**Endocrine,** *vol. 25, no. 2, 147–154, November 2004* 0969–711X/04/25:147–154/$25.00 

# Src Tyrosine Kinase Regulates CYP17 Expression and Androstenedione Secretion in Theca-Enriched Mouse Ovarian Cells

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**Src tyrosine kinase belongs to a non-receptor tyrosine kinase family and has been shown to be involved in G protein–coupled receptor desensitization and internalization. Stimulation of ovarian thecal cells with luteinizing hormone (LH) activates adenylyl cyclase via a G protein–coupled LH receptor leading to an increase in cAMP. Subsequently, cAMP activates protein kinase A (PKA) that increases steroidogenesis. In order to evaluate the role of Src in thecal cell steroidogenesis, a pharmacological approach was utilized by treating a population of mouse ovarian theca-enriched cells (TEC) in vitro with two Src inhibitors, geldanamycin (GA) and herbimycin A (HA). Treatment of TEC with either GA or HA increased basal androstenedione secretion without alteration of cAMP. In the presence of forskolin, GA and HA treatment further increased androstenedione secretion. RT-PCR analysis of RNA from cells treated with GA for 8, 24, and 48 h revealed that GA increased cytochrome P450 17α-hydroxylase/lyase (CYP17) mRNA at 48 h. CYP17 promoter activity also increased after treatment of cells with GA and after co-transfection with a Src dominant negative plasmid. Inhibition of PKA using H89 blocked the effect GA and HA on androstenedione secretion. These results indicate that the pharmacological inhibitors of Src, GA and HA, tested in vitro increased thecal CYP17 promoter activity, CYP17 mRNA, and androstenedione secretion. In addition, GA and HA induced thecal androstenedione secretion may be cAMP independent but possibly requires PKA.**

**Key Words:** Androstenedione; theca; ovary; steroidogenesis; cAMP.

## Introduction

Previous studies have shown that in vitro lipopolysaccharide (LPS or also called endotoxin) inhibited luteinizing hormone (LH)-stimulated thecal-interstitial cell androgen secretion in the rat *(1)*. The inhibitory effect of LPS could be blocked by addition of herbimycin, HA *(1)*, a drug with the ability to inhibit tyrosine kinases with Src selectivity *(2)*. Thus, Src tyrosine kinase appeared to be present in the ovary and yet its role was unknown. In addition, previous studies have shown that inhibition of Src pharmacologically using HA-treated rat thecal-interstitial cells increased LH-stimulated cAMP above that of LH alone *(1)*. In that study *(1)*, treatment with HA alone did not increase cAMP but a significant increase in androstenedione was observed *(3)*. Herbimycin was also very effective in reducing phosphodiesterase activity in LH-stimulated rat thecal-interstitial cells and was likely causal in the increased cAMP and steroidogenesis *(3)*.

Two studies related to Src regulation of steroidogenesis have utilized Leydig cells lines, which are embryologic homologs of theca *(3,4)*. Inhibition of Src in mouse MA-10 Leydig tumor cells and mouse TM3 Leydig cells through expression of a dominant negative Src kinase was associated with increased LH-stimulation of cAMP (MA10 and TM3 cells) and progesterone (MA10 cells) *(3,4)*. The increase in cAMP also coincided with a decrease in phosphodiesterase activity in the cell lines as observed in rat thecal-interstitial cells *(3,4)*. In a human adrenal cell line, inhibition of Src has been shown to increase mRNA of steroid acute regulatory protein (StAR), cytochrome P450 side chain cleavage enzyme, and 17α-hydroxylase-lyase (CYP17) *(5)*.

The purpose of the present study was to investigate whether CYP17 promoter activity and mRNA were increased in theca-enriched populations of mouse ovarian cells by pharmacological inhibitors that block Src tyrosine kinase and thus provide insight into the mechanism of increased ovarian androgen. The mouse model was of particular interest because of its utility for gene targeting in future studies. We report in this study using theca-enriched mouse ovarian dispersates (TEC) that pharmacological inhibition of Src increased CYP17 promoter activity and mRNA and androgen secretion.

Received August 23, 2004; Revised November 4, 2004; Accepted November 8, 2004.

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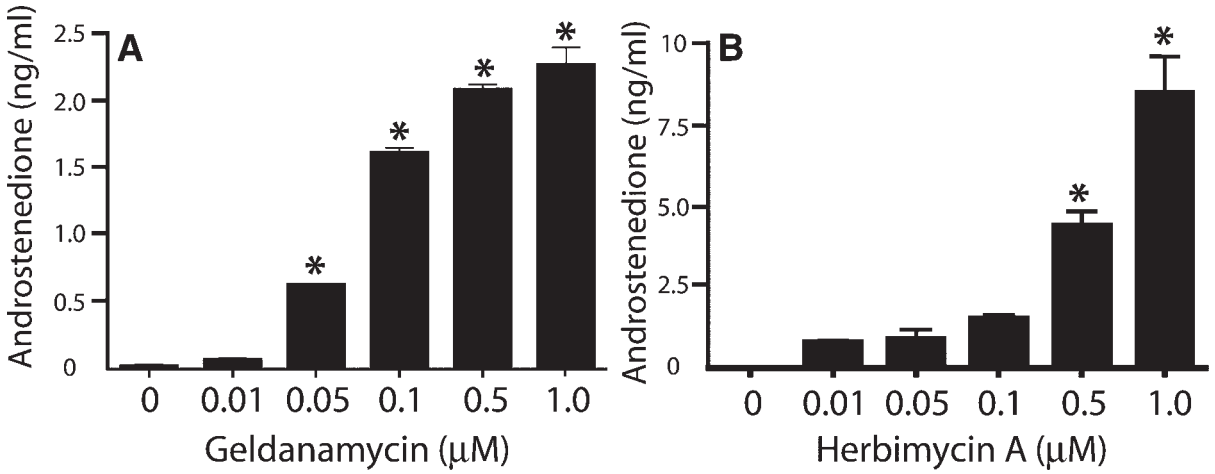



**Fig. 1.** Effects of various doses of geldanamycin and herbimycin A, ranging from 0.01 to 1 μ*M* final concentrations on androstenedione levels in the culture media of theca-enriched cells after 48 h. One-way ANOVA indicated $p \leq 0.05$ for GA and HA treatments. Asterisks indicate $p \leq 0.05$ vs "0" control by Tukey's test. Where no error bars are present, the standard errors were too small to appear.

## Results

### *Effects of Geldanamycin (GA) and Herbimycin (HA) on TEC Steroidogenesis and cAMP (Figs. 1 and 2)*

Effective concentrations of GA and HA in stimulating androstenedione were determined by dose responses (Fig. 1). GA at 0.1 μ*M* and HA at 0.5 μ*M* significantly and consistently increased androstenedione secretion and thus those concentrations of GA and HA were utilized throughout the remainder of the study. The 0.05 μ*M* GA also increased androstenedione above controls (0) but not as consistently as 0.1 μ*M* (Fig. 1). A time course at 24, 48, and 72 h revealed that the maximal secretion of steroids and cAMP was at 48 h and thus 48 h was used throughout the studies (data not shown).

When compared with controls, GA (Fig. 2A) and HA (Fig. 2B) increased androstenedione and decreased progesterone (but only HA significantly) without affecting cAMP or 17α-hydroxyprogesterone. Forskolin (FK), an adenylyl cyclase stimulator, significantly increased 17α-hydroxyprogesterone, progesterone, and cAMP accumulation (but not androstenedione) above control levels (Figs. 2A,B). Treatment with FK and GA (or HA) resulted in further increases in cAMP and androstenedione, whereas this combination either had no effect (HA + FK) on or slightly increased (GA + FK) 17α-hydroxyprogesterone (Figs. 2A,B). GA and HA, each alone, which reduced progesterone, also reduced FK-stimulated progesterone (Figs. 2A,B).

### *Effects of H89, a Protein Kinase A (PKA) Inhibitor, on TEC Steroidogenesis and cAMP (Fig. 3)*

H89 blocked the stimulatory effect of HA (Fig. 3A) and GA (Fig. 3B) on androstenedione secretion. H89 alone had no statistically significant effect on androstenedione accumulation when compared with the controls. GA, HA, and H89, each alone, reduced progesterone secretion. H89 reduced cAMP (Figs. 3A,B) but only significantly shown in Fig. 3B.

### *CYP17 Promoter Activity in TEC (Fig. 4)*

Because GA increased TEC secretion of androstenedione, the effects of GA on CYP17 promoter activity were investigated. GA increased CYP17 promoter activity by two- to threefold. TEC were also co-transfected with a Src dominant negative (DN) plasmid to suppress Src activity and this treatment also increased CYP 17 promoter activity by approx threefold.

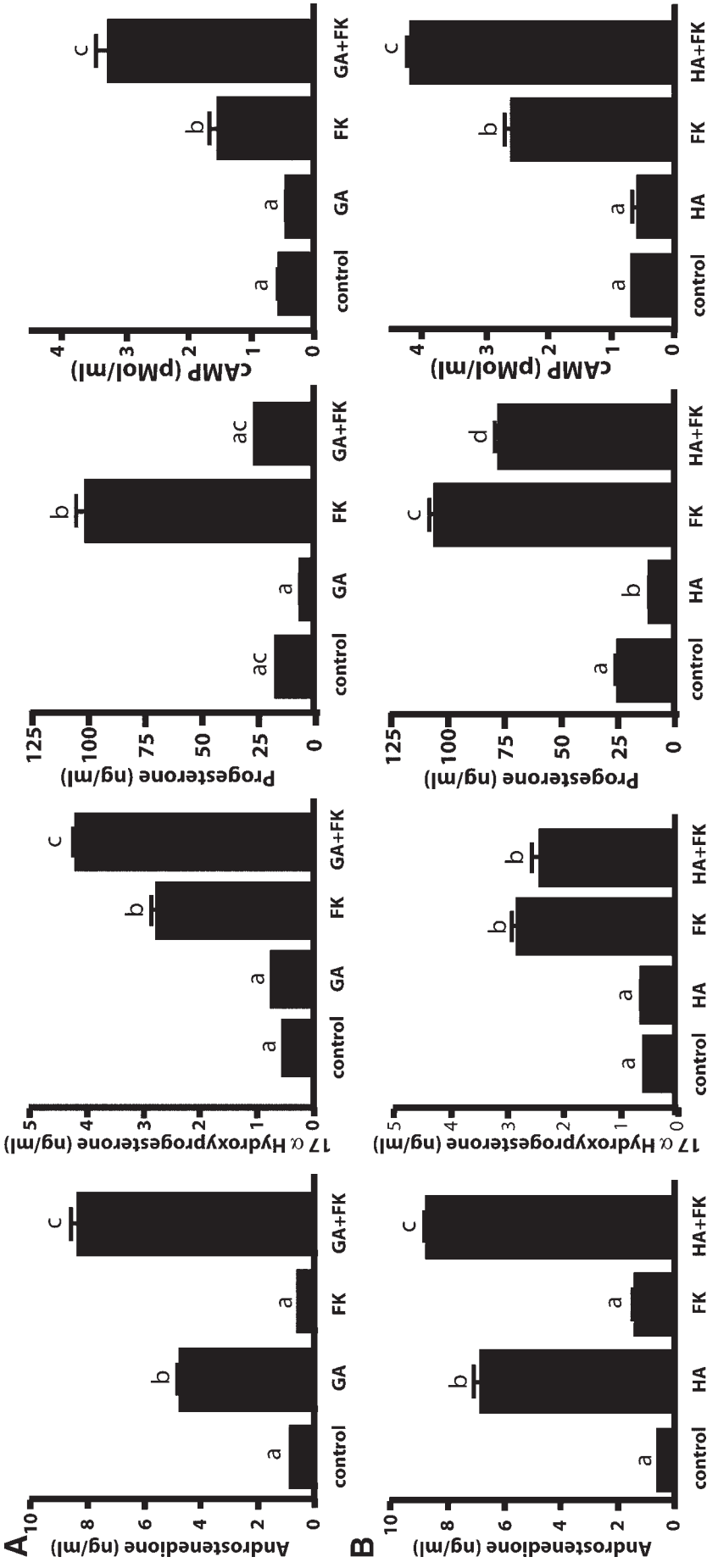
### *Effects of GA on CYP17 mRNA and 3β-HSD (Fig. 5)*

Steady-state levels of 3β-HSD mRNA did not change after GA treatment but CYP17 mRNA which was not detectable at 8 h and 24 h after GA treatment was detectable at 48 h.

## Discussion

A significant finding of this study was that the two pharmacological inhibitors of Src (GA and HA) increased androstenedione accumulation in mouse thecal cultures. These data implicate Src as a potential control point for thecal androgen synthesis since both CYP17 promoter activity and CYP17 mRNA were increased when Src was inhibited. The present data also support previous studies on the adrenal, whereby inhibition of Src tyrosine kinase increased androgens *(5)*. In the adrenal studies, inhibition of Src was associated with an increase in steroidogenic acute regulatory (StAR) protein and mRNA as well as cholesterol side-chain cleavage (CYP11A). Treatment of adrenal cells with a cAMP ago-

**Fig. 2.** *(Opposite page)* Androstenedione, 17α-hydroxyprogesterone, progesterone, and cAMP levels in media from theca-enriched cells treated in vitro for 48 h with (**A**) GA (0.1 μ*M*) and (**B**) HA (0.5 μ*M*) with and without Forskolin (FK, 10 μ*M*). Within each graph, Tukey's test revealed that bars with the different letters were statistically different ($p \leq 0.05$) from each other.


A
B
Androstenedione (ng/ml)
17 α Hydroxyprogesterone (ng/ml)
Progesterone (ng/ml)
cAMP (pMol/ml)
control
GA
FK
GA+FK
HA
HA+FK
a
b
c
d
ac

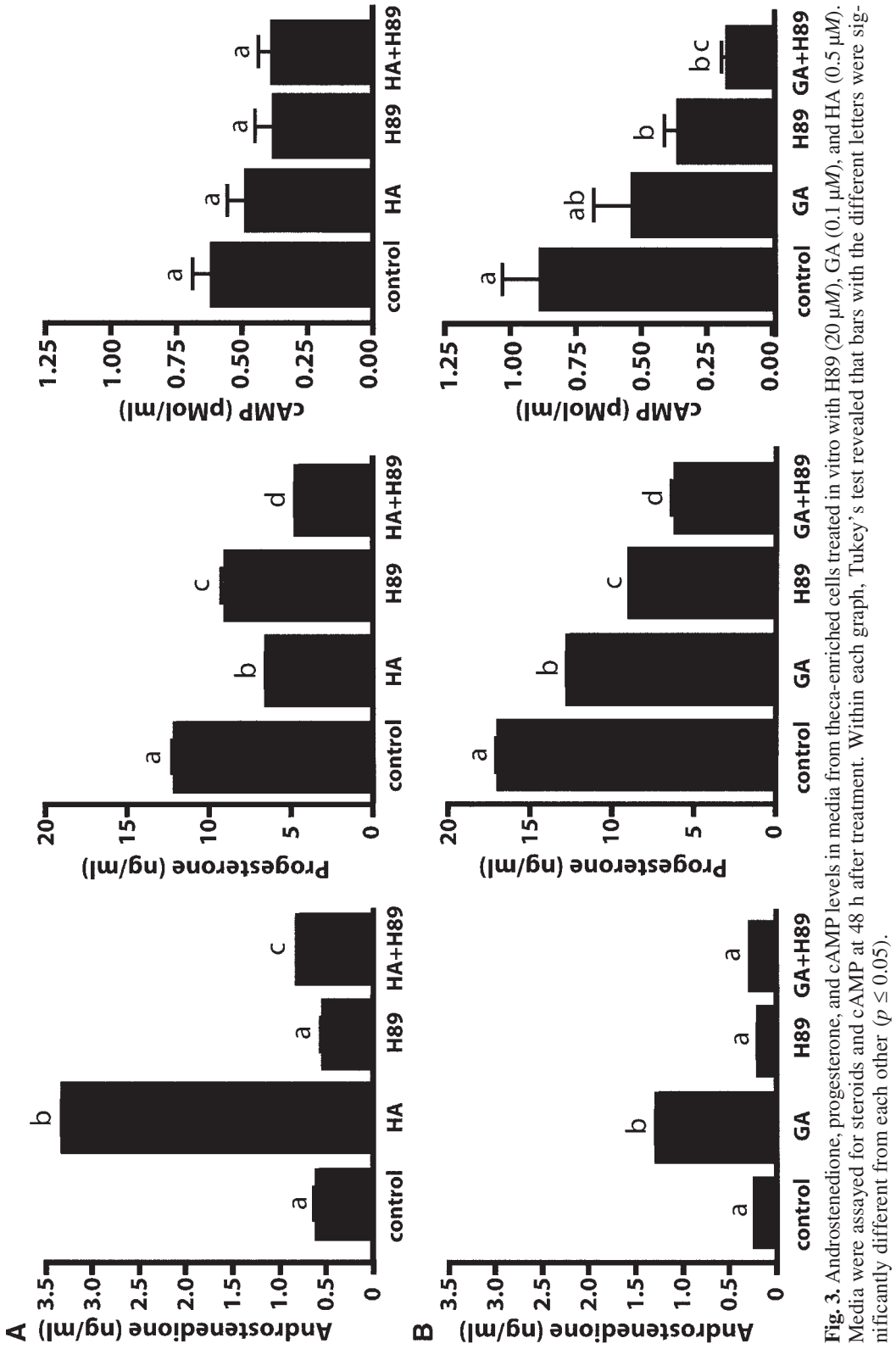



**Fig. 3.** Androstenedione, progesterone, and cAMP levels in media from theca-enriched cells treated in vitro with H89 (20 μ*M*), GA (0.1 μ*M*), and HA (0.5 μ*M*). Media were assayed for steroids and cAMP at 48 h after treatment. Within each graph, Tukey's test revealed that bars with the different letters were significantly different from each other ($p \leq 0.05$).

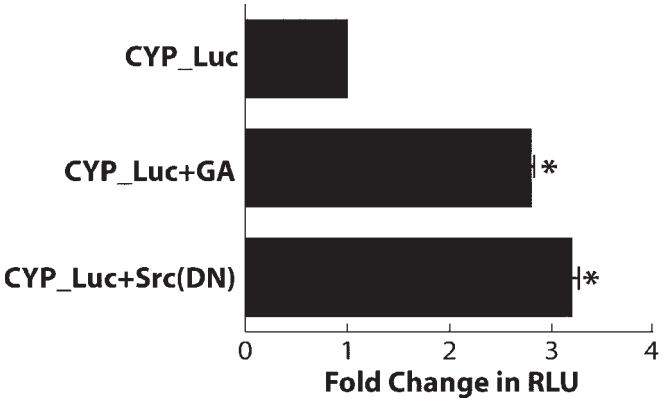



**Fig. 4.** Effects of geldanamycin (0.1 μ*M*, GA) on cytochrome P450 17α-hydroxylase promoter activity in theca-enriched cells after 48 h. Cells were treated with vehicle (CYP_Luc), CYP_Luc + GA (GA, 0.1 μ*M*) and CYP_Luc + Src (DN), Src dominant negative. Asterisks indicate $p \leq 0.05$ compared to CYP_Luc by Tukey's test.

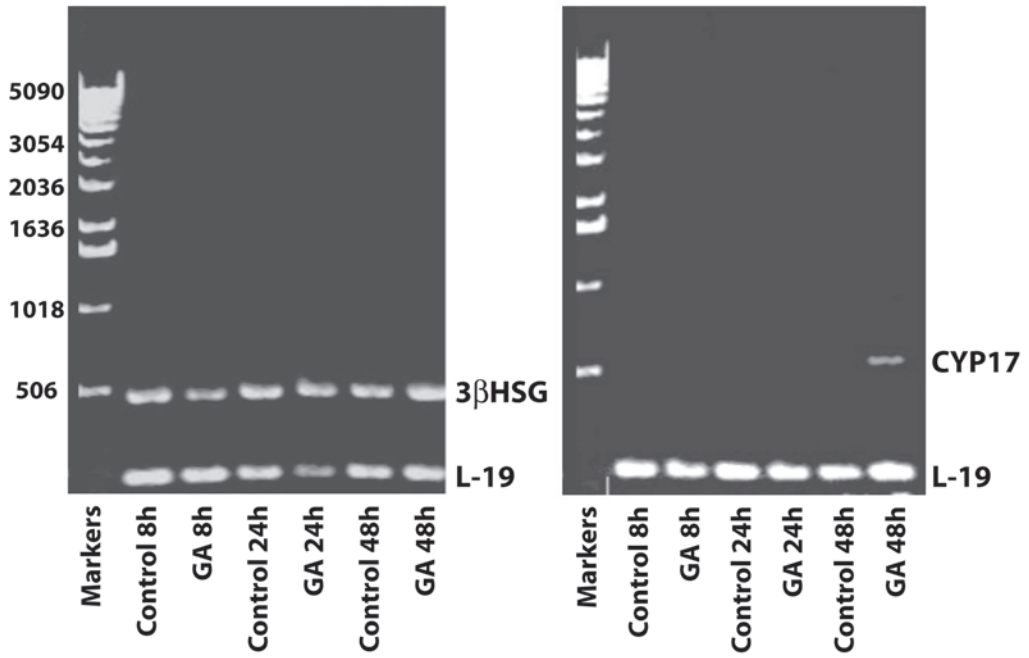



**Fig. 5.** Effects of geldanamycin (0.1 μ*M*, GA) on the expression level of cDNA reverse transcribed from mRNA of theca enriched cells, collected after 8, 24, and 48 h of treatment. 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17α-hyroxylase/lyase (CYP17) and L-19 (an internal control). This is a representative of two determinations using a different preparation of cells for each.

nist also increased dehydroepiandrosterone sulfate (DHEAS) and treatment with a Src inhibitor and dibutyryl cAMP, in combination, further increased the production of DHEAS *(6)* presumably through activation of 17α hydroxylase. The present studies using TEC extend and support the adrenal studies especially since androstenedione increased along with CYP17 mRNA and its promoter activity.

Both drugs, HA and GA, increased androstenedione while progesterone decreased (Fig. 2). CYP17 promoter activity and mRNA also increased and thus the enzyme, 17α-hydroxylase/lyase, likely converted progesterone to androstenedione. Thus, the decline in progesterone can be explained by its conversion to androstenedione. Levels of 17α-hydroxyprogesterone did not change with treatment throughout this period (Fig. 2). 17α-Hydroxyprogesterone is likely to be shuttled quickly to androstenedione and thus levels remain static as progesterone moves through this pathway while being converted to androstendione.

Previous studies using different model systems such as MA-10 and TM3 cell lines and rat theca revealed that HA *(3)* and a Src dominant negative plasmid *(4)* significantly increased LH-stimulated steroidogenesis by elevating cAMP, which correlated with reduction of phosphodiesterase activity. The present study did not observe an increase in mouse thecal cAMP secretion after HA treatment (Fig. 2B), which is consistent with that observed for rat theca *(3)*, but androstenedione was increased in both studies. In addition, in the present study, in the presence of FK (which increased cAMP),

GA and HA further increased cAMP (Fig. 2). Similar increases were observed in MA-10, TM3, and rat thecal cells with Src inhibitors and agents that increased cAMP such as luteinizing hormone, isobutylmethylxanthine, and forskolin.

One potential mechanism by which GA and HA may have increased androstenedione is through stimulation of PKA. H89 (a PKA inhibitor) completely blocked the stimulatory effect of GA and HA on TEC androstenedione secretion (Fig. 3). The question arises as to how PKA might be activated if not through a cAMP-dependent mechanism. Whether GA or HA could directly stimulate PKA or alter downstream effectors of the cAMP pathway is unknown. GA and HA did not activate PKA in an in vitro cell free assay (unpublished observation). Possibly, endogenous factors other than cAMP-stimulated PKA leading to cAMP-like responses *(7–9)*. Consideration must also be given to the nonspecificity of these pharmacological inhibitors especially since both of the inhibitors (HA and GA) bind heat shock protein-90, which is required not only for Src activation but other kinase activities as well *(10)*.

GA increased CYP17 mRNA at 48 h in theca-enriched cells (Fig. 5). Although RT-PCR is not one of the best ways to quantify mRNA levels, the result had more qualitative value, as CYP17 mRNA was present only in samples derived from TEC treated with GA for no less than 48 h. The androstenedione levels from conditioned media also had increased by that time (Figs. 1–3). The effect of GA appeared to be somewhat specific because 3β-HSD mRNA did not change over the course of treatment ranging from 8 h to 48 h (Fig. 5). The mechanism by which GA would increase CYP17 mRNA is unknown but may involve PKA because inhibition of PKA with H89 blocked the increase in androstenedione, which depends on CYP17 mRNA. Whether the increase in CYP17 mRNA induced by GA would have been blocked by H89 was not tested.

GA increased CYP17 promoter activity (Fig. 4) indicating that blockage of Src activity may lead to increased CYP17 mRNA. The increase in CYP17 mRNA and CYP17 promoter activity were likely responsible for the increased androstenedione secretion. However, androgens have been shown to inhibit CYP17 promoter activity *(11)*. Previous studies using MA-10 Leydig tumor cells have shown that androstenedione inhibits cAMP-induced activation of the CYP17 promoter via an androgen-receptor-dependent mechanism *(10,11)*. Apparently, in the TEC model androstenedione did not inhibit promoter activity since it was elevated (Fig. 2) when promoter activity was also increased (Fig. 4); however, we cannot rule out that other androgens may mediate this inhibitory effect.

Studies using a human adrenal cell line (H295R) have shown that transcriptional activation of CYP17 depends on complex formation among p54 (nrb)/NonO, protein-associated splicing factor, and steroidogenic factor-1 (SF-1) *(13)* and phosphatase activity *(14)*. PKA activated a phosphatase that dephosphorylated SF-1 and increased transcription of CYP17 *(14)*. Human CYP17 contains cAMP response elements (CREs) and thus is activated by cAMP unlike mouse CYP17 that does not contain CREs *(12)*. In the human, PKA activation induces binding of cAMP response element binding protein (CREB) to the two CRE sites in the promoter of mitogen-activated protein kinase phosphatase-1 (MKP-1) and forms, together with E-box-bound USF proteins, a complex able to promote transcription of MKP-1 *(15)*. The Ras/ERK pathway appears to be critical for the regulation of the MKP-1 protein turnover and presumably involves phosphorylation of serine residues 359 and 364 preventing the ubiquitin-mediated degradation *(16)*.

Other mechanisms may be operative in regulation of mouse thecal CYP17 promoter activity and androgen secretion. A possible explanation for this could be that Src inhibition may stimulate CYP17 promoter activity (Fig. 4) leading to accumulation of CYP17 mRNA (Fig. 5). Stimulation of the cAMP pathway when Src is inhibited may stabilize the CYP17 mRNA leading to increased CYP17 protein synthesis and hence more androgen production. The increased androgen eventually may shut off the promoter activity via feed back inhibition *(11)*.

The role of Src in follicular development is unknown, but the present and previous studies place Src as a potential centerpiece in the regulation of follicular estradiol. Possibly, Src regulates the secretion of thecal androstenedione *(3,4,17,18)*, a precursor of estradiol, during follicular development. Activation of Src reduces responsiveness of thecal cells to LH, whereas inhibition of Src enhances responses of thecal cells to LH. Thus, it appears that Src modifies responses of LH on thecal cells. Estradiol, a major steroid regulating follicular development, has been proposed to utilize Src as an intracellular mediator of signaling *(19)*. Inhibition of Src may inhibit the ability of estrogen to stimulate gene expression since Src plays a role in phosphorylating estrogen receptor and estrogen receptor must be phosphorylated in order to bind to its response element *(20)*. Thus, as a follicle develops to the antral stage and produces estradiol, Src may regulate thecal steroidogenesis and responsiveness of the ovary to estradiol.

Collectively, these results indicate that the pharmacological inhibitors of Src (GA and HA) tested in vitro, increase thecal androgen secretion. Src may regulate the level of CYP17 mRNA and promoter activity and control, in part, follicular steroidogenesis.

## Materials and Methods

### *Hormones and Chemical*

Src tyrosine kinase inhibitors, geldanamycin (GA) and herbimycin A (HA), were obtained from Calbiochem (La Jolla, CA). The PKA inhibitor, H89, was obtained from Bio-

mol Research Laboratories Inc. (Plymouth Meeting, PA). Medium M-199 was obtained from Gibco BRL. The following reagents were purchased from Sigma (St. Louis, MO): penicillin G/streptomycin and forskolin (FK). Lipofectamine Plus was acquired from Invitrogen (Grand Island, NY). Oligonucleotides required for CYP17 promoter–reporter constructs and those required for detection of CYP17 and 3β-HSD cDNAs were obtained from Integrated DNA Technologies (Coralville, IA). The luciferase reporter assay system was obtained from Promega (Madison, WI). Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific (St. Louis, MO).

### *Animals*

C57BL6 mice were purchased from Harlan Inc. (Indianapolis, IN) and maintained as a breeding colony at Kansas University Medical Center. Mice were kept under controlled temperature (25 ± 2°C) and daily lighting (12-h light and 12-h dark). Food and water were provided *ad libitum*. The Institutional Animal Care and Use Committee approved all studies.

### *Theca Enrichment*

Mice were sacrificed on d 25 of age (d 1 = day of birth) and the ovaries were cleaned of adhering tissues. Day 25 was chosen because ovulation had not yet occurred and a homogeneous population of follicles was present in the ovaries. Follicles were punctured with a 27-gauge needle and the extruded granulosa cells were discarded. The ovarian remnants were removed and placed in a collagenase (CLS1, Worthington Biochemical Corporation, Lakewood, NJ) and DNAse mixture (4 mg collagenase/mL, 10 μg DNAse/mL, and 1 mg BSA/mL in M199 containing 25 m*M* HEPES with a final pH of 7.4) for 10 min at 37°C to disperse the ovarian cells (called theca-enriched cells, TEC). The TEC contain theca, interstitial, stromal, and granulosa cells as well as other cell types. Then the TEC were further dispersed by sequential passage through 18-, 20-, and then 22-gauge syringe needles at 10-min intervals. After the first 10-min incubation, the cells were passed once through an 18-gauge needle attached to a 1-cc syringe. After a second 10-min incubation, the dispersate was passed once through a 20-gauge needle, and this was followed by a 10-min incubation in the collagenase-DNAse solution. Finally, the dispersates were passed once through a 22-gauge needle, M199 was added, and the TEC were washed three times. The dispersion method is similar to that previously described *(21)*. Ovarian cell viability and cell number were determined by trypan blue exclusion. The cell viability was consistently greater than 90%. The TEC were then placed in the culture medium consisting of Medium-199 containing Hank's salts, 25 m*M* HEPES, 2 m*M* L-glutamine, 0.1% BSA, 1% fetal bovine serum (FBS), 100 U penicillinG/mL, and 100 μg streptomycin/mL and ITS (Sigma, St. Louis, MO) whose constituents are insulin 5 μg/mL, transferrin 5 μg/mL, and selenium 5 ng/mL (ITS, Sigma, St. Louis, MO).

### *Cell Culture*

TEC were allowed to attach overnight (40,000 viable cells per well in a 96-well plate) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air in 0.2 mL culture media after which the wells were rinsed twice to remove unattached cells. Then fresh media and treatments were added as described in the results section. Specific treatments were performed in replicates of three and each experiment was repeated at least three independent times with a different preparation (pool) of cells. The cell culture method was essentially that of Roby et al. *(21)* and was performed for 48 h because this was the earliest time for maximal secretion of steroids and cAMP.

### *Isolation of Total RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

TEC were plated on a 6-well culture plate at a density of $1.5 \times 10^6$ viable cells/well in 2 mL of culture medium. The TEC were then allowed to attach overnight in a humid environment of 5% $CO_2$ and 95% air at 37°C. The following day, culture media were removed and fresh media and treatments were added. The cells were treated with GA (0.1 μ*M* in 0.1% DMSO) for 8, 24, and 48 h. There were three replicate wells per time point. The replicate wells were pooled for preparing RNA and the experiment was performed on two separate occasions. Total RNA was then isolated with Trizol Reagent (Life Technologies Inc., Rockville, MD) and quantified by spectrophotometric measurement at 260 nm. One microgram total RNA was reverse transcribed using random hexamer primers and the resulting cDNA was used for PCR analysis. Specific primers for CYP17, 3β-HSD, and L19 (control) were designed using the Mac Vector program and produced by IDT Technologies (Coralville, IA); the sequences were 5'-CGC CTT TGC GGA TAG TAG TAG C-3' (sense) and 5'-AGG ATC CAG TTC AGC ACA GAG C-3' (anti-sense) for CYP17; 5'-GGA GCT GCC TGG TGA CTG GA-3' (sense) and 5'-GCC TTC TCA GCC ATC TTT TTG C-3' (anti-sense) for 3βHSD; and 5'-CTG AAG GTC AAA GGG AAT GTG-3' (sense) and 5'-GGA CAG AGT CTT GAT GAT CTC-3' (anti-sense) for L19.

### *Transfection and Luciferase Assay*

A CYP17 1.05 kbp promoter–reporter construct (CYP_Luc) was made by using 5'-GGC CTC GAG GGC AGA TGG CCA GCT GT and 5'-GGC GAG CTC TAT TGG CAT TGC GTC CC as primers to amplify the sequence from mouse liver genomic DNA. The promoter–reporter construct contained *Sac*I and *Xho*I sites, and was subsequently inserted into pGL3 basic vector *(22)*. Src dominant negative (DN) plasmid was obtained from Upstate Biotechnology (New York, NY; cat. no. 21-154). TEC were plated in 24-well culture plates at a density of $6 \times 10^4$ viable cells/

well/mL media and cultured overnight as described above to allow for attachment. The next morning cells were rinsed and placed in serum-free medium without antibiotics for 30 min prior to transfection. Transfection medium (200 µL of M-199 containing no antibiotics) contained 0.4 µg total plasmid DNA [CYP_Luc or Src(DN)] and β-galactosidase plasmid and 12 µL Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). Cells were transfected with plasmids for 18 h using Lipofectamine Plus at 37°C in 5% $CO_2$ in air. The serum-free media were replaced with media containing 1% serum and then geldanamycin was added for 48 h. Cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), and harvested with Reporter Lysis Buffer (Promega, Madison, WI). Luciferase and β-galactosidase activities were measured with the Luciferase Assay System (Promega) and Galacto-Light Plus (Applied Biosystems, Bedford, MA), respectively, using a plate luminometer. Luciferase activities were normalized to β-galactosidase activities.

### *Steroid and cAMP Measurement*

Culture media were collected and progesterone, 17α-hydroxyprogesterone, and androstenedione were measured in unextracted conditioned media by radioimmunoassay (RIA) as described previously *(23)*. cAMP was measured in media, using a commercial EIA kit obtained from Assay Designs Inc. (Ann Arbor, MI). Previous studies have shown that media concentrations of cAMP parallel cellular concentrations *(3)*. The intra- and interassay coefficients of variation for the progesterone, androstenedione, cAMP, and 17α-hydroxyprogesterone assays were less than 11% based on three assays for each using duplicate determinations. The sensitivity of each assay was 50 pg/mL. The specificity of the assays has been previously reported for progesterone *(24)*, androstenedione *(25)*, 17α-hydroxprogesterone *(26)*, and cAMP (Assay Designs, Inc, Ann Arbor, MI, cat. no. 901-066). For cAMP cross reactivity was determined at the 50% binding point and found to be 0.3% for AMP, 0.1% for ATP, and less than 0.001% for cGMP, GMP, GTP, cUMP, and CTP.

### *Statistics*

Data were analyzed by one-way ANOVA. If the ANOVA indicated significant effects with a $p \leq 0.05$, then specific differences between means were detected by Tukey's test and were also considered significant if $p \leq 0.05$.

## Acknowledgments

This work was supported by, a Specialized Cooperative Center Program in Reproductive Research from the NICHD (HD 33994, PFT), a NIH grant from the National Center for Research Resources (RR-16475) to support a Kansas Biomedical Research Infrastructure Network, the Andrew W. Mellon Foundation, and a KUMC Biomedical Research Training Fellowship (GC).

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