The Src tyrosine kinase pathway regulates thecal CYP17 expression and androstenedione secretion

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Abstract In order to evaluate the role of Src tyrosine kinase in thecal cell steroidogenesis, a pharmacological approach was utilized by treating enriched populations of mouse ovarian theca-interstitial cells in vitro with a direct Src kinase inhibitor, PP2. Inhibition of Src with PP2 increased both basal and forskolin-stimulated androstenedione secretion, and increased cytochrome P450 17- α hydroxylase-lyase (CYP17) promoter activity and steady state mRNA. PP2 did not change thecal levels of StAR mRNA. Inhibition of mitogen-activated protein kinase kinase, a downstream regulator of Src activity, using PD98059 also increased forskolin-stimulated secretion of androstenedione above forskolin alone, but had no effect on basal secretion of androstenedione. Src inhibition increased mitogen-activated protein kinase phosphatase-1 protein and decreased phosphorylation of SF-1, which correlated with increased CYP17 promoter activity and mRNA levels. These results implicate Src tyrosine kinase in the regulation of CYP17 and thecal androgen secretion.

Keywords Androstenedione · Theca · CYP17 · Src tyrosine kinase · MAP kinase · SF-1

Introduction

Src tyrosine kinase is a non receptor kinase involved in a variety of cell signaling events, regulating both cell growth [1, 2] and differentiation [3], and cell death and migration [4]. A recent study has shown that in vitro inhibition of Src tyrosine kinase using indirect pharmacological inhibitors such as herbamycin-A and geldanamycin increased thecal secretion of androstenedione in the mouse [5]. Earlier studies using dominant negative Src as well as indirect pharmacological inhibitors of Src with rat thecal-interstitial cells, MA10 Leydig tumor cells, and a TM3 Leydig cell line demonstrated increased LH-stimulated cAMP and steroidogenesis [6–10]. Indirect pharmacological inhibitors of Src such as geldanamycin prevent binding of heat shock protein 90 (hsp90) to Src and thus increase Src turnover [11]. The indirect inhibitors have numerous nonspecific actions such as blocking Raf–hsp90 interactions and increasing the turnover of Raf [12]. Raf has been shown to be present in steroidogenic cells [13, 14].

Direct inhibitors of Src tyrosine kinase, such as PP2 and SU6656, bind directly to Src and inactivate it [15]. The effects of direct pharmacological inhibition of Src on ovarian androgen secretion have not been investigated. However, in adrenalcortical carcinoma derived cells, inhibition of Src using PP2 resulted in increased conversion of progesterone to 17 α -hydroxyprogesterone, increased CYP17 mRNA expression, and increased DHEA production [16, 17]. Several additional studies in adrenal cells have further described the control of CYP17 gene expression and the role of SF-1 [18, 19]. SF-1, phosphorylated via

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the ERK pathway, is unable to transactivate CYP17. Upon cAMP activation, PKA-mediated phosphorylation and activation of the dual-specificity phosphatase MKP-1 resulted in the dephosphorylation of SF-1 and increased SF-1-mediated transactivation of CYP17 [20, 21]. Similar studies in theca are limited but implicate a similar mechanism. Studies using theca derived from normal and polycystic (PCOS) ovaries indicate inhibition of ERK correlates with increased CYP17 and androgen production [22]. Although the phosphorylation status of SF-1 was not assessed in that study, CYP17 may have been increased via reduced phosphorylation of SF-1 when ERK was inhibited. Thus, blockage of the MEK/ERK pathway should have similar effects as Src inhibition on androgen secretion. The present study utilized enriched populations of mouse theca-interstitial cells to assess *in vitro* effects of (1) PP2, a direct inhibitor of Src, on steroidogenesis, and cAMP secretion, StAR, CYP11A1, 3 β -HSD, and CYP17 gene expression, (2) the MEK inhibitor PD98059 on steroidogenesis and cAMP secretion, and (3) Src inhibition on CYP17 promoter activity, and SF-1, phosphorylated SF-1 (pSerSF-1), and MKP-1 protein expression.

We report in this study that direct inhibition of Src or inhibition of a downstream effector, MEK, increased basal and forskolin-stimulated androstenedione secretion in mouse theca-interstitial cells. Src inhibition also increased CYP17 mRNA expression, CYP17 promoter activity, and increased MKP-1 but decreased pSerSF-1 protein levels, which correlated with increased androstenedione secretion.

Materials and methods

Hormones and chemicals

Src tyrosine kinase inhibitors, PP2 and SU6656, the MEK inhibitor, PD 98059, and mSF-1 were purchased from Calbiochem (La Jolla, CA). Phospho-Ser/Thr specific antibodies were purchased from Cell Signaling Technology (Boston, MA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). The following reagents were purchased from Sigma (St. Louis, MO): penicillin G/streptomycin, forskolin, dimethylsulfoxide, and ITS (insulin 5 mg/ml, transferrin 5 mg/ml, and selenium 5 ng/ml). Lipofectamine Plus, all liquid culture media, and Trizol Reagent were from Invitrogen (Grand Island, NY). Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). The Luciferase Reporter Assay System (pGL3 basic), Reporter Lysis Buffer, and Luciferase Assay System were obtained from Promega (Madison, WI). Galacto-Light Plus, SYBR green, and TaqMan PCR reagents were obtained from Applied Biosystems (Bedford, MA). The Src dominant negative

plasmid was obtained from Upstate Biologicals (Charlottesville, VA). Collagenase (CLS1) and DNase were obtained from Worthington Biochemical Corporation (Lakewood, NJ). MKP-1 antibody was from Santa Cruz (Santa Cruz, CA). cAMP assay kits were obtained from Assay Designs Inc. (Ann Arbor, MI).

Animals

C57BL/6 mice (Harlan Inc., Indianapolis, IN) were maintained as a breeding colony at the University of Kansas Medical Center. Mice were maintained in rooms under controlled temperature ($25 \pm 2^\circ\text{C}$) and daily lighting (12-h light and 12-h dark). Food and water were provided *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee.

Enrichment of mouse ovarian theca-interstitial cells and cell culture

Theca-interstitial cells were collected from mice on days 26–28 of age and cultured as previously described [5]. Briefly, ovaries were cleaned of connective tissues, granulosa cells were expressed and discarded, and the ovarian remnants were digested in a collagenase and DNase mixture. The ovaries were further dispersed by sequential passing of the cell preparation through 18-, 20-, and then 22-gauge syringe needles during the digestion period. Following dispersal, washing, and assessment of viability (greater than 90%), the theca-interstitial cells were placed in Medium-199 containing Hanks, 25 mM Hepes, 2 mM L-glutamine, 0.1% BSA, 1% FBS, ITS, 100 U penicillin/G/ml, and 100 μg streptomycin/ml. Theca-interstitial cells were added to tissue culture plates (6-well 1.5×10^6 viable cells/well/2 ml media; 96-well 40,000 viable cells/well/0.2 ml media) and were cultured overnight to allow for cellular attachment. After overnight incubation the wells were rinsed twice to remove unattached cells and fresh media and treatments were added. Cell attachment after overnight incubation ranged from 90% to 93%. Inhibitors or diluent controls were added 2 h prior to treatment with forskolin. Treatments were performed in triplicate and each experiment was repeated on at least three independent occasions with different preparations of cells. Data presented represent results from a single experiment and are representative of the results from at least three independent experiments.

Isolation of total RNA and real time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from theca-interstitial cells following culture using Trizol reagent and following the

manufacturer's instructions. Total RNA (3 µg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase as described by the manufacturer. The resulting cDNA was diluted 10-fold in sterile water and aliquots were subjected to quantitative real-time PCR. PCR primer pairs and probes for the analysis were designed with Primer Express 3.0. Real-time PCR was carried out on an Applied Biosystems HT7900 Sequence Detector using standard methods [23]. To account for differences in starting material, mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probe reagents were used. The SYBR green reagent was used to detect amplicons of the mouse StAR, CYP11A1, and CYP17 cDNAs. Primers used were the following: Mouse StAR (NM_011485.4, forward: CCGGAGCAGAGTGGTGTCA; reverse: CAGTGGATG AAGCACCATGC), mouse CYP11A1 (NM_019779, forward: CCAGTGTCCCCATGCTCAAC; reverse: TGCAT GGTCTTCCAGGTCT), mouse CYP17 (NM_007809, forward: GATCTAAGAAGCGCTCAGGCA; reverse: GG GCACTGCATCACGATAAA), mouse 3βHSD (NC_000069.5, forward: CAATCTGAAAGGTACCCAGAAC CT; reverse AGATGAAGGCTGGCACACTTG). Primer concentrations for each target cDNA were determined empirically. Agarose gel electrophoresis and analysis of the multicomponent data for all samples using the Dissociation Curves 1.0 program (Applied Biosystems) indicated the presence of a single PCR product. The experimental and GAPDH PCRs were carried out in separate tubes in triplicate. The relative amounts of experimental and GAPDH PCR products were determined by comparison to a standard curve generated by serial dilution of a sample containing high levels of the target amplicons that was also run in triplicate. An arbitrary value of template was assigned to the highest standard and corresponding values to the subsequent dilutions and these relative values were plotted against the threshold value for each dilution to generate a standard curve. The relative amount for each experimental triplicate (i.e., unknown) and GAPDH triplicate was assigned an arbitrary value based on the slope and y-intercept of the standard curve. The average of the experimental triplicate was divided by the average of the GAPDH triplicate and the resulting normalized values (relative expression) were used for statistical analysis.

Transfection and luciferase assay

The CYP17 390 bp promoter-reporter construct was made by initial amplification of 1.05 kb of the promoter using two primers: 5'-GGC CTC GAG GGC AGA TGG CCA GCT GT and 5'-GGC GAG CTC TAT TGG CAT TGC GTC CC, as previously reported [5], followed by restriction digestion at *NheI* (−393) and *XhoI* (−3) sites and insertion into the pGL3 basic vector. The final promoter

was sequenced and compared to previously published sequences to ensure accuracy [24]. Theca-interstitial cells were plated in 24-well culture plates (6×10^4 viable cells/well/ml), cultured overnight, and rinsed to remove unattached cells. Serum-free medium without antibiotics was added for 30 min prior to transfection. Transfection medium (200 µl of M-199 without antibiotics) contained 0.4 mg total plasmid DNA and β-galactosidase with or without an Src dom neg and 12 µl Lipofectamine/Plus. Cells were transfected with plasmids for 3 h and then media were removed and replaced with media containing 1% FBS and treatments as described in the Results for an additional 6, 24, or 48 h. Cells were then washed twice with phosphate-buffered saline (PBS, pH 7.4), and harvested with Reporter Lysis Buffer. Luciferase and β-galactosidase activities were measured with the Luciferase Assay System and Galacto-Light Plus, respectively, using a plate luminometer. Luciferase activities were normalized to β-galactosidase activities.

Immunoprecipitation and Western Blot

SF-1 specific antibody was linked to agarose G beads at 4°C for 1 h. The slurry of beads was washed three times in RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Nonidet P40, 0.1% SDS, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 µg aprotinin/ml, 5 µg leupeptin/ml, and 5 µg pepstatin/ml) to remove unbound antibody. Cells were lysed in RIPA buffer, insoluble material was removed by centrifugation, and the protein concentration was measured using a Bradford method (BioRad Labs, Hercules, CA). Five hundred micrograms of cell lysate was incubated with the agarose/SF-1 antibody beads, followed by washing to remove unbound protein. For immunoblotting, the beads containing proteins were subjected to SDS–PAGE and electro-transferred to PVDF membranes [25]. Membranes were incubated in 5% nonfat dry milk in 10 mM Tris, 150 mM PBS, 0.05% Tween 20, pH 8.0 (TBST) and incubated with phospho-Ser/Thr PKA substrate specific antibody (1:1,000) overnight at 4°C. The phospho-Ser/Thr PKA substrate antibody was generated against a synthetic phospho-PKA substrate peptide and recognizes phosphorylated substrates of PKA, PKC, and PKG (Cell Signaling Technology). Phosphorylation of SF-1 by PKA and other mechanisms has been described [26, 27]. Following overnight incubation, the membranes were washed with TBST, incubated with horseradish peroxidase-conjugated secondary antibody (1:2,500) and the bands were visualized by enhanced chemiluminescence. After membrane stripping, immunoblotting for β-actin was carried out and served as an internal loading control. Cell lysates from different treatment groups were used and

probed for MKP-1 in a similar manner as described above but using MKP-1 antibody at a 1:500 dilution.

Steroid and cAMP measurement

Progesterone and androstenedione were measured in unextracted culture media by radioimmunoassay (RIA) as previously described [28]. cAMP was measured in culture media, using an enzyme immunoassay kit obtained from Assay Designs Inc. A previous study from our laboratory has shown that concentrations of cAMP in media parallel concentrations within the cells [8]. The intra- and inter-assay coefficients of variation for all the assays were less than 11% based on three assays for each with duplicate determinations of each standard.

Statistics

Data were analyzed by 1- or 2-way ANOVA as appropriate to detect statistically significant effects. Statistical differences between means were detected by Fisher's test if the ANOVA indicated a significant difference ($P \leq 0.05$).

Results

Effects of the Src specific inhibitor PP2 on theca-interstitial cell steroidogenesis and cAMP (Fig. 1)

Dose response studies revealed that 10 μ M PP2 was maximally effective in stimulating theca-interstitial cell androstenedione secretion after 48 h when combined with 10 μ M forskolin (data not shown). Thus, 10 μ M PP2 and 10 μ M forskolin were chosen for the remaining studies.

The Src specific inhibitor PP2 stimulated basal thecal-interstitial androstenedione accumulation in culture media after 24 h, and this level was maintained at the 48-h time point. The stimulatory effect of PP2 alone on basal androstenedione accumulation was not observed at the 6-h

time point. PP2 alone had no effect on basal progesterone or cAMP accumulation in the media at any time point examined. As anticipated, forskolin increased the accumulation of progesterone, androstenedione, and cAMP in the media. The effects of forskolin alone were significant 6 h after treatment, maximal at 24 h, and maintained at the 48-h time point. The effects of PP2 on forskolin-stimulated steroid and cAMP were variable, dependent on time and hormone. Media levels of progesterone and cAMP were lower in the forskolin plus PP2 treated cultures compared to the forskolin-treated cultures after 24 h of treatment. However, addition of PP2 to forskolin had no effect on forskolin-stimulated progesterone or cAMP accumulation at 6 and 48 h. In contrast to the effects of inhibition of Src on forskolin-stimulated cAMP and progesterone, PP2 dramatically and significantly enhanced forskolin-stimulated androstenedione accumulation. Androstenedione levels were elevated at 24 h and were increased further at 48 h.

Effects of PD98059, a MEK inhibitor, on theca-interstitial cell steroidogenesis and cAMP (Fig. 2)

MEK is central in the ERK pathway and is downstream of Src. Thus, inhibition of the ERK pathway using the MEK inhibitor PD98059 was hypothesized to have similar effects on steroidogenesis and cAMP as the Src inhibitor PP2. Similar to PP2, treatment with PD98059 (25 μ M) had no effect on basal accumulation of progesterone, or cAMP. In contrast to the effects of PP2, PD98059 did not stimulate basal androstenedione accumulation in the media at 48 hours after treatment. Co-treatment with forskolin and PD98059 resulted in 2–3-fold higher accumulation of androstenedione compared to treatment with forskolin alone. This was similar to the increased androstenedione accumulation following PP2 treatment although the magnitude of increase was not as great (compare Figs. 1b and 2b). Effects of forskolin plus PD98059 on the accumulation of progesterone and cAMP were similar to PP2 treatment; both were lower when comparing forskolin with PD98059 to forskolin alone.

Fig. 1 In vitro effects of PP2 (10 μ M) on basal and forskolin (10 μ M)-stimulated accumulation of progesterone (a), androstenedione (b), and cAMP (c) in media from ovarian theca-interstitial cells at

6, 24, and 48 h after treatment. * $P < 0.05$ compared to control, # $P < 0.01$ compared to forskolin. Some error bars are too small to be observed on the graph

Fig. 2 In vitro effects of the MEK inhibitor PD98059 (25 μ M) and forskolin (10 μ M) on accumulation of progesterone (a), androstenedione (b), and cAMP (c) in media from mouse theca-interstitial cells

48 h after treatment. PD98059 was added to the culture 2 h before the addition of forskolin. * $P \leq 0.05$ compared to control, # $P \leq 0.05$ compared to forskolin

Effects of PP2 treatment on theca-interstitial cell StAR, CYP11A1, 3β HSD, and CYP17 mRNA (Fig. 3)

Expression of StAR, CYP11A1, 3β HSD, and CYP17 transcripts was analyzed in the theca-interstitial cultures after 6, 24, and 48 h of treatment using real time RT-PCR normalized to GAPDH. The relative expression of StAR mRNA was increased in response to forskolin treatment at 6, 24, and 48 h. Treatment with PP2 had no effect on basal or forskolin-stimulated StAR mRNA. CYP11A1 mRNA was increased in response to forskolin treatment at the 24- and 48-h time points. PP2 treatment alone increased CYP11A1 mRNA at 24 h but not 48 h, and PP2 treatment did not alter forskolin-stimulated CYP11A1 mRNA levels. CYP11A1 mRNA levels were not changed by any treatment at the 6-h time point. Levels of 3β HSD mRNA were different in response to treatment only at the 48-h time point. Both forskolin treatment alone and PP2 treatment alone increased 3β HSD mRNA levels. The combination of forskolin and PP2 resulted in higher levels of 3β HSD mRNA compared to forskolin alone but levels not different than PP2 alone. 3β HSD mRNA levels were not altered by any treatment at the 6- and 24-h time points. Forskolin treatment stimulated a transient increase in CYP17 mRNA; levels were increased after 6 and 24 h but not after 48 h of treatment. Treatment of thecal-interstitial cells with PP2 alone resulted in significant stimulation of CYP17 mRNA levels at all three times measured. CYP17 mRNA levels after treatment with the combination of forskolin and PP2 were not different from the elevated levels stimulated by PP2 alone.

Effects of Src pathway inhibitors on CYP17 promoter activity in theca-interstitial cells (Figs. 4 and 5)

Effects of Src inhibitors PP2 and SU6656, the MEK inhibitor PD98059, and a Src dominant negative expression

plasmid on CYP17 promoter activity were investigated using a 0.39 kb CYP17-luc promoter-reporter construct. Forskolin treatment alone had no effect on CYP17 promoter activity at 48 h. PP2, SU6656, and PD98059 treatments and transfection with an Src dominant negative plasmid increased CYP17 promoter activity at 48 h by 2–3-fold (Fig. 4). Co-treatment with the inhibitors plus forskolin resulted in reduced CYP17 promoter activity compared to that stimulated by the inhibitors alone. A time course of 6, 24, and 48 h after treatment with forskolin, PP2, or forskolin plus PP2 revealed that at the time points examined forskolin alone had no effect on CYP17 promoter activity (Fig. 5). CYP17 promoter activity following PP2 treatment was increased at 6 h, reached a maximum at 24 h, and then declined, but still remained increased compared to control, by 48 h after treatment. Co-treatment of theca-interstitial cells with forskolin and PP2 resulted in a transient increase in CYP17 promoter activity. CYP17 promoter activity was maximal at 6 h after treatment with forskolin plus PP2 and declined thereafter to nearly basal levels by 48 h.

Effects of the Src inhibitor PP2 on steroidogenic factor-1 (SF-1) and mitogen-activated protein kinase phosphatase-1 (MKP-1) in theca-interstitial cells (Fig. 6)

Forskolin had no effect on theca-interstitial cell protein levels of SF-1 or its phosphorylated form (pSer), as determined by immunoprecipitation with anti-SF-1 and subsequent western blotting with phospho-Ser/Thr PKA substrate specific antibody; however, PP2 reduced the pSer form of SF-1 but not total SF-1. A similar reduction in pSer was observed following the combination treatment of forskolin and PP2.

Forskolin treatment slightly increased MKP-1 protein levels as indicated by a faint signal 48 h after treatment.

Fig. 3 In vitro effects of the Src inhibitor PP2 (10 μ M) and forskolin (10 μ M) on the relative expression of mouse theca-interstitial StAR, CYP11A1, 3 β HSD, and CYP17 mRNA. mRNA levels were determined by quantitative PCR and were normalized by GAPDH mRNA after 6, 24, and 48 h of treatment. * P < 0.05 compared to control, # P < 0.01 compared to forskolin. Some error bars are too small to be observed on the graph

Treatment with PP2 significantly increased MKP-1 protein and this increase remained when the cells were treated with the Src inhibitor PP2 in combination with forskolin.

Fig. 4 In vitro effects of PP2 (10 μ M), SU6656 (20 μ M), Src dominant negative, and PD98059 (25 μ M) on CYP17 promoter activity in the presence and absence of forskolin (10 μ M) in mouse theca-interstitial cells at 48 h after treatment. * P $\leq$ 0.05 compared to control, empty vector, # P $\leq$ 0.05 compared to treatment with the same compound without forskolin

Fig. 5 A time course of the in vitro effects of PP2 (10 μ M) on CYP17 promoter activity in the presence and absence of forskolin (10 μ M) on mouse theca-interstitial cells at 6, 24, and 48 h after treatment. * P $\leq$ 0.05 compared to control, empty vector, # P $\leq$ 0.05 compared to PP2

Discussion

The significant finding of this in vitro study was that specific pharmacological inhibition of Src resulted in increased basal and forskolin-stimulated androstenedione synthesis by mouse theca-interstitial cells. Increased androstenedione was associated with increase expression of CYP17 mRNA and promoter activity. Thus, there is likely a significant interaction between the Src pathway and cAMP-mediated thecal androgen secretion.

Previous studies in human adrenal cells demonstrated that inhibition of Src using PP2 in vitro increased production of androgens [16, 17]. Increased basal DHEA production in the case of the adrenal coincided with increased expression of StAR, CYP11A1, and CYP17 [16]. Our findings in theca-interstitial cells also indicate

Fig. 6 In vitro effects of the Src inhibitor PP2 (10 μ M) in the presence and absence of forskolin (10 μ M) on steroidogenic factor-1 (SF-1), the phosphorylated form of SF-1 (pSer), and MKP-1 protein levels determined by Western blotting at 48 h after treatment of theca-interstitial cells. β -actin was used as a loading control

Inhibition of Src with PP2 increased basal androgen production associated with a significant increase in CYP17 expression, only a transient increase in CYP11A1 expression, and increased 3 β HSD mRNA only after 48 h of culture. In contrast to the studies in the adrenal [16], basal expression of StAR mRNA in mouse theca-interstitial cells was not changed by PP2 treatment. In contrast to effects in the adrenal cells, combination of elevated cAMP (forskolin treatment) and Src inhibition did not result in reduced 3 β HSD. Thus under the conditions of elevated cAMP, adrenal and thecal cells respond differently to inhibition of Src. Interestingly, the effects of Src inhibition in theca appeared to be directed specifically at androgen production, and not as a general stimulation of steroidogenesis as a whole. Although both basal and forskolin-stimulated androstenedione were increased, neither basal nor forskolin-stimulated progesterone or cAMP were increased by PP2 treatment. In fact, both forskolin-stimulated progesterone and cAMP were transiently decreased by the co-treatment with the Src inhibitor PP2. PCR studies further substantiated a specific effect of Src inhibition. As anticipated, forskolin treatment of theca-interstitial cells resulted in increased expression of StAR mRNA [29, 30] and inhibition of Src did not change expression of StAR. In contrast to StAR, CYP17 mRNA expression, both basal and forskolin-stimulated, was increased following PP2 treatment at all time points examined. Thus it would appear that Src kinase functions to inhibit androgen production by mouse theca. This is similar to the finding in porcine theca where PDGF treatment in vitro resulted in stimulation of Src kinase activity and inhibition of androgen production [31].

Several studies indicate Src and MEK/ERK signaling pathways interact [32, 33] and a role for MEK/ERK

signaling in regulating androgen synthesis has previously been established [29, 34, 35]. In mouse theca-interstitial cells the MEK inhibitor PD98059 augmented forskolin-stimulated androstenedione production similar to inhibition of Src by PP2. However, inhibition of MEK did not alter basal steroid production and this effect differed from PP2. Thus, it would appear that more than one signaling cascade is involved in Src-mediated effects on thecal androgen production. Under conditions where intracellular cAMP is elevated, as during forskolin treatment, Src signaling involves MEK/ERK and when intracellular cAMP is not elevated, under basal conditions, Src-mediated effects are independent of MEK/ERK.

Previous studies using MA10 and TM3 Leydig cell lines revealed that herbimycin, a tyrosine kinase inhibitor with Src selectivity, or transfection with a dominant negative Src resulted in increased steroidogenesis together with elevated cAMP [6, 8]. Further studies demonstrated that stimulation of Src activity resulted in reduced steroidogenesis. In porcine thecal cells PDGF-mediated stimulation of Src activity resulted in reduced gonadotropin-stimulated steroidogenesis [36]. In addition, transfection of MA10 and TM3 Leydig cell lines with a temperature sensitive constitutively active Src mutant also resulted in inhibition of cAMP-stimulated steroidogenesis [6, 8]. Thus, increases in Src activity are likely to reduce LH-stimulated thecal androgen secretion and decreases in Src activity increase LH-stimulated thecal androgen secretion.

Interesting differences in the effects of direct (the present study) and indirect [5] inhibitors of Src on cAMP secretion are noted. Inhibitors of Src that bind directly to Src and inactivate it did not increase accumulation of cAMP in the media. However, indirect inhibitors of Src that bind hsp90 and further inhibit phosphodiesterase activity increase cAMP secretion and steroidogenesis [5, 6, 8]. The reasons for these differences are not understood. However, it is interesting to note that the direct inhibition of Src enhanced androgen secretion when forskolin was added (leading to increased cAMP), and this was similar to the effect of indirect inhibitors (herbamycin-A and geldanamycin) that inherently increased cAMP [5].

Inhibition of Src enhanced CYP17 promoter activity in a transient manner. Promoter activity was highest 24 h after treatment. However, accumulation of CYP17 mRNA as measured by qPCR remained elevated at 48 h after treatment. Thus inhibition of Src may further enhance androgen production by promoting the stabilization of CYP17 mRNA. Although we currently have no specific data to support this hypothesis, it is interesting to note that in other systems Src kinase plays a role in increasing mRNA stability [37, 38]. Contrary to our expectation, co-stimulation with forskolin and PP2 suppressed the increase in CYP17 promoter activity induced by PP2. In addition, CYP17

promoter activity was increased by PP2 at 6 h but was eventually reduced after 24 and 48 h of co-stimulation with forskolin and PP2. At the same time androstenedione was increased at 6 h and was increased further at 48 h. Again these data do not directly correlate with PCR studies and may indicate effects on mRNA stability. Additionally, based on our data it appears the regulation of CYP17 promoter activity and subsequent downstream accumulation of androgen are not events regulated in parallel. The significant control of CYP17 gene expression, posttranslational modifications such as phosphorylation [39, 40], and the many co-factors involved in the ultimate enzyme activity [41] are all places for regulation and possible interaction, either directly or indirectly with Src. It is interesting to note that although forskolin treatment alone had no effect on promoter activity, forskolin did alter PP2-stimulated promoter activity, indicating some interaction between the signaling pathways in mouse theca cells. Although not investigated in the present study, early response to PP2 may be related to effects on classical early response genes such as *fos* and *jun*. *Fos* has been shown to play a role in the regulation of both CYP17 and androgen production [42]. Further it has been postulated that increased androgen production and increased CYP17 expression in polycystic ovary syndrome may be due to decreased levels of *fos* expression [42]. The ability of the Src inhibitor PP2 to inhibit *fos* in several different systems has been demonstrated [43, 44]. Thus, regulation of CYP17 appears to be key in mouse thecal steroidogenesis. As anticipated, forskolin treatment of mouse theca resulted in increases in cAMP and steroid accumulation (Figs. 1 and 2). However, at the same time forskolin treatment resulted in only modest changes in CYP17 mRNA levels as determined by qPCR (Fig. 3) and no measurable changes in CYP17 reporter activity (Figs. 4 and 5). It is interesting to note McAllister et al. previously reported in human theca that basal and forskolin-stimulated CYP17 promoter activity was not detected without co-transfection with SF-1 [30]. Although not tested in the present study, a similar need may be present in mouse theca. Why forskolin-stimulated changes in CYP17 promoter activity were not observed is not clear; however, these data indicate CYP17 promoter activity, mRNA stability, and enzyme activity are likely regulated via different mechanisms.

The regulation of CYP17 gene expression is complex; however, a definitive role for SF-1 has been described [45–47]. In human theca, expression of a CYP17 promoter construct was not detectable without co-expression of SF-1 [30]. SF-1 binding to the rat CYP17 promoter in vitro was reduced when SF-1 was phosphorylated [47]. Further studies in human adrenal cells demonstrated dephosphorylation of SF-1 enhanced CYP17 transcriptional activation [48, 49]. Later studies in adrenal cells demonstrated that

mitogen-activated protein kinase phosphatase-1 (MKP-1), a dual specificity phosphatase, increased transcription of CYP17 and that reduction of ERK1/2 also increased transcription of CYP17 [21]. Our present data indicate a similar mechanism may be operational in mouse thecal cells. Inhibition of the ERK pathway using Src or MEK inhibitors coupled with a simultaneous increase in cAMP (using forskolin) would be expected to increase PKA activity and subsequent accumulation of MKP-1 protein and dephosphorylation of SF-1. SF-1 would bind to the promoter region of CYP17 and activate transcription. The latter events would increase promoter activity of CYP17, and increase CYP17 mRNA and thecal androgen secretion.

Collectively, these results indicate that direct pharmacological inhibitors of Src increased basal and forskolin-stimulated thecal androgen. In addition, inhibition of Src appears to act primarily at the level of CYP17 in the regulation of ovarian thecal androgen secretion.

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