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Transforming growth factor-α inhibition of luteinizing hormone-stimulated androgen production by ovarian theca-interstitial cells: mechanism of action

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We have previously demonstrated that TGFα inhibits theca-interstitial cell (TIC) androgen production by specifically blocking LH stimulation of 17α-hydroxylase/C_{17-20} lyase ($P450_{17\alpha}$) activity. The purpose of the present studies was to examine the mechanism by which this block occurs. TIC were isolated from hypophysectomized immature rats by Percoll gradient centrifugation and cultured up to 6 days in serum-free medium with LH (0–100 ng/ml) and TGFα (0–100 ng/ml). When freshly isolated TIC were treated with TGFα alone (100 ng/ml) there was no change in PKA activity from basal levels. LH (100 ng/ml) stimulated a significant increase in PKA activity that was abolished by TGFα. TGFα did not diminish LH stimulation of cAMP production. TGFα alone did not alter the basal expression of cholesterol side-chain cleavage ($P450_{SCC}$), 3β-hydroxysteroid dehydrogenase (3β-HSD) or $P450_{17\alpha}$ mRNAs. LH stimulated dose-related increases in $P450_{SCC}$ (80-fold), 3β-HSD (5-fold) and $P450_{17\alpha}$ (35-fold) mRNAs. Concomitant treatment with TGFα (100 ng/ml) inhibited LH stimulation of $P450_{17\alpha}$ mRNA >90% and $P450_{SCC}$ mRNA 35% while 3β-HSD mRNA was stimulated 2-fold. Time course studies demonstrated that the effects of TGFα were present at 2 days in culture. At 4 and 6 days in culture there were small, if any, increases in mRNA levels stimulated by LH. There were no significant effects of TGFα at 4 or 6 days. Our data demonstrate that TGFα inhibition of TIC androgen production involves suppression of $P450_{SCC}$ and $P450_{17\alpha}$ mRNA expression by inhibiting LH stimulation of PKA activity.

Keywords: TGFα; theca; ovary; rat; androgen

Introduction

Transforming growth factor-α (TGFα) is a 50 amino acid peptide (Derynk, 1990) that shares structural homology (Derynk, 1990) with epidermal growth factor (EGF) and activates the same cell surface receptor (Massague, 1983). EGF/TGFα receptors are present on both theca and granulosa cells (GC) in the ovary (Buck & Schomberg, 1991; Fujinaga *et al.*, 1992). Expression of the TGFα gene appears primarily in the theca cells (Kudlow *et al.*, 1987) but also in GC (Yeh *et al.*, 1993). TGFα is secreted by ovarian thecal cells (Skinner *et al.*, 1987; Bendell *et al.*, 1988; Lobb *et al.*, 1988; Skinner & Coffey, 1988) and has been identified in follicular fluid (Hsu *et al.*, 1987; Skinner *et al.*, 1987; Skinner & Coffey, 1988). Taken together with the observation that TGFα has been immunolocalized in actively growing follicles (Lobb *et al.*, 1989; Chegini & Williams, 1992) these data indicate that TGFα may be involved in regulating proliferation of theca and granulosa cells in developing follicles (Lobb *et al.*, 1988; Skinner & Coffey, 1988; Roberts & Skinner, 1991).

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In addition to growth-promoting effects, there is considerable evidence that TGFα is an autocrine/paracrine regulator in the ovarian follicle. Treatment of GC with EGF has been shown to increase FSH binding (May *et al.*, 1987), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 20α-hydroxysteroid dehydrogenase (20α-HSD) activities (Jones *et al.*, 1982). In contrast, treatment with EGF or TGFα inhibits the stimulatory effects of FSH on estrogen production (Hsueh *et al.*, 1981; Jones *et al.*, 1982; Schomberg *et al.*, 1983; Adashi *et al.*, 1987; Bendell & Dorrington, 1990) and LH binding (Mondschein & Schomberg, 1980). Thus, it appears that TGFα promotes proliferation of GC but inhibits the differentiation of GC to the estrogenic phenotype that occurs in dominant follicles.

In the theca, EGF (Erickson & Case, 1983) and TGFα (Roberts & Skinner, 1991; Weitsman & Magoffin, 1993) have been shown to inhibit LH/hCG stimulation of androgen production. Studies of steroid metabolism by the TIC demonstrated that TGFα specifically inhibits LH stimulation of 17α-hydroxylase/C_{17-20} lyase ($P450_{17\alpha}$) activity (Weitsman & Magoffin, 1993). The purpose of the present studies was to examine the mechanism by which TGFα inhibits $P450_{17\alpha}$ activity in ovarian TIC.

Results

Effect of TGFα on LH-stimulated cAMP-dependent protein kinase activity

In previous studies we demonstrated that TGFα inhibited 8-Br-cAMP-stimulated TIC androgen production approximately 70% indicating that the mechanism of TGFα involved inhibition of the LH/cAMP signalling pathway at a step distal to cAMP production (Weitsman & Magoffin, 1993). In TIC, the cAMP-dependent protein kinases (PKA) play a key role in mediating the stimulation of differentiation and androgen production (Magoffin, 1989). We therefore tested the effects of TGFα on PKA activity. As shown in Figure 1, untreated TIC contained a measurable level of basal PKA activity. Treatment of the cells with TGFα alone (100 ng/ml) did not alter the basal PKA activity. LH (100 ng/ml) caused a significant increase in PKA activity ($P<0.01$) that was markedly inhibited (70%) in the presence of TGFα (Figure 1).

The inhibition of LH-stimulated PKA activity could be the result of a direct effect on PKA or it could be the consequence of diminished activation of PKA due to decreased cAMP production. To help distinguish between these possibilities we measured the effect of TGFα on LH stimulation of cAMP production (Figure 2). In the absence of TGFα, LH stimulated a dose-dependent increase in cAMP production. Although TGFα alone had no effect on basal cAMP production, there was a modest increase in cAMP production at 10 and 30 ng/ml of LH. The ED_{50} for LH stimulation (12.3 ± 2.5 ng/ml) was unchanged by TGFα treatment. These data demonstrate that the decrease in PKA activity caused by TGFα was not due to diminished cAMP production.

Effect of TGFα on steroidogenic enzyme mRNA expression

The inhibition of PKA activity by TGFα would be expected to decrease the expression of steroidogenic enzyme mRNAs in response to LH stimulation. To further understand the mechanism of TGFα action we examined the effects of TGFα on LH-stimulated expression of the genes encoding the key enzymes required for androgen production.

At 2 days in culture LH stimulated a dose-related increase in $P450_{SCC}$ mRNA that reached 80-fold at 10 ng/ml of LH (Figure 3). In the presence of TGFα there was a small increase ($P < 0.05$) in $P450_{SCC}$ mRNA at very low concentrations of LH (0.1 ng/ml) but LH stimulation of $P450_{SCC}$ mRNA was inhibited by approximately 35% at concentrations of LH $\geqslant$ 10 ng/ml. There was no effect of TGFα on the ED_{50} for LH stimulation of $P450_{SCC}$ mRNA (4.7 ± 0.4 ng/ml).

As shown in Figure 4, LH stimulated a dose-related increase in 3β-HSD mRNA that reached a maximum of 5-fold. In contrast to its effect on $P450_{SCC}$ mRNA, TGFα augmented the increase in 3β-HSD mRNA levels stimulated by LH approximately 2-fold. The ED_{50} for LH stimulation of 3β-HSD mRNA (4.5 ± 0.5 ng/ml) was not altered by TGFα.

Treatment of the cultured TIC with increasing concentrations of LH for 2 days stimulated a dose-dependent increase in $P450_{17\alpha}$ mRNA (Figure 5) that was 35-fold greater than basal levels at 100 ng/ml of LH. The ED_{50} for LH stimulation of $P450_{17\alpha}$ mRNA was 15.1 ± 0.5 ng/ml. Similar to the effect on $P450_{SCC}$ mRNA TGFα caused a small increase in $P450_{17\alpha}$ mRNA at very low concentrations of LH (0.1 ng/ml). In contrast to the augmentation of LH-stimulated 3β-HSD mRNA levels and the partial inhibition of LH-stimulated $P450_{SCC}$ mRNA levels, TGFα (100 ng/ml) completely blocked the LH-stimulated increase in $P450_{17\alpha}$ mRNA expression (Figure 5).

As shown in Figure 6, there was no significant effect of TGFα alone on $P450_{SCC}$, 3β-HSD or $P450_{17\alpha}$ mRNA levels in the cultured TIC at any concentration tested (0.1–100 ng/ml).

Time course of TGFα effects on steroidogenic enzyme mRNA expression

In previous publications (Magoffin, 1989; Magoffin & Weitsman, 1993a,b,c; Weitsman & Magoffin, 1993) we have shown that the maximal stimulation of androgen production and steroidogenic enzyme mRNA expression occurs at 2 days in this culture model. To determine if TGFα altered the time course of steroidogenic enzyme mRNA expression we tested the effects of TGFα over a 6 day period. As shown in Figure 7, TGFα alone (100 ng/ml) did not alter basal $P450_{SCC}$

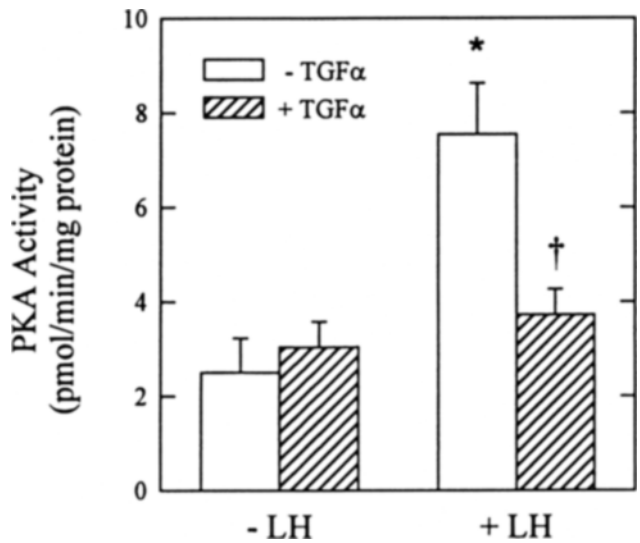

Figure 1 Effect of TGFα on basal and LH-stimulated protein kinase A activity. Purified TIC (2×10^6 viable cells/well) were incubated (37°C) with and without TGFα (100 ng/ml) and LH (100 ng/ml) for 30 min. After the incubation the cells were extracted and the supernatant was assayed for PKA activity. PKA activity was normalized to the protein content of the cell pellet. Data are the mean ± SEM of three experiments. *$P < 0.01$ *vs* control; †$P < 0.01$ *vs* LH

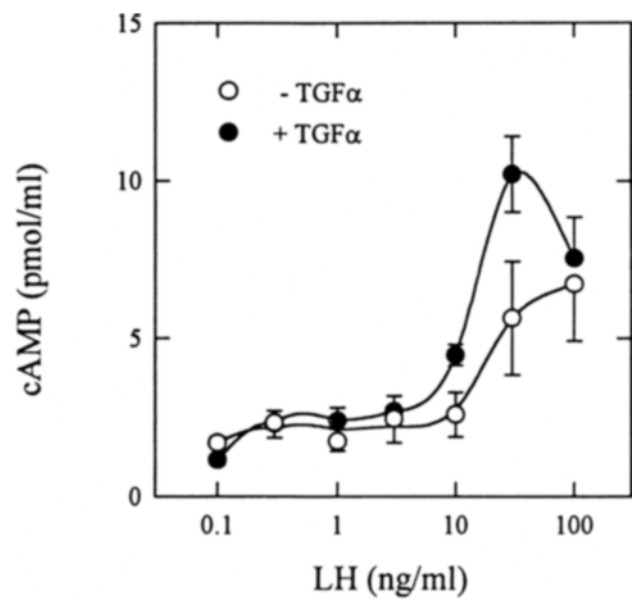

Figure 2 Effect of TGFα on LH stimulation of cAMP production by TIC. Purified TIC 2×10^6 viable cells/well) were cultured for 2 days with and without TGFα (100 ng/ml). The TIC were then incubated (60 min) with increasing concentrations of LH (0–100 ng/ml) in the presence of IBMX. The data are the mean ± SEM of four experiments

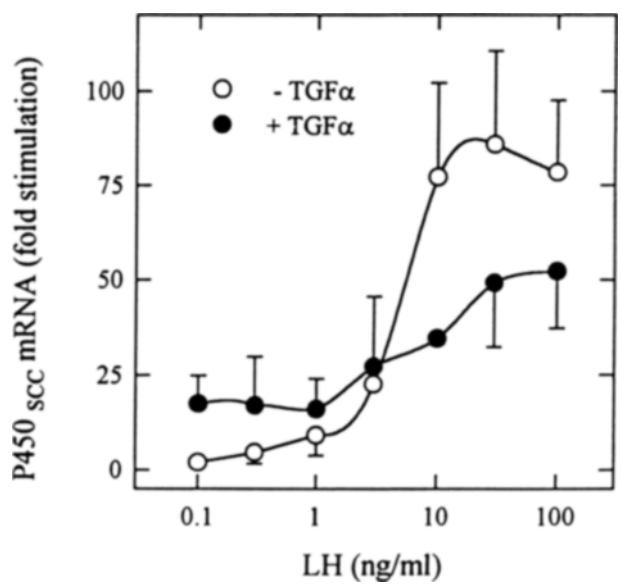

Figure 3 Effect of TGFα on LH stimulation of $P450_{SCC}$ mRNA expression in TIC. Isolated TIC (3×10^4 viable cells/well) were cultured with increasing concentrations of LH (0–100 ng/ml) in the presence and absence of TGFα (100 ng/ml) for 2 days. $P450_{SCC}$ mRNA was measured in cytoplasmic extracts of the TIC by specific RT-PCR assay. The data were normalized to β-actin. Data are the mean ± SEM of three experiments

mRNA expression up to 6 days in culture. LH (100 ng/ml) stimulated an approximately 80-fold increase in $P450_{SCC}$ mRNA levels that decreased 90% at 4 days and returned to basal levels at 6 days. TGFα caused the expected 35% inhibition of LH-stimulated $P450_{SCC}$ mRNA levels at 2 days but did not alter LH-stimulated $P450_{SCC}$ mRNA expression at 4 or 6 days.

There was no significant change in 3β-HSD mRNA levels as a result of treatment with TGFα alone throughout the 6 day culture period (Figure 8). LH (100 ng/ml) stimulated a 5-fold increase in 3β-HSD mRNA levels at 2 days but did not increase 3β-HSD mRNA over basal levels at 4 or 6 days. Concomitant treatment with TGFα plus LH did not significantly alter 3β-HSD mRNA levels from those stimulated by LH alone.

As shown in Figure 9, LH (100 ng/ml) stimulated the expected 35-fold increase in $P450_{17\alpha}$ mRNA levels at 2 days but did not stimulate $P450_{17\alpha}$ mRNA over basal levels at 4 or

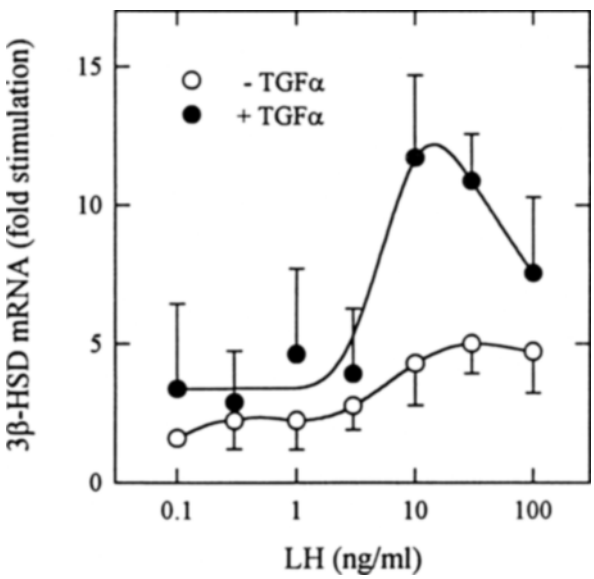

Figure 4 Effect of TGFα on LH stimulation of 3β-HSD mRNA expression in TIC. Isolated TIC (3×10^4 viable cells/well) were cultured with increasing concentrations of LH (0–100 ng/ml) in the presence and absence of TGFα (100 ng/ml) for 2 days. 3β-HSD mRNA was measured in cytoplasmic extracts of the TIC by specific RT-PCR assay. The data were normalized to β-actin. Data are the mean ± SEM of three experiments

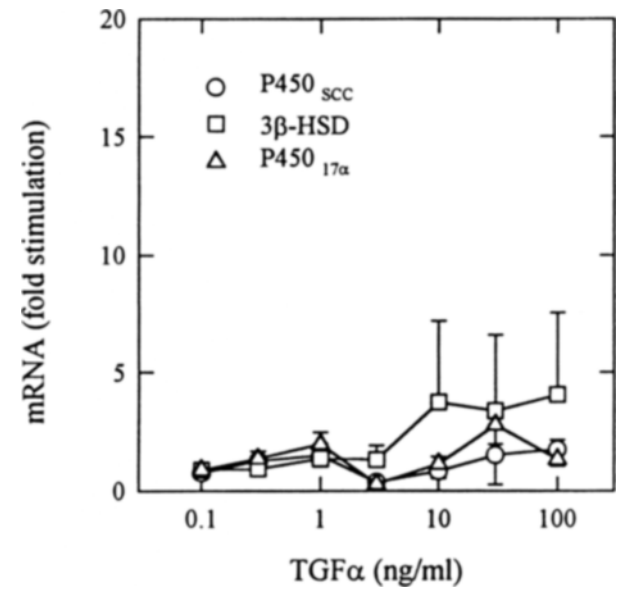

Figure 6 Effect of TGFα on steroidogenic enzyme mRNA expression in TIC. Isolated TIC (3×10^4 viable cells/well) were cultured with increasing concentrations of TGFα (0–100 ng/ml) for 2 days. $P450_{SCC}$ 3β-HSD and $P450_{17\alpha}$ mRNA were measured in cytoplasmic extracts of the TIC by specific RT-PCR assays. The data were normalized to β-actin. Data are the mean ± SEM of three experiments

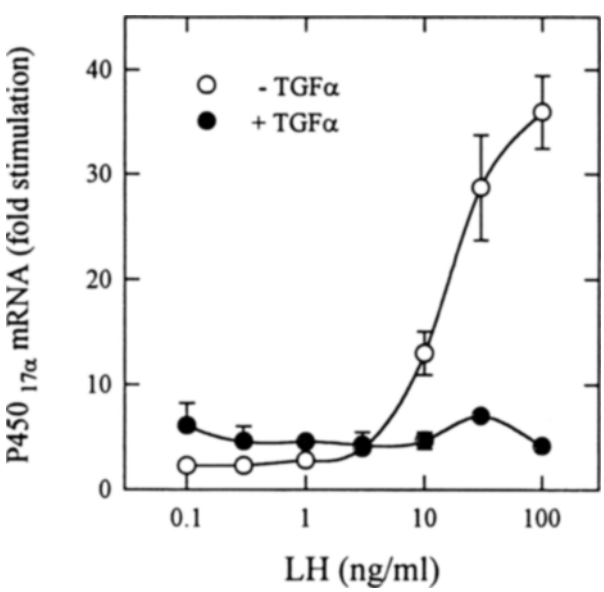

Figure 5 Effect of TGFα on LH stimulation of $P450_{17\alpha}$ mRNA expression in TIC. Isolated TIC (3×10^4 viable cells/well) were cultured with increasing concentrations of LH (0–100 ng/ml) in the presence and absence of TGFα (100 ng/ml) for 2 days. $P450_{17\alpha}$ mRNA was measured in cytoplasmic extracts of the TIC by specific RT-PCR assay. The data were normalized to β-actin. Data are the mean ± SEM of three experiments

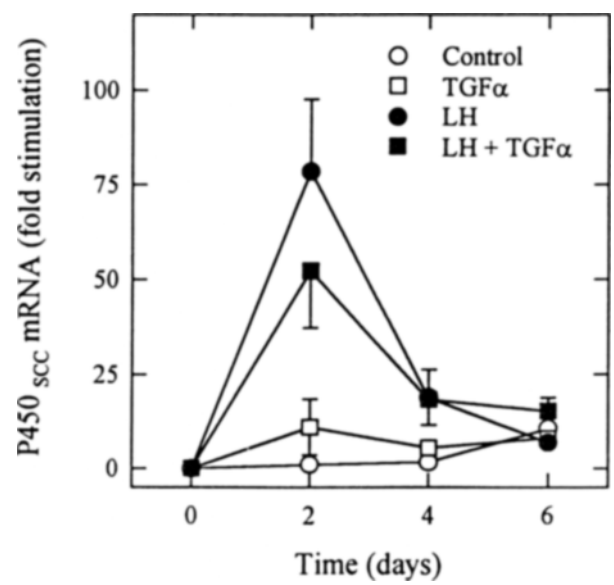

Figure 7 Time course of TGFα effects on LH stimulation of $P450_{SCC}$ mRNA expression in TIC. Purified TIC (3×10^4 viable cells/well) were cultured up to 6 days with and without LH (100 ng/ml) and TGFα (100 ng/ml). At 2 day intervals replicate groups of wells were extracted for mRNA measurements. In the remaining cultures the medium was changed and fresh hormones were added. $P450_{SCC}$ mRNA was measured by specific RT-PCR assay and normalized to β-actin. Data are the mean ± SEM of three experiments

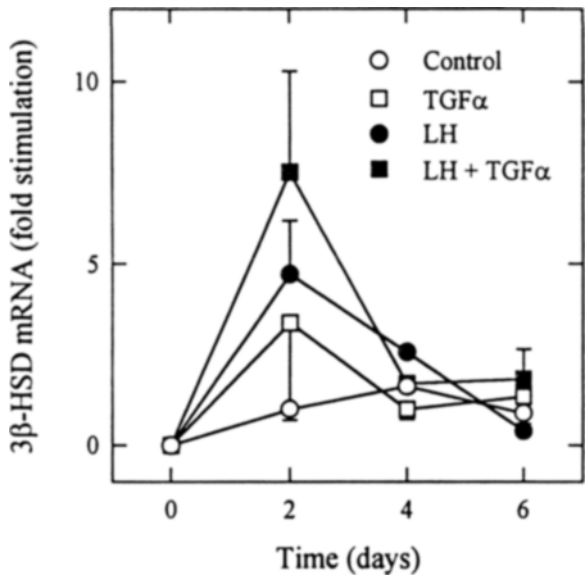

Figure 8 Time course of TGFα effects on LH stimulation of 3β-HSD mRNA expression in TIC. Purified TIC (3×10^4 viable cells/well) were cultured up to 6 days with and without LH (100 ng/ml) and TGFα (100 ng/ml). At 2 day intervals replicate groups of wells were extracted for mRNA measurements. In the remaining cultures the medium was changed and fresh hormones were added. 3β-HSD mRNA was measured by specific RT-PCR assay and normalized to β-actin. Data are the mean ± SEM of three experiments

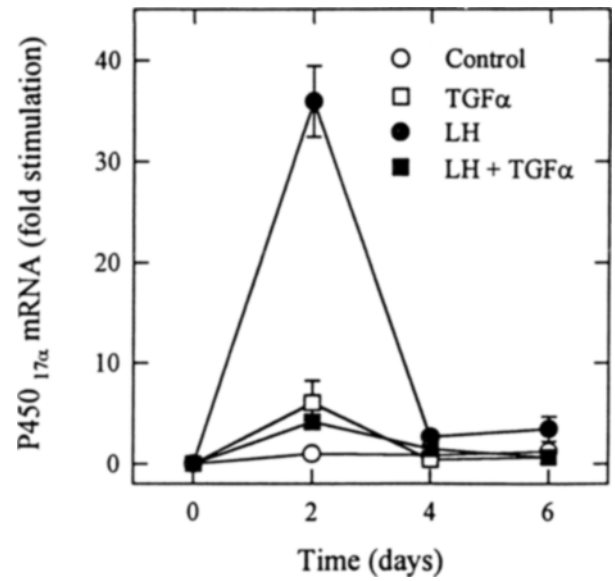

Figure 9 Time course of TGFα effects on LH stimulation of $P450_{17\alpha}$ mRNA expression in TIC. Purified TIC (3×10^4 viable cells/well) were cultured up to 6 days with and without LH (100 ng/ml) and TGFα (100 ng/ml). At 2 day intervals replicate groups of wells were extracted for mRNA measurements. In the remaining cultures the medium was changed and fresh hormones were added. $P450_{17\alpha}$ mRNA was measured by specific RT-PCR assay and normalized to β-actin. Data are the mean ± SEM of three experiments

6 days. TGFα (100 ng/ml) did not alter $P450_{17\alpha}$ mRNA expression alone but in combination with LH, dramatically reduced LH stimulation of $P450_{17\alpha}$ mRNA.

Discussion

Our previous studies demonstrated that TGFα is a potent inhibitor of LH-stimulated androgen production by ovarian TIC (Weitsman & Magoffin, 1993). Studies of the metabolism of radiolabeled progesterone demonstrated that TGFα treatment blocks $P450_{17\alpha}$ activity (Weitsman & Magoffin, 1993). The present studies extend these observations by demonstrating that TGFα causes a specific inhibition of the LH stimulation of $P450_{17\alpha}$ mRNA expression. This effect appears to be largely the result of a block in the stimulation of PKA activity by LH. In light of our present data demonstrating that LH stimulation of cAMP production was not decreased by TGFα and our previous findings that TGFα inhibited the stimulatory effects of reagents that increase intracellular cAMP concentrations (Weitsman & Magoffin, 1993), the inhibitory effect of TGFα appears to be directly on the PKA enzyme itself.

The mechanism of LH stimulation of androgen production in TIC involves increases in the expression of $P450_{SCC}$, 3β-HSD, and $P450_{17\alpha}$ mRNA and translation of the mRNAs into functional proteins (Magoffin & Erickson, 1994). It has been well documented that the isolated TIC contain only basal levels of $P450_{SCC}$, 3β-HSD and $P450_{17\alpha}$ mRNA when they are first place into culture (Magoffin & Erickson, 1988, 1994; Magoffin, 1989). Although the TIC respond to acute LH stimulation with large increases in cAMP production, steroid hormone production by the TIC is not immediately stimulated by LH (Magoffin, 1989). Sustained stimulation with LH is required before $P450_{SCC}$ 3β-HSD and $P450_{17\alpha}$ mRNA begin to increase and the TIC become steroidogenically responsive to LH (Magoffin & Weitsman, 1993a,b,c). Furthermore, LH stimulates TIC differentiation and steroidogenesis through activation of either or both isoforms of PKA (Magoffin, 1989). Our present observations are consistent with the hypothesis that TGFα prevents cAMP from fully activating the PKA isoenzymes in TIC and thereby prevents LH from stimulating expression of $P450_{17\alpha}$ mRNA and protein production. The TIC are capable of producing progesterone (Weitsman & Magoffin, 1993) but because $P450_{17\alpha}$ activity is lacking the TIC cannot metabolize progestins to androgens in the presence of TGFα.

The effects of TGFα on steroidogenic enzyme mRNA in TIC cannot be fully explained by inhibition of PKA activity alone. The differential effects appear to be the result of a combination of regulatory mechanisms. The TGFα receptor contains intrinsic tyrosine kinase activity as does the type I insulin-like growth factor receptor (Ulrich & Schlessinger, 1990). We have previously shown that insulin-like growth factor-I (IGF-I) alone does not activate PKA (Magoffin & Weitsman, 1994) or affect $P450_{17\alpha}$ mRNA (Magoffin & Weitsman, 1993a) in TIC, but it stimulates the expression of $P450_{SCC}$ (Magoffin & Weitsman, 1993c) and 3β-HSD mRNA (Magoffin & Weitsman, 1993b) in the absence of LH. Clearly the effects of TGFα on TIC steroidogenesis are different from the effects of IGF-I but the responses of the steroidogenic enzyme mRNAs to TGFα indicate that there may be certain common regulatory responses between IGF-I and TGFα. The fact that TGFα augments LH stimulation of 3β-HSD mRNA expression in a manner very similar to IGF-I suggests that TGFα and IGF-I interact with the LH-PKA signalling pathway through a common protein tyrosine kinase mediated mechanism. There also appear to be differences in the responsiveness of the $P450_{SCC}$ and $P450_{17\alpha}$ genes to the effects of TGFα. LH stimulation of both $P450_{SCC}$ and $P450_{17\alpha}$ mRNA was inhibited by TGFα but the inhibitory effect on $P450_{17\alpha}$ mRNA was greater than on $P450_{SCC}$ mRNA. The molecular basis for this difference is not evident but it is clear that IGF-I also regulates $P450_{SCC}$ and $P450_{17\alpha}$ mRNA expression differently (Magoffin & Weitsman, 1993a,c). It is possible that other intraovarian regulators may play a role in mediating these differential responses.

A physiological role for TGFα in the ovary has yet to be proven, however the data support the concept that TGFα

may be important in promoting proliferation of ovarian cells in developing follicles. This concept is supported by the localization of TGFα to growing follicles (Lobb *et al.*, 1989; Chegini & Williams, 1992). TGFα mRNA is expressed in both the theca (Kudlow *et al.*, 1987) and granulosa cells (GC) (Yeh *et al.*, 1993) but TGFα has only been shown to be secreted by TIC (Skinner *et al.*, 1987; Skinner & Coffey, 1988) suggesting a paracrine role with respect to GC and an autocrine role with respect to the TIC. In GC TGFα stimulates proliferation (Bendell *et al.*, 1988; Skinner & Coffey, 1988; Bendell & Dorrington, 1990) and FSH binding (May *et al.*, 1987) but inhibits FSH-stimulated differentiated functions such as estrogen production (Jones *et al.*, 1982; Schomberg *et al.*, 1983; Adashi *et al.*, 1987; Bendell & Dorrington, 1990; von Melchner *et al.*, 1992) and induction of LH receptors (Mondschein & Schomberg, 1980). Similar effects are observed in TIC. TGFα increases TIC proliferation (Skinner & Coffey, 1988; Roberts & Skinner, 1991) but inhibits androgen production (Roberts & Skinner, 1991; Weitsman & Magoffin, 1993). Taken together, these data suggest that TGFα may stimulate the proliferation of the TIC and GC during the growth phase of follicle development while suppressing differentiated functions. In the TIC this mechanism may play an important role in preventing precocious secretion of androgens. Elevated androgen levels have been associated with an increased incidence of atresia (Louvet *et al.*, 1975), decreased ovulation rates (Scaramuzzi *et al.*, 1977), and cessation of ovulation in polycystic ovarian disease (Yen, 1980). Thus, TGFα may play a key role in the normal physiology of developing follicles. Further research will be required to define the precise nature of that role.

Materials and methods

Reagents

Highly purified ovine LH (G3-330BR) was generously provided by Dr Harold Papkoff, University of California, San Francisco. TGFα was obtained from Collaborative Research Inc. (Bedford, MA, USA). McCoy's 5a medium, Medium 199, penicillin-streptomycin solution, L-glutamine solution, bovine serum albumin and trypan blue stain were obtained from Gibco (Santa Clara, CA, USA). Collagenase (CLS; 144 U/mg) was obtained from Worthington (Freehold, NJ, USA). cAMP [^{125}I]RIA kit and [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from DuPont NEN (Boston, MA, USA). Protein kinase A assay system, m-MLV reverse transcriptase, vanadyl ribonucleoside complexes and proteinase K were obtained from Gibco BRL (Gaithersburg, MD, USA). RNAsin was obtained from Promega (Madison, WI, USA). Reagents for polymerase chain reactions including Taq DNA polymerase were obtained from Perkin-Elmer Cetus (Norwalk, CT, USA).

Animals

Immature Sprague-Dawley rats were hypophysectomized by Harlan Industries (Indianapolis, IN, USA) at 21 days of age. They were given 5% dextrose and food *ad libitum*. Five days after hypophysectomy the rats were euthanised by CO_2 inhalation followed by cervical dislocation as approved by the CSMC Institutional Animal Care and Use Committee. The ovaries were collected in ice cold medium 199 containing 25 mM HEPES and 1 mg/ml BSA.

Cell culture

Ovarian cells were enzymatically dispersed as previously described (Magoffin & Erickson, 1982). The TIC were purified from the whole ovarian dispersate by Percoll gradient centrifugation (Magoffin & Erickson, 1988). An aliquot of the purified TIC suspension was counted in a hemacytometer and the viability was determined by trypan blue exclusion. In the studies where cAMP production and PKA activity were measured the TIC were cultured (2×10^6 viable cells/well) in 6-well tissue culture plates (Falcon, Beckton Dickinson Labware, Lincoln Park, NJ, USA) containing 2 ml of McCoy's 5a medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine with and without TGFα (0–100 ng/ml) and LH (0–100 ng/ml). In studies where mRNA was measured the TIC were cultured (3×10^4 viable cells/well) in 96-well tissue culture plates (Falcon) containing 200 μl of McCoy's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine with and without TGFα (0–100 ng/ml) and LH (0–100 ng/ml). The medium was collected and frozen (−20°C) at 2 day intervals and was replaced with fresh medium and the appropriate hormones at the original concentrations. TIC were cultured in a humidified 95% air, 5% CO_2 atmosphere up to 6 days.

Determination of cAMP production

TIC were cultured with and without TGFα (100 ng/ml) for 2 days. The TIC were then incubated (37°C) with LH (0–100 ng/ml) in the presence of 3-isobutyl-1-methylxanthine (1 mM) for 60 min. After the incubation the 96-well plates containing TIC and medium were heated in a boiling water bath (5 min) and the medium was frozen (−20°C) until the cAMP content of the medium was measured using the cAMP [^{125}I]radioimmunoassay system (NEN).

Protein kinase A activity

TIC were incubated (37°C) with and without TGFα (100 ng/ml) and LH (100 ng/ml) for 30 min. After the incubation the TIC were first washed with PBS buffer, then extracted in PKA extraction buffer (5 mM EDTA, 50 mM Tris, pH = 7.5). The TIC were homogenized and PKA activity in the supernatant was quantitated by phosphorylation of kemptide in the presence and absence of PKI (6-22) amide using the Protein Kinase A assay system (Gibco BRL) according to the manufacturer's protocol. The cell pellets were resuspended in 250 μl of PBS buffer and the protein content was measured in duplicate 100 μl samples by the method of Bradford (1976). PKA activity was standardized to protein content to control for plating and extraction variation.

Measurement of mRNA

mRNA was measured by reverse transcription (RT) of cytoplasmic RNA extracts followed by amplification of specific cDNAs by polymerase chain reaction (PCR) as previously described (Magoffin & Weitsman, 1993a,b,c). Cytoplasmic extracts were prepared from cultured TIC by lysing and scraping the cells from the culture wells in 50 μl/well of ice cold RNA extraction buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH = 8.0, 0.5% NP-40, 1 mM DTT and 20 mM vanadyl ribonucleoside complexes). The extracts from four replicate wells were pooled. The extracts were digested with proteinase K, extracted with phenol/chloroform and precipitated with isopropanol. The pellet was resuspended in 20 ml RNase-free water then frozen (−80°C). Aliquots of RNA (4 μl for $P450_{SCC}$ and $P450_{17\alpha}$ 3 μl for 3β-HSD and 1 μl for β-actin) were transcribed into cDNA. The appropriate control templates (Magoffin & Weitsman, 1993a,b,c) were included in each reaction then amplified by PCR for 25 cycles. The amplification products were ethanol precipitated and digested with the appropriate restriction enzyme to cut the control products, then separated on a 2% agarose gel. The DNA was visualized with ethidium bromide staining and the bands were cut from the gel and counted in a scintillation counter. The c.p.m. in the bands amplified from the cellular mRNA were normalized to the c.p.m. from the bands amplified from the control DNA to control for PCR varia-

tions. The coefficients of variation for measurement of $P450_{SCC}$, 3β-HSD, $P450_{17\alpha}$ and β-actin mRNA were 8.1%, 9.9%, 12.1% and 6.3% respectively. The data were normalized to β-actin to control for procedural variations.

Statistical analysis

Dose response curves were analysed and the ED_{50} values were calculated using the Allfit program (De Lean *et al.*, 1978). Differences between treatment groups were determined by unpaired t test or, for multiple comparisons, by one way analysis of variance followed by Student–Newman–Keuls test. The level of significance was considered to be $P \leqslant 0.05$.

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