Role of transforming growth factor-β1 in gene expression and activity of estradiol and progesterone-generating enzymes in FSH-stimulated bovine granulosa cells

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Abstract

Survival and inhibitory factors regulate steroidogenesis and determine the fate of developing follicles. The objective of this study was to determine the role of transforming growth factor-β1 (TGFβ1) in the regulation of estradiol-17β (E2) and progesterone (P4) secretion in FSH-stimulated bovine granulosa cells. Granulosa cells were obtained from 2 to 5 mm follicles and cultured in serum-free medium. FSH dose (1 and 10 ng/ml for 6 days) and time in culture (2, 4, and 6 days with 1 ng/ml FSH) increased E2 secretion and mRNA expression of E2-related enzymes cytochrome P450 aromatase (CYP19A1) and 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1), but not HSD17B7. TGFβ1 in the presence of FSH (1 ng/ml) inhibited E2 secretion, and decreased mRNA expression of FSH receptor (FSHR), CYP19A1, and HSD17B1, but not HSD17B7. FSH dose did not affect P4 secretion and mRNA expression of 3β-hydroxysteroid dehydrogenase (HSD3B) and α-glutathione S-transferase (GSTA), but inhibited the amount of steroidogenic acute regulatory protein (STAR) mRNA. Conversely, P4 and mRNA expression of STAR, cytochrome P450 side-chain cleavage (CYP11A1), HSD3B, and GSTA increased with time in culture. TGFβ1 inhibited P4 secretion and decreased mRNA expression of STAR, CYP11A1, HSD3B, and GSTA. TGFβ1 modified the formation of granulosa cell clumps and reduced total cell protein. Finally, TGFβ1 decreased conversion of androgens to E2, but did not decrease the conversion of estrone (E1) to E2 and pregnenolone to P4. Overall, these results indicate that TGFβ1 counteracts stimulation of E2 and P4 synthesis in granulosa cells by inhibiting key enzymes involved in the conversion of androgens to E2 and cholesterol to P4 without shutting down HSD17B reducing activity and HSD3B activity.

Introduction

In cattle, follicle development occurs in waves consisting of rhythmic emergence and growth of a new cohort of antral follicles (Ireland et al. 2000). During this process, the proportion of follicles undergoing atresia increases dramatically with follicular diameter and the rate of atresia doubles between 2 and 8 mm (Lussier et al. 1987). At the final growth phase, most follicles are atretic and only one follicle (exceptionally two) is selected to become dominant and continue to grow until ovulation (Fortune et al. 2001). Many factors are involved in follicular development, including gonadotropins, steroid hormones, cytokines, and other endocrine, paracrine, and autocrine factors. These factors induce cell death or survival and thus determine the fate of the growing follicle.

Gonadotropin stimulation of responsive follicles is associated with increased synthesis of estradiol-17β (E2) and progesterone (P4), the two key steroid hormones associated with the development of the ovolatory follicle (Price et al. 1999, Kolibianakis et al. 2005). E2 is produced by granulosa cells and is important for granulosa cell growth, attenuation of granulosa cell apoptosis, and positive and negative feedback regulation of the hypothalamic–pituitary–ovarian axis (Kolibianakis et al. 2005). In ruminants and humans, gonadotropin stimulation of E2 requires luteinizing hormone (LH) stimulation of theca cells to produce androgens, mostly as androstenedione (A4), and follicle-stimulating hormone (FSH)-mediated conversion of theca-derived A4 to E2 in granulosa cells (Fortune 1986, Hillier et al. 1994). A4 is converted to estrone (E1) by cytochrome P450 aromatase (CYP19A1) and then into E2 by 17β-hydroxysteroid dehydrogenase (HSD17B7) reducing enzymes (Hillier et al. 1994, Mindnich et al. 2004). Granulosa cells also produce P4, which is necessary for the induction of ovulation (Drummond 2006). The production of P4 involves transformation of cholesterol to pregnenolone (P5) by the cytochrome P450 side-chain cleavage (CYP11A1) followed by the conversion of P5 to P4 by 3β-hydroxysteroid dehydrogenase (HSD3B). In addition, α-glutathione S-transferase (GSTA) is
expressed in bovine granulosa cells and codes for a protein with high HSD3B activity (Rabahi et al. 1999, Raffalli-Mathieu et al. 2007). In vivo, most of the steroidogenic enzyme genes are not expressed in granulosa cells of small bovine antral follicles (0.4–4 mm; Bao & Garverick 1998). In granulosa cells, expression of CYP19A1 and CYP11A1 gradually increases during follicle growth, and HSD3B is first observed in non-atretic follicles at ~8 mm (Xu et al. 1995, Bao et al. 1997). These data indicate that the timely expression of steroidogenic enzymes at distinct periods of follicular development is important for the selection of the dominant follicle and successful ovulation. A better characterization of the regulation of E2 and P4 synthesis in granulosa cells will improve our understanding of follicle development.

In recent years, much attention has focused on the role of the members of the transforming growth factor-β (TGFB) superfamily acting as paracrine and autocrine factors to modulate ovarian function and fertility (Knight & Glister 2006). TGFB1, which is the most extensively studied member of this family, is present in granulosa and theca cells and in the vascular system of the ovary in many species (Nilsson et al. 2003, Juengel & McNatty 2005), and has been shown to either stimulate or inhibit E2 and P4 synthesis. For instance, TGFB1 stimulated E2 and P4 secretion from rodent granulosa cells (Zachow et al. 1999, Knight & Glister 2006), whereas TGFB1 inhibited granulosa cell secretion of E2 and P4 from pigs and ruminants (Chang et al. 1996, Wandji & Fortune 1996; Ford & Howard 1997, Juengel et al. 2004, Ouellette et al. 2005). These reports detailing the effect of TGFB1 on ovarian cells appear to be highly dependent on the species studied, stage of follicle differentiation, and the presence of different growth factors as co-treatments, and the mechanism of TGFB1 action in granulosa cells is not clear. In vivo, the complexities of hormonal interactions with the cytokines produced by ovarian cells limit our investigations of steroidogenesis in follicle development. Fortunately, E2 secretion can be maintained for several days in bovine granulosa cells cultured in vitro with FSH in serum-free conditions (Gutierrez et al. 1997). This in vitro model mimics the gradual increase in E2 secretion seen in the growing follicles and can be used to identify the key factors regulating steroidogenesis in granulosa cells. Therefore, the objective of the present study was to determine the effect of TGFB1 on E2 and P4 synthesis and the corresponding expression and activity of key steroidogenic enzymes in FSH-stimulated bovine granulosa cells.

**Results**

**Effect of FSH dose and time in culture**

In granulosa cells cultured for 6 days, FSH at 1 and 10 ng/ml had no effect on P4 synthesis, but significantly increased E2 secretion (Fig. 1A and F). FSH decreased steroidogenic acute regulatory protein (STAR) mRNA level, increased CYP11A1 (only at the 10 ng/ml dose), and did not affect HSD3B and GSTA mRNA levels (Fig. 1B–E). For E2 secretion, FSH had no effect on HSD17B7 mRNA level but increased CYP19A1 and HSD17B1 mRNA levels (Fig. 1H–J). In the presence of FSH at 1 ng/ml, both P4 and E2 accumulation increased from days 2 to 6 (Fig. 2A and F). In agreement with increased secretion of P4 with time in culture, mRNA expression of the P4-related enzymes STAR, CYP11A1, HSD3B, and GSTA also increased with time (Fig. 2B–E). For E2-related enzymes, time in culture had no effect on HSD17B7 mRNA level but CYP19A1 and HSD17B1 mRNA levels increased with time (Fig. 2H–J).

**Effects of TGFB1 on secretion of steroid hormones and mRNA expression and activity of steroidogenic enzymes**

Since P4 and E2 secretion was stimulated in granulosa cells cultured for 6 days with 1 ng/ml FSH (see above), we examined the effect of TGFB1 on steroid hormones and mRNA expression of steroidogenic enzymes under this condition. Addition of TGFB1 caused a significant dose-dependent inhibition of P4 and E2 secretion (Fig. 3). To determine enzymatic activities, the tritiated steroid hormone precursors of A4, testosterone (T), E1, and P5 were converted to the corresponding radiolabeled product (E2 and P4) confirming that the CYP19A1, reducing HSD17B, and HSD3B activities measured were specific. When granulosa cells were cultured with 0.5 ng/ml TGFB1, there was a significant inhibition of combined CYP19A1 and HSD17B activity as measured by the conversion of [3H]A4 to [3H]E2 (Fig. 4A), although the intermediary product [3H]E1 was not detected. Both doses of TGFB1 caused a significant inhibition in the CYP19A1 activity measured by the conversion of [3H]T to [3H]E2 (Fig. 4B). TGFB1 did not alter HSD17B reducing activity or HSD3B activity (Fig. 5). Corresponding to the decreased secretion of P4 and E2, TGFB1 caused a significant inhibition in the mRNA expression of STAR, CYP11A1, HSD3B, GSTA, CYP19A1, HSD17B1, and FSHR (Fig. 6). However, HSD17B7 mRNA was not changed by the treatment of TGFB1 (Fig. 6I).

**Cell morphology and total cell protein**

Granulosa cells were seeded onto wells and cultured for 6 days in serum-free medium. The granulosa cells initially formed tightly packed aggregates from day 2, which enlarged with time in culture (data not shown). When TGFB1 was added to FSH-stimulated cells, granulosa cell clumps were smaller and appeared more spherical than control cells (Fig. 7D). Total cell protein was 30 and 38% higher in the 1 and 10 ng/ml FSH-treated groups compared with the control group without FSH (Fig. 7A). By contrast, in the presence of 1 ng/ml FSH, the highest dose of TGFB1 (0.5 ng/ml) decreased total cell protein by 23% compared...
with the control group without TGFβ1 (Fig. 7B). Furthermore, in the absence of FSH, 6 days of treatment with 0.1, 0.5, or 1 ng/ml TGFβ1 had no significant effect on total cell protein (data not shown).

Discussion
The regulation of E2 and P4 production in granulosa cells is critical for ovarian follicle growth. The synthesis of E2 from androgens requires aromatization by CYP19A1. In agreement with previous studies (Gutierrez et al. 1997, Silva & Price 2000, Sahmi et al. 2004), the physiological dose of FSH used in the present study (1 ng/ml) stimulated E2 secretion and abundance of CYP19A1 and HSD17B1 mRNA. Under this stimulatory condition, TGFβ1 caused a marked inhibition of E2 secretion and CYP19A1 activity, due to decreased expression of CYP19A1 mRNA. These findings concur with those obtained in extragonadal tissues where TGFβ1 was shown to inhibit CYP19A1 activity, CYP19A1 mRNA, and CYP19A1 protein levels in cultured human fetal hepatocytes, trophoblast cells, and adipose stroma cells (Simpson et al. 1989, Rainey et al. 1992, Luo et al. 2002). The conversion of A4 to E2 also requires HSD17B reducing activity; however, TGFβ1 did not affect HSD17B activity in the present study. TGFβ1 did inhibit HSD17B1 expression, suggesting that another enzyme may be contributing to total HSD17B activity. Another enzyme known to convert E1 to E2 is HSD17B7 (Krazeisen et al. 1999, Krusche et al. 2001), and in the
present study this isoform was detected in bovine granulosa cells and was not inhibited by TGFB1. In rodent and rabbit ovaries, this isoform is present only in the corpus luteum and was first reported as the prolactin receptor-associated protein (Nokelainen et al. 1998, Krusche et al. 2001, Risk et al. 2005). In humans, \textit{HSD17B7} transcript was found in the ovaries of non-pregnant, but not pregnant, women (Krazeisen et al. 1999). The mRNA expression of \textit{HSD17B7} was not affected by TGFB1 in agreement with a lack of the effect of TGFB1 on \textit{HSD17B} activity. FSH dose and time in culture also did not affect the expression of \textit{HSD17B7} mRNA in the present study. For \textit{HSD17B7} activity, a 1000-fold excess of unlabeled \textit{E1} had to be added and incubation time had to be shortened to obtain comparable conditions of substrate excess, indicating that \textit{HSD17B} reducing activity is very high in cultured bovine granulosa cells. The high \textit{HSD17B} activity could explain why the \textit{CYP19A1} product [3H]E1 was undetectable in the presence or absence of TGFB1.

**Figure 2** Effect of time in culture (2, 4, and 6 days) on secretion of \textit{E2} and \textit{P4} and mRNA expression of steroidogenic enzymes in bovine granulosa cells. Granulosa cells were cultured in serum-free medium with 1 ng/ml FSH starting at day 0. (A and F) The data represent secretion of \textit{E2} and \textit{P4} during the last 48 h of culture. (B–E and H–J) Abundance of \textit{STAR}, \textit{CYP11A1}, \textit{HSD3B}, \textit{GSTA}, \textit{HSD17B7}, \textit{CYP19A1}, and \textit{HSD17B1} mRNA was measured by semi-quantitative RT-PCR and normalized to the housekeeping gene \textit{I1B15}. Data are means ± S.E.M. of three separate culture replicates. Asterisk indicates that the mean is significantly different from control at day 2 (P<0.05, one-way ANOVA, with Dunnett’s test). (G) Representative agarose gel from two replicates showing PCR products for each steroidogenic enzyme and constitutively expressed housekeeping gene \textit{I1B15}.
In developing antral follicles, a positive E\textsubscript{2}/P\textsubscript{4} ratio must be maintained and it is critical to limit P\textsubscript{4} secretion until the time of ovulation induction, because premature increase in P\textsubscript{4} is associated with follicular atresia (Ireland & Roche 1982, Irving-Rodgers et al. 2003). In agreement with the above, a low dose of FSH stimulated E\textsubscript{2} but not P\textsubscript{4} in the present study and CYP11A1 was only stimulated by the highest dose of FSH (10 ng/ml). Additionally, the present study is the first report showing that FSH down-regulates the expression of STAR in vitro. This finding may explain why STAR is undetectable in the granulosa of healthy antral follicles at any size in vivo (Soumano & Price 1997, Bao et al. 1998). In our time-course experiments, in the presence of low dose of FSH, the production of P\textsubscript{4} increased as well as the expression of STAR, CYP11A1, HSD3B, and GSTA. These findings are in agreement with a previous study (Sahmi et al. 2004), which showed an increase with time in HSD3B and CYP11A1. Overall, these results indicate that P\textsubscript{4} increases spontaneously in cultured granulosa cells and that readily available stores of cholesterol are present in granulosa cells, which can be transformed to P\textsubscript{4} by CYP11A1, HSD3B, and GSTA. Similar to the effect of TGF\textbeta\textsubscript{1} on E\textsubscript{2} synthesis, TGF\textbeta\textsubscript{1} also inhibited the progestin synthetic pathway. TGF\textbeta\textsubscript{1} inhibited P\textsubscript{4} secretion and abundance of mRNA encoding STAR, CYP11A1, HSD3B, and GSTA, which are required for sustained production of P\textsubscript{4} from cholesterol. These data are consistent with the effects of TGF\textbeta\textsubscript{1} on STAR, CYP11A1, and HSD3B in thecal, adrenocortical, and adrenal tumor cells among different species (Rainey et al. 1991, Cherradi et al. 1995, Naaman-Reperant et al. 1996, Attia et al. 2000, Herrmann et al. 2002), and this is the first time that TGF\textbeta\textsubscript{1} has been shown to inhibit mRNA encoding GSTA. Surprisingly, despite the reduced abundance of HSD3B and GSTA mRNA, TGF\textbeta\textsubscript{1} did not affect total HSD3B activity. Similarly, TGF\textbeta\textsubscript{1} did not significantly alter HSD3B activity in the rat fetal testis (Gautier et al. 1997). TGF\textbeta\textsubscript{1} may have stabilized the corresponding enzyme proteins of HSD3B and GSTA or, alternatively, other unknown gene products with HSD3B activity may be involved.

In agreement with previous morphological studies (Gutierrez et al. 1997, Marssters et al. 2003), FSH increases cell number. In this model, it has been suggested that after dispersion, granulosa cells revert to...
a less mature phenotype, re-establish cell–cell communications, and proliferate in the presence of FSH (Gutierrez et al. 1997, Marsters et al. 2003). The moderate increase in total cell protein observed in the presence of FSH could indicate that FSH stimulated proliferation or, alternatively, that FSH prevented apoptosis and increased cell survival. FSH and E2 are known to act as survival factors to induce granulosa cell proliferation and prevent apoptosis (Gutierrez et al. 1997, Yang & Rajamahendran 2000, Jiang et al. 2003, Quirk et al. 2004). In the present study, TGFβ1 caused visible differences in the morphology of granulosa cell clumps, which appeared smaller and more spherical than those in FSH-treated controls. TGFβ1 at the highest dose also caused a slight decrease in total granulosa cell protein. It is unclear at the present time whether the TGFβ1-induced reduction in steroidogenesis is a result or cause of the change in cell proliferation or survival. But, in the absence of FSH, TGFβ1 did not significantly affect total granulosa cell protein. Similarly, TGFβ1 alone had no effect on DNA synthesis in cultured bovine granulosa cells (Lerner et al. 1995). Therefore, the effects of TGFβ1 on granulosa cells could be due to inhibition of the cell-surviving activity of FSH and/or mediated through a loss of E2-stimulated cell survival (Yang & Rajamahendran 2000, Quirk et al. 2004). Ongoing experiments in our laboratory will test this hypothesis, by investigating the effects of TGFβ1 on proliferation and apoptosis of bovine granulosa cells cultured with or without FSH.

We conclude that TGFβ1 plays an inhibitory role in E2 and P4 steroidogenesis in granulosa cells cultured in conditions where E2 and P4 secretion is being stimulated, and that TGFβ1 counteracts the stimulation of mRNA encoding steroidogenic enzymes. As TGFβ1 inhibited FSH mRNA levels and inhibited FSH-induced CYP19A1 and HSD17B1 but not HSD17B7, we propose that the inhibitory effects of TGFβ1 on FSH-stimulated E2 secretion may be due at least in part to the inhibition of FSHR (Fig. 8). These selective inhibitory effects suggest that TGFβ1 may be acting in a physiological manner to limit the amount of E2 and P4 produced by the granulosa cells without totally shutting down the steroidogenic potential (i.e., HSD17B reducing activity and HSD3B activity are unaffected). By acting in this manner, the physiological role of TGFβ1 may be to limit FSH-stimulated growth and differentiation of granulosa cells and play an active role in determining the fate of the developing follicle toward ovulation or atresia.

Materials and Methods

Experimental design

As it has not been established whether FSH stimulates all the steroidogenic enzyme genes in granulosa cells, we first determined the effect of FSH by culturing cells as described below with graded doses of FSH (0, 1, or 10 ng/ml) for 6 days. Based on these data, the development of gene expression during culture was assessed by culturing cells with 1 ng/ml FSH for 2, 4, or 6 days, and the cells were recovered at each time point for the extraction of RNA. Expression of the estrogenic enzyme genes was the highest on day 6; therefore, all subsequent experiments with TGFβ1 were performed for 6 days in the presence of 1 ng/ml FSH.

Cell culture

Cell culture was performed essentially as described by Gutierrez et al. (1997). Briefly, ovaries were collected at a local abattoir from adult cows irrespective of the stage of the estrous cycle and transported to the laboratory at 37 °C in PBS containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and fungiczone (1 μg/ml). Follicles of 2–5 mm in diameter were dissected from the ovaries, and granulosa cells were isolated mechanically by rinsing sections of follicle walls repeatedly through a disposable pipette. The granulosa cell suspension was filtered through a 150 mesh steel sieve (Sigma) to remove oocytes.

The cells were seeded onto 24-well tissue culture plates (Corning Glass Works, Corning, NY, USA) at a density of 10⁶ viable cells (tested by Trypan blue exclusion) in 1 ml α-MEM with l-glutamine containing sodium bicarbonate (10 mM),
HEPES (20 mM), non-essential amino acid mix (1.1 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml), protease-free BSA (0.1%), sodium selenite (4 ng/ml), transferrin (2.5 μg/ml), Aβ (100 nM; all from Sigma), ovine FSH (1 ng/ml; oFSH, AFP-5332B; NIDDKD, Torrance, CA, USA), insulin (10 ng/ml; Invitrogen), and graded doses of recombinant active human TGFβ1 (R&D Systems, Minneapolis, MN, USA) starting on the first day of culture. Cultures were maintained at 37 °C in 5% CO₂ for 6 days, with 700 μl medium being replaced every 2 days.

At the end of culture, the medium was collected and frozen for subsequent steroid assay and granulosa cells were collected for RNA extraction or lysed for total protein measurement. Total cell protein was extracted by the addition of 200 μl of 1 M NaOH to each well for 2 h at room temperature, followed by neutralization with 200 μl of 1 M HCl. Protein concentrations were measured using the Bio-Rad micro-assay (Bio-Rad). All experiments were performed with at least three independent cell cultures, and three to five wells per treatment were pooled for each assay.
Steroid assays

Culture medium samples were assayed for E_2 as reported previously (Bélanger et al. 1990), but without C-18 column extraction. Cross-reaction of A_4 and E_1 with the E_2 assay was less than 0.1% (Bélanger et al. 1980). P_4 was measured in duplicate as described (Lafrance & Goff 1985). Intra- and inter-assay coefficients of variation were less than 15% for both assays. The sensitivity of the E_2 and P_4 assays was 8 and 32 pg per tube respectively. The steroid hormone concentrations were corrected for cell number by normalization to total cell protein per well.

RT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen), according to the manufacturer's instructions, and treated with DNase (Qiagen). The RT reaction was performed on 1 μg total RNA with Omniscript enzyme (Qiagen). Gene expression was measured by semi-quantitative PCR. The primers used were those described previously for cyclophilin (1B15; Bettegowda et al. 2006), CYP11A1 (Vanselow et al. 2004), HSD3B (Vanselow et al. 2004), CYP19A1 (Sahmi et al. 2004), and FSHR (Ndiaye et al. 2005). Sense (5'-TTGTGCAGAGTTGGCAGATTCT-3') and
antisense (5'-AGGAATCGCTCGGTGTAAGTA-3') primers for HSD17B1 were designed based on the bovine sequence (NM_001102365) with a product size of 287 bp. Sense (5'-CGTTGCTGGAGAAGATGATG-3') and antisense (5'-ACCAGAGGATGAGAGCTG-3') primers for HSD17B7 were designed based on the bovine sequence (XM_581467) with a product size of 789 bp. Sense (5'-GACAGGCTACATCAGCGAGTT-3') and antisense (5'-ATGTCGACCGAGCTGATGTT-3') primers for STAR were designed based on the bovine sequence (XM_001250261) with a product size of 248 bp. Sense (5'-GATGGAGGATCCTGCTGCT-3') and antisense (5'-GACTGTCGACTCTGGCCTTGG-3') primers for GSTA were designed based on the bovine sequence (BTU49179) with a product size of 528 bp. Forward and reverse primers used in the PCRs were located in different exons to avoid amplification of any residual genomic DNA. The PCR products for each gene were sequenced to confirm the identity of the gene. PCR was performed under the following conditions: (1) initial denaturation at 94 °C for 3 min; (2) amplification cycles of denaturation at 94 °C for 45 s, annealing for 45 s at 60 °C (CYP11A1, HSD3B, and FSHR) or 64 °C (HSD17B1, HSD17B7, GSTA), or for 30 s at 62 °C (STAR, 1B15 and CYP19A1); (3) elongation at 72 °C for 1 min; and (4) final elongation at 72 °C for 5 min. Optimal cycle number for amplification during the exponential phase was determined for each gene. The reactions were performed for 31 cycles for STAR, 35 cycles for CYP11A1, 34 cycles for HSD3B, 25 cycles for GSTA, 29 cycles for CYP19A1, 36 cycles for HSD17B1 and HSD17B7, 37 cycles for FSHR, and 27 cycles for 1B15. The PCR products were separated on 2% agarose gels containing 0.001% ethidium bromide and visualized under u.v. light. Quantification of band intensities was performed with NIH Image J software (http://rsb.info.nih.gov/nih-image/). Target gene mRNA abundance was expressed relative to 1B15 mRNA abundance.

**Enzyme activity assays**

Short-term incubation of tritiated steroid hormone precursors was conducted at the end of day 6 of culture to measure specific enzyme activities. On day 6, all the medium of each well was removed and replaced with fresh medium without A4 but containing 6–11 nM (corresponding to 1.6 × 10^6 disintegrations per minute (DPM)/ml medium) of either [3H]E1, [3H]A4, [3H]T, or [3H]P4, and 0.01, or 0.05 ng/ml TGFBI. Cells were incubated for 1.5 or 3 h at 37 °C. At the end of the incubation, the medium was recovered and frozen at −20 °C until analysis of steroid metabolism by thin layer chromatography (TLC) as described previously (Godin et al. 1999). Briefly, steroids were extracted from the medium using diethyl ether and resolved on DC-Alufolien neutral (type E) paper plates (Whatman, Maidstone, Kent, England) in toluene:acetone (4:1). Each TLC plate contained [3H]E1, [3H]A4, [3H]E2, [3H]T, and [3H]P4 as standards. A culture medium control was performed by incubating tracer in culture medium without cells, and was used to obtain background radioactivity that was subtracted from product counts. After migration of samples, the TLC plates were exposed to phosphor screens designed for tritium detection, and tritiated steroid metabolites were localized with a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). The rate of production of specific steroid metabolites was quantified by scraping the corresponding sample and background spots from the TLC plate and counting the radioactivity using PCS scintillation fluid (Amersham).

Activity assays were validated by determining the conditions required to maintain excess substrate. In preliminary time-course experiments, 1.6 × 10^6 DPM of [3H]precursor (6–11 nM) were incubated with granulosa cells for 3 and 6 h. The amount of conversion of [3H]A4 and [3H]T precursors to [3H]E2 was similar with 29 and 58% conversion after 3 and 6 h respectively, and thus an incubation time of 3 h was selected to measure the rate of conversion of [3H]A4 and [3H]T to [3H]E2. For HSD17B reducing activity, the amount of conversion of [3H]E1 precursor to [3H]E2 was 92 and 98% after 3 and 6 h respectively. Therefore, to obtain experimental conditions of excess precursor, increasing concentrations of unlabeled E1 (10⁻⁷, 10⁻⁶, 10⁻⁵ M) were added to the 8.8 pmol/ml of [3H]E1, and incubation time was reduced to 1.5 h. This resulted in 6, 20, and 30% conversion of [3H]E1 to [3H]E2 respectively, and therefore 10⁻⁶ M unlabeled E1 was added to all [3H]E1 incubations to quantify HSD17B reducing activity. For HSD3B activity, the amount of conversion of [3H]P2 precursor to [3H]P4 was 37, 69, 79, and 90% conversion after 1.5, 3, 4.5, and 6 h respectively, and thus an incubation time of 1.5 h was selected to measure the rate of conversion of [3H]P2 to [3H]P4.

**Statistical analysis**

Statistical analysis was performed using the JMP software (SAS Institute, Cary, NC, USA). Data are presented as means ± S.E.M. The data were normally distributed as verified using the Shapiro–Wilk test. One-way ANOVA was used to test the main effect of FSH, time, and TGFB1 on the measured parameters. Differences between treatment dose of FSH or TGFBI with the 0 dose control or between 4- and 6-day cultures with the 2-day culture group were identified with Dunnett’s test.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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