKA inhibitor on INSIG1 was then examined. The cultured cells were preincubated with PRKA inhibitor (H89 [10 µM]) for 1h, followed by hCG (50 ng/ml) stimulation for 4 h. Whole-cell lysates were analyzed for INSIG1 protein as described in *Materials and Methods*. The results in Figure 3B show that the cells treated with hCG plus PRKA inhibitor exhibited a two-fold increase in INSIG1 protein level compared with those treated with hCG alone. Thus, PRKA inhibition abrogated the inhibitory effect of hCG on INSIG1.

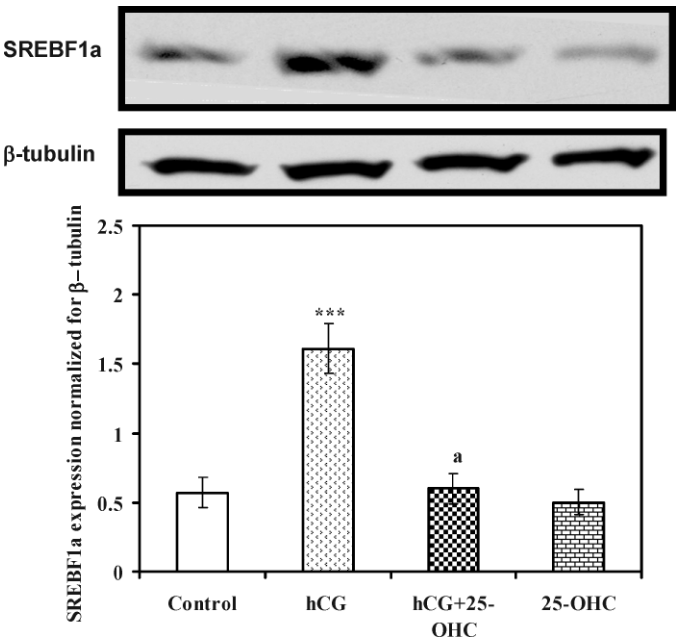

FIG. 6. Effect of hCG and 25-OHC on SREBF1a protein expression. Cells were pretreated with 25-OHC (10 µg/ml) for 1 h, followed by hCG (50 ng/ml) for 4 h. Whole-cell lysates were analyzed for the active form of SREBF1a by Western blot analysis (top panel). The graph of the densitometric scans is shown in the bottom panel. Protein loading was monitored by stripping and reprobing the same blot with antibody for β-tubulin. The blot is representative of one experiment, and the graph represents the mean of three experiments. Error bars represent the mean ± SE. *** $P < 0.001$ vs. control. a, Represents significant differences ($P < 0.001$) compared with hCG.

Effect of AGM on hCG-Stimulated SREBF1a Protein Expression

The stimulatory effect of hCG on SREBF1a could be attributed to a direct stimulation of the hCG-mediated signaling pathways or an indirect effect produced in response to cholesterol depletion brought about by stimulation of steroidogenesis. To examine this, we used AGM, which is known to inhibit steroidogenesis, thereby preventing cholesterol depletion. The ability of hCG to induce SREBF1a with or without AGM pretreatment was then examined. The results in Figure 4A show a significant increase in SREBF1a protein level in response to hCG. However, in cells treated with hCG plus AGM, the stimulatory effect of hCG was significantly reduced. Because the stimulatory effect was not completely abolished, these results also suggest that SREBF1a might be regulated through both cholesterol depletion and direct activation independent of cholesterol depletion.

Effect of AGM on hCG-Stimulated Androstenedione Production

To show that AGM is exerting its expected effect on hCG-stimulated androstenedione production, T-I cells were pretreated with AGM (5 µg/ml) for 1 h, followed by stimulation with hCG (50 ng/ml) for 4 h. The incubation media were collected and quantified for androstenedione by RIA. The results in Figure 4B show that, while androstenedione production was increased approximately six-fold in hCG-stimulated theca cells compared with the control, AGM significantly decreased this hCG-stimulated response. The AGM alone had no effect on basal androstenedione production. These results show that AGM exerted an inhibitory effect on androstenedione production as expected.

Effect of hCG and AGM on Cholesterol Content

To evaluate the effect of hCG and AGM on total and free cholesterol, cultured T-I cells were pretreated with AGM (5 µg/ml) for 1 h, followed by the addition of hCG (50 ng/ml) for 4 h. Cells were lysed with 1% Triton X-100 in choloroform, and total and free cholesterol contents were estimated as described in *Materials and Methods*. The results in Figure 5, A and B, show that treatment with hCG significantly reduced the total and free cholesterol content. However, pretreatment with AGM abolished the effect of hCG on total and free cholesterol levels. These results suggest that hCG treatment increases the

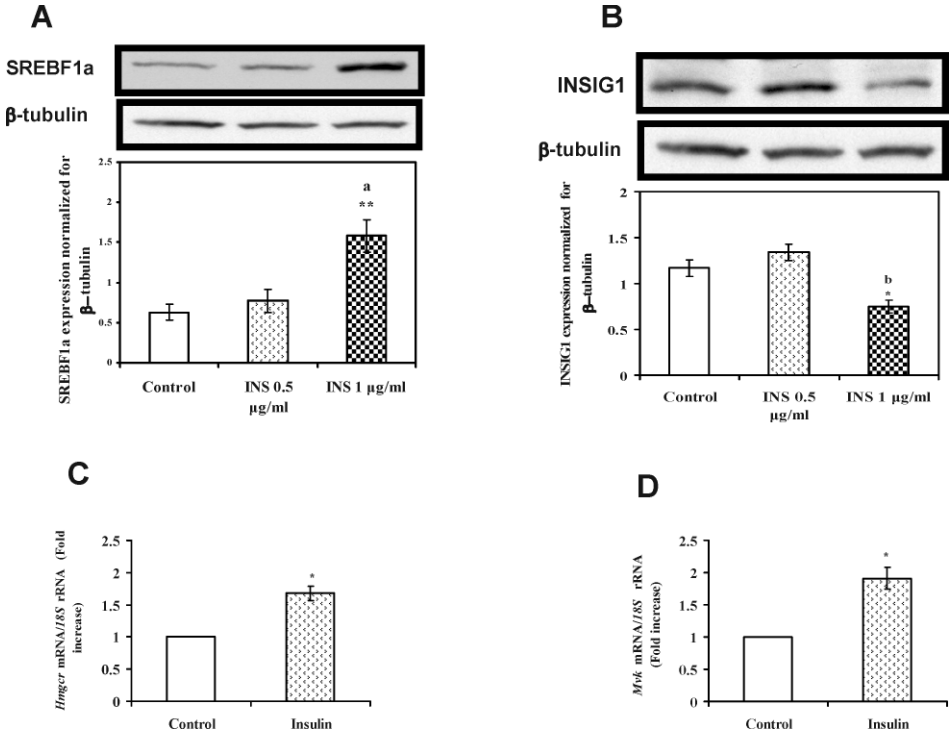

FIG. 7. Insulin-mediated regulation of SREBF1a and its target genes. Cells were treated with different concentrations of insulin (0, 0.5, and 1 μg/ml) for 4 h. Whole-cell lysates were analyzed for the expression of the active form of SREBF1a (**A**) and INSIG1 (**B**) by Western blot analysis (top panels). The graphs of the densitometric scans are shown in the bottom panels of **A** and **B**. Protein loading was monitored by stripping and reprobing the same blot with antibody for β-tubulin. Blots are representative of one experiment, and the graphs represent the mean of three experiments. Error bars represent the mean ± SE. **C** and **D**) The T-I cells were treated with insulin (1 μg/ml) for 4 h. Total RNA was reverse transcribed, and the resulting cDNA was subjected to real-time PCR using predesigned primers and probes for rat *Hmgcr* and *Mvk* as described in *Materials and Methods*. **C**) The graph represents the change in *Hmgcr* mRNA expression normalized for *18S* rRNA. **D**) The graph represents the change in *Mvk* mRNA expression normalized for *18S* rRNA. Error bars represent the mean ± SE of two independent experiments, triplicate determinations in each, n = 6. * $P < 0.05$ and ** $P < 0.01$ vs. control. a, Represents significant differences ($P < 0.05$) compared with insulin (INS) 0.5 μg/ml. b, Represents significant differences ($P < 0.01$) compared with INS 0.5 μg/ml.

utilization of cellular cholesterol as a result of increased conversion to androgens.

Effect of 25-OHC on hCG-Stimulated SREBF1a Protein Level

To further delineate the regulation of SREBF1a, cultured cells were pretreated with 25-OHC for 1 h, followed by the addition of hCG (50 ng/ml) for 4 h. Cells were harvested, and Western blot analysis was performed using anti-SREBF1a antibody. The results show that pretreatment with 25-OHC prevented the increase in the active form of SREBF1a seen in response to hCG treatment alone (Fig. 6). These results suggest that in T-I cells SREBF1a is regulated by cellular cholesterol. This supports the earlier experiments that showed that the effect of hCG on SREBF1a levels might proceed through cholesterol depletion brought about by its conversion to androgens.

Effect of Insulin on SREBF1a and INSIG1 Protein Level

As mentioned in the *Introduction*, T-I cells are also a target for insulin to increase androgen production. Furthermore, insulin has been shown to increase cholesterol accumulation in T-I cells [33]. Because SREBF1a has an important role in the biosynthesis and uptake of cholesterol, the effect of insulin on SREBF1a was examined. Cultures of T-I cells were treated with different doses of insulin (0, 0.5, and 1 μg/ml) for 4 h. Whole-cell lysates were analyzed for SREBF1a protein level by Western blot analysis. The results show that insulin at a concentration of 1 μg/ml significantly increased the active form of SREBF1a compared with the control (Fig. 7A).

To examine the possible effect of insulin on INSIG levels, T-I cells were treated with different concentrations of insulin (0, 0.5, and 1 μg/ml) for 4 h, and whole-cell lysates were analyzed for INSIG1 protein levels. The results in Figure 7B show that insulin treatment at a concentration of 1 μg/ml significantly reduced the INSIG1 protein level compared with the control. Thus, insulin-mediated reduction in INSIG1 may be associated with the increase in the active form of SREBF1a. To examine if the insulin stimulation of the active form of SREBF1a was accompanied by a corresponding increase in the transcription of SREBF target genes, cells were treated with insulin (1 μg/ml) for 4 h, followed by total RNA isolation and real-time PCR. *Hmgcr* and *Mvk* mRNA levels showed an increase in response to insulin treatment (Fig. 7, C and D, respectively). These results suggest that the increase in the active form of SREBF1a produced by insulin is accompanied by an increase in the transcription of SREBF1a target genes.

Effect of 25-OHC on Insulin-Stimulated SREBF1a Protein Level

To assess the regulation of SREBF1a, cultured cells were pretreated with 25-OHC (10 μg/ml) for 1 h, followed by the addition of insulin (1 μg/ml) for 4 h. Cells were lysed, and Western blot analysis was performed using anti-SREBF1a antibody. The results show that pretreatment with 25-OHC did not cause a decrease in the insulin-induced SREBF1a levels

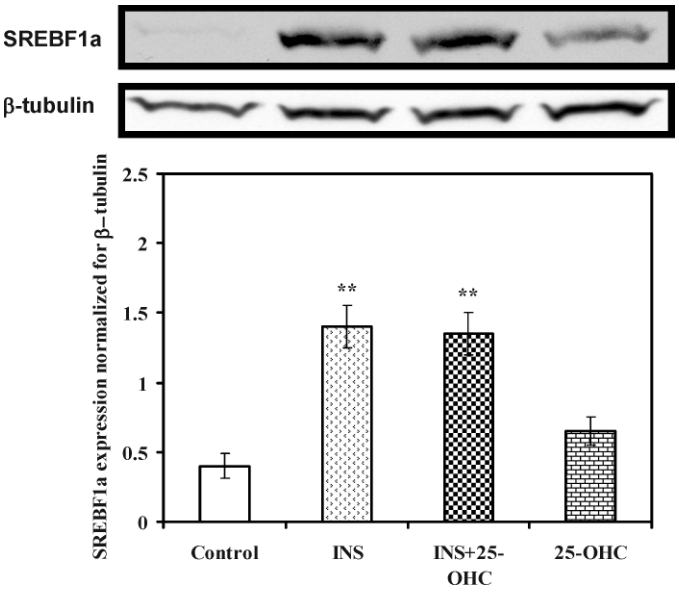

FIG. 8. Effect of insulin and 25-OHC on SREBF1a protein expression. Cells were pretreated with 25-OHC (10 μg/ml) for 1 h, followed by insulin (1 μg/ml) for 4 h. Whole-cell lysates were analyzed for the active form of SREBF1a by Western blot analysis (top panel). The graph of the densitometric scans is shown in the bottom panel. Protein loading was monitored by stripping and reprobing the same blot with antibody for β-tubulin. The blot is representative of one experiment, and the graph represents the mean of three experiments. Error bars represent the mean ± SE. ** $P < 0.01$ vs. control. INS, insulin.

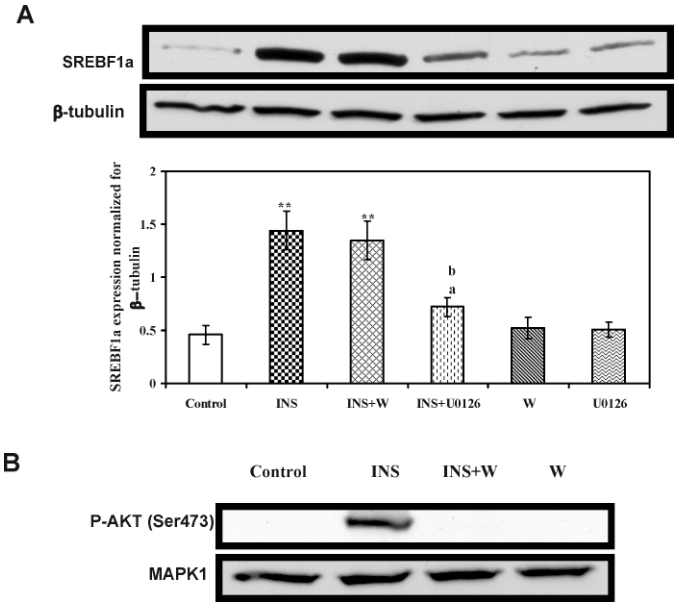

FIG. 9. Effect of PIK3 and MAP kinase inhibitors on SREBF1a protein expression in T-I cells. Cells were pretreated with the PIK3 inhibitor wortmannin (100 nM) for 30 min or the MAP2K inhibitor U0126 (10 μM) for 1 h, followed by insulin (1 μg/ml) for 4 h. Whole-cell lysates were analyzed for the expression of the active form of SREBF1a using Western blot analysis. The graph of the densitometric scans is shown in **A**. Protein loading was monitored by stripping and reprobing the same blot with antibody for β-tubulin. The blot is representative of one experiment, and the graph represents the mean of three experiments. Error bars represent the mean ± SE. ** $P < 0.01$ vs. control. a, Represents significant differences ($P < 0.05$) compared with insulin (INS). b, Represents significant differences ($P < 0.05$) compared with INS + wortmannin (W). **B**) The T-I cells were pretreated with the PIK3 inhibitor wortmannin (100 nM) for 30 min, followed by insulin (1 μg/ml) for 30 min. Western blot analysis was performed with antibody for phosphorylated AKT, and the same blot was stripped and reprobed with antibody for total MAPK1 to normalize for protein loading. The blot represents one of two independent experiments.

(Fig. 8). These findings suggest that insulin-mediated SREBF1a expression appears to be independent of sterol metabolism.

Effect of PIK3 and MAP Kinase Inhibitors on Insulin-Stimulated SREBF1a Protein Expression

Because insulin signaling occurs through phophatidylinositol 3-kinase and MAP kinase pathways, inhibitors of these two pathways were used to determine the signaling pathway involved in insulin-mediated activation of SREBF1a. Cultured cells were treated with PIK3 inhibitor wortmannin (100 nM) for 30 min or with MAP2K inhibitor U0126 (10 μM) for 1 h, followed by treatment with insulin (1 μg/ml) for 4 h. The results show that inhibiting PIK3 did not block the increase in SREBF1a in response to insulin, whereas inhibition of the MAP kinase pathway caused a reduction of SREBF1a levels, suggesting that insulin activates SREBF1a through a MAP kinase-dependent pathway (Fig. 9A).

The inhibitory potential of the PIK3 inhibitor wortmannin was verified by examining the phosphorylation of AKT under insulin-stimulated conditions. Cells were pretreated with wortmannin (100 nM) for 30 min, followed by the addition of insulin (1 μg/ml) for 30 min. Whole-cell lysates were analyzed for AKT (Ser 473) phosphorylation by Western blot analysis. The results in Figure 9B show that cells treated with insulin increased phosphorylation of AKT, whereas pretreatment with wortmannin completely abolished insulin-mediated phosphorylation of AKT.

DISCUSSION

It is well established that LH and insulin stimulate androgen biosynthesis in T-I cells [8, 9, 11, 15, 34, 35]. The steroidogenic cells derive cholesterol through de novo synthesis and by uptake of plasma-derived cholesterol [11, 25, 36]. Because SREBFs are well-known principal transcriptional regulators of cholesterol biosynthesis, the present study examined the possible regulation of SREBF1a and INSIG1 in response to hCG and insulin. Our results show that the increase in the active form of SREBF1a in response to hCG treatment was accompanied by a corresponding increase in the mRNA levels of SREBF target genes such as *Hmgcr* and *Mvk*. The hCG treatment also produced a decrease in the level of INSIG1, which is a repressor of SREBF processing. When the cellular demand for cholesterol rises, the SCAP-SREBF complex detaches from the INSIG1 and localizes in the Golgi. INSIG1 is then rapidly ubiquitinated and degraded [26, 37, 38]. In the present study, we show that the INSIG1 level is significantly reduced in response to hCG, suggesting that the decreased level of INSIG1 facilitates the conversion of the inactive SREBF precursor to the active form. Furthermore, LH/hCG might have a direct or an indirect stimulatory effect on SREBF1a, as inclusion of AGM, an inhibitor of CYP11A1, did not completely reduce the hCG response. Thus, the increase in SREBF1a might result from a depletion of cholesterol caused by increased conversion of cholesterol to androgens, as well as direct activation by hCG independent of its effect on cholesterol depletion. In addition, hCG treatment produced depletion of cellular total and free cholesterol as expected due to increased conversion of cholesterol to androgens. To provide further evidence that SREBF1a is regulated by cellular

cholesterol, pretreatment with 25-OHC abrogated the hCG-mediated increase in SREBF1a. Our results are consistent with previous studies that have shown the involvement of SREBF in cellular processes related to steroid biosynthesis such as regulation of the steroidogenic acute regulatory protein gene [39, 40], low-density lipoprotein receptor expression in porcine granulosa cells [41], and promoter activity of the rat high-density lipoprotein receptor, scavenger receptor class B member I (SCARB1) [42].

Because activation of PRKA has been shown to be involved in LH/hCG-stimulated steroidogenesis [43–45], the role of PRKA in stimulating SREBF1a in response to hCG was examined. Our results show that PRKA inhibitor completely blocked the stimulatory effect of hCG on SREBF1a. Furthermore, a combination of PRKA inhibitor and hCG treatment increased the INSIG1 level. These results suggest that hCG stimulates SREBF1a through PRKA activation. In addition, we have shown that insulin stimulation of SREBF1a appears to be mediated through the MAPK3 pathway, as inhibition of the MAPK3 pathway (but not the PIK3 pathway) reduced the insulin response.

The regulation of SREBF1a in T-I cells by insulin has particular significance because hyperinsulinemia is closely associated with hyperandrogenic conditions such as PCOS [1, 4]. In this context, our previous investigations have shown that insulin increases the expression of SCARB1 in T-I cells [33]. Furthermore, the findings by Lopez and McLean [42] that SREBF1a binds to the promoter elements of SCARB1 suggest that the stimulatory effect of insulin on cholesterol transport in T-I cells might be mediated through SREBF1a. On the basis of our results, we speculate that in hyperinsulinemic states T-I cells may respond to insulin by up-regulating SREBF1a. This increase in SREBF1a then causes increased transcription of SRE-responsive genes, which are involved in both the synthesis and uptake of cholesterol. Thus, increased availability of cholesterol in T-I cells in response to hCG and insulin might contribute to chronically elevated androgen levels. Furthermore, insulin might have a direct stimulatory effect on SREBF1a, independent of changes in cellular cholesterol levels, as the addition of 25-OHC did not cause a decrease in the insulin-induced SREBF1a levels.

In summary, the findings reported herein support that hCG and insulin stimulate the active form of SREBF1a. Furthermore, the elevated levels of androgens seen in hyperandrogenic conditions in response to LH and insulin may be mediated by an increase in SREBF1a, resulting in increased cholesterol accumulation in T-I cells.

ACKNOWLEDGMENTS

We express our appreciation to Helle Peegal, Dr. Pradeep Kayampilly, Dr. Anil K Nair, Dr. Bindu Menon, and Dr. Thippeswamy Gulappa for their critical reading of the manuscript and valuable comments. We thank the University of Michigan Hybridoma Core for SREBF1a monoclonal antibody generation.

REFERENCES

1. Barbieri R. Polycystic ovarian disease. Annu Rev Med 1991; 42:199–204.
2. Dunaif A, Thomas A. Current concepts in the polycystic ovary syndrome. Annu Rev Med 2001; 52:401–419.
3. Magoffin DA. The ovarian androgen-producing cells: a 2001 perspective. Rev Endocr Metab Disord 2002; 3:47–53.
4. Azziz R. Androgen excess is the key element in polycystic ovary syndrome. Fertil Steril 2003; 80:252–254.
5. Cara JF, Rosenfield RL. Insulin-like growth factor I and insulin potentiate luteinizing hormone-induced androgen synthesis by rat ovarian thecal-interstitial cells. Endocrinology 1988; 123:733–739.
6. Caubo B, DeVinna RS, Tonetta SA. Regulation of steroidogenesis in cultured porcine theca cells by growth factors. Endocrinology 1989; 125: 321–326.
7. Magarelli PC, Zachow RJ, Magoffin DA. Developmental and hormonal regulation of rat theca-cell differentiation factor secretion in ovarian follicles. Biol Reprod 1996; 55:416–420.
8. Duleba AJ, Spaczynski RZ, Olive DL, Behrman HR. Effects of insulin and insulin-like growth factors on proliferation of rat ovarian theca-interstitial cells. Biol Reprod 1997; 56:891–897.
9. Duleba AJ, Spaczynski RZ, Olive DL, Behrman HR. Divergent mechanisms regulate proliferation/survival and steroidogenesis of theca-interstitial cells. Mol Hum Reprod 1999; 5:193–198.
10. Zhang G, Garmey JC, Veldhuis JD. Interactive stimulation by luteinizing hormone and insulin of the steroidogenic acute regulatory (StAR) protein and 17alpha-hydroxylase/17,20-lyase (CYP17) genes in porcine theca cells. Endocrinology 2000; 141:2735–2742.
11. Wu Q, Sucheta S, Azhar S, Menon KMJ. Lipoprotein enhancement of ovarian theca-interstitial cell steroidogenesis: relative contribution of scavenger receptor class B (type I) and adenosine 5′-triphosphate-binding cassette (type A1) transporter in high-density lipoprotein-cholesterol transport and androgen synthesis. Endocrinology 2003; 144:2437–2445.
12. Kwintkiewicz J, Spaczynski RZ, Foyouzi N, Pehlivan T, Duleba AJ. Insulin and oxidative stress modulate proliferation of rat ovarian theca-interstitial cells through diverse signal transduction pathways. Biol Reprod 2006; 74:1034–1040.
13. Tajima K, Orisaka M, Mori T, Kotsuji F. Ovarian theca cells in follicular function. Reprod Biomed Online 2007; 15:591–609.
14. Schirman-Hildesheim TD, Gershon E, Litichever N, Galiani D, Ben-Aroya N, Dekel N, Koch Y. Local production of the gonadotropic hormones in the rat ovary. Mol Cell Endocrinol 2008; 282:32–38.
15. Towns R, Azhar S, Peegel H, Menon KMJ. 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. Endocrine 2005; 27:269–277.
16. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 1997; 89:331–340.
17. Goldstein JL, DeBose-Boyd RA, Brown MS. Protein sensors for membrane sterols. Cell 2006; 124:35–46.
18. Bengoechea-Alonso MT, Ericsson J. SREBP in signal transduction: cholesterol metabolism and beyond. Curr Opin Cell Biol 2007; 19:215–222.
19. Hua X, Wu J, Goldstein JL, Brown MS, Hobbs HH. Structure of the human gene encoding sterol regulatory element binding protein-1 (SREBF1) and localization of SREBF1 and SREBF2 to chromosomes 17p11.2 and 22q13. Genomics 1995; 25:667–673.
20. Osborne TF. Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. J Biol Chem 2000; 275:32379–32382.
21. Osborne TF. CREating a SCAP-less liver keeps SREBPs pinned in the ER membrane and prevents increased lipid synthesis in response to low cholesterol and high insulin. Genes Dev 2001; 15:1873–1888.
22. Espenshade PJ. SREBPs: sterol-regulated transcription factors. J Cell Sci 2006; 119:973–976.
23. Eberlé D, Hegarty B, Bossard P, Ferré P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie 2004; 86:839–848.
24. Brown MS, Goldstein JL. Sterol regulatory element binding proteins (SREBPs): controllers of lipid synthesis and cellular uptake. Nutr Rev 1998; 56:S54–S75.
25. Azhar S, Reaven E. Scavenger receptor class BI and selective cholesteryl ester uptake: partners in the regulation of steroidogenesis. Mol Cell Endocrinol 2002; 195:1–26.
26. Sun LP, Seemann J, Goldstein JL, Brown MS. Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Insig renders sorting signal in Scap inaccessible to COPII proteins. Proc Natl Acad Sci U S A 2007; 104:6519–6526.
27. Edwards PA, Tabor D, Kast HR, Venkateswaran A. Regulation of gene expression by SREBP and SCAP. Biochim Biophys Acta 2000; 1529: 103–113.
28. Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS. Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell 2002; 110:489–500.
29. Rawson RB, Zelenski NG, Nijhawan D, Ye J, Sakai J, Hasan MT, Chang TY, Brown MS, Goldstein JL. Complementation cloning of S2P, a gene encoding a putative metalloprotease required for intramembrane cleavage of SREBPs. Mol Cell 1997; 1:47–57.

30. Yabe D, Brown MS, Goldstein JL. Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins. Proc Natl Acad Sci U S A 2002; 99:12753–12758.
31. Janowski BA. The hypocholesterolemic agent LY295427 up-regulates INSIG-1, identifying the INSIG-1 protein as a mediator of cholesterol homeostasis through SREBP. Proc Natl Acad Sci U S A 2002; 99:12675–12680.
32. Towns R, Menon KMJ. The role of cyclic AMP response element binding protein in transactivation of scavenger receptor class B type I promoter in transfected cells and in primary cultures of rat theca-interstitial cells. Mol Cell Endocrinol 2005; 245:23–30.
33. Li X, Peegel H, Menon KMJ. Regulation of high density lipoprotein receptor messenger ribonucleic acid expression and cholesterol transport in theca-interstitial cells by insulin and human chorionic gonadotropin. Endocrinology 2001; 142:174–181.
34. Cara J. Insulin-like growth factors, insulin-like growth factor binding proteins and ovarian androgen production. Horm Res 1994; 42:49–54.
35. Munir I, Yen HW, Geller DH, Torbati D, Bierden RM, Weitsman SR, Agarwal SK, Magoffin DA. Insulin augmentation of 17alpha-hydroxylase activity is mediated by phosphatidyl inositol 3-kinase but not extracellular signal-regulated kinase-1/2 in human ovarian theca cells. Endocrinology 2004; 145:175–183.
36. Sekar N, Garmey JC, Veldhuis JD. Mechanisms underlying the steroidogenic synergy of insulin and luteinizing hormone in porcine granulosa cells: joint amplification of pivotal sterol-regulatory genes encoding the low-density lipoprotein (LDL) receptor, steroidogenic acute regulatory (stAR) protein and cytochrome P450 side-chain cleavage (P450scc) enzyme. Mol Cell Endocrinol 2000; 159:25–35.
37. Radhakrishnan A, Ikeda Y, Kwon HJ, Brown MS, Goldstein JL. Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. Proc Natl Acad Sci U S A 2007; 104:6511–6518.
38. Gong Y, Lee JN, Lee PC, Goldstein JL, Brown MS, Ye J. Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake. Cell Metab 2006; 3:15–24.
39. Shea-Eaton WK, Trinidad MJ, Lopez D, Nackley A, McLean MP. Sterol regulatory element binding protein-1a regulation of the steroidogenic acute regulatory protein gene. Endocrinology 2001; 142:1525–1533.
40. Christenson LK, Osborne TF, McAllister JM, Strauss JF. Conditional response of the human steroidogenic acute regulatory protein gene promoter to sterol regulatory element binding protein-1a. Endocrinology 2001; 142:28–36.
41. Sekar N, Veldhuis JD. Involvement of Sp1 and SREBF1a in transcriptional activation of the LDL receptor gene by insulin and LH in cultured porcine granulosa-luteal cells. Am J Physiol Endocrinol Metab 2004; 287: E128–E135.
42. Lopez D, McLean MP. Sterol regulatory element-binding protein-1a binds to cis elements in the promoter of the rat high density lipoprotein receptor SR-BI gene. Endocrinology 1999; 140:5669–5681.
43. Menon KMJ, Gunaga KP. Role of cyclic AMP in reproductive processes. Fertil Steril 1974; 25:732–750.
44. Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss JF III, Amsterdam A. The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. J Biol Chem 2001; 276:13957–13964.
45. Bogan RL, Niswender GD. Constitutive steroidogenesis in ovine large luteal cells may be mediated by tonically active protein kinase A. Biol Reprod 2007; 77:209–216.