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Gonadotropin-Releasing Hormone and Gonadal Steroids Regulate Transcription Factor mRNA Expression in Primary Pituitary and Immortalized Gonadotrope Cells

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Abstract

Hormonal regulation of pituitary gonadotropin gene expression has been attributed to gonadotropin-releasing hormone (GnRH)-mediated stimulation of immediate early gene expression and gonadal steroid interactions with their respective nuclear receptors. A number of orphan nuclear receptors including steroidogenic factor 1, liver receptor homologue 1, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1, and chicken ovalbumin upstream promoter-transcription factors I/II as well as the GATA family members, GATA2 and GATA4, have also been implicated in transcriptional regulation of the gonadotropin genes. We hypothesized that hormonally mediated changes in these latter transcription factors may provide an additional mechanism for mediating hormonal effects beyond the more classically appreciated pathways. In these studies, we demonstrate significant regulation of orphan nuclear receptor and GATA messenger RNA levels by GnRH, dihydrotestosterone, estradiol, and progesterone in both cultured primary pituitary cells and gonadotrope-derived cell line, LβT2. These results advance our understanding of the complex mechanisms by which GnRH and steroid hormones achieve precise regulation of anterior pituitary function.

Keywords

GnRH, gonadal steroids, pituitary, transcription factor

Introduction

Normal sexual maturation and adult reproductive function depend upon the correct temporal and quantitative biosynthesis and secretion of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), by the gonadotrope subpopulation of the anterior pituitary gland. Luteinizing hormone and FSH act in the gonads to stimulate both hormonal production and the development of mature gametes. Gonadotropin gene expression is precisely regulated by input from the hypothalamic peptide, gonadotropin-releasing hormone (GnRH), as well as by gonadal steroid feedback acting at the hypothalamus and directly on the gonadotropes.

Gonadotropin-releasing hormone binds to a specific G-protein-coupled membrane receptor, GnRHR, to activate the protein kinase C, protein kinase A, and calcium intracellular signaling pathways. These pathways, in turn, increase expression of the immediate early genes jun, fos, and early growth response gene 1 (Egr-1) as well as phosphorylation of cyclic adenosine monophosphate response-element binding protein, ultimately stimulating gonadotropin gene expression.[1-7] Although the details differ across reports, it is clear that each of these signaling pathways differentially impacts expression of the individual gonadotropin subunits.[8-10]

In contrast to GnRH, gonadal steroid effects are mediated via interactions with nuclear receptors, specifically the estrogen receptors (ERα or ERβ), progesterone receptors (PR-A or PR-B), and androgen receptor (AR).[11-16] The steroid receptors then bind DNA promoter cis-elements directly or via tethering through transcription factor complexes to activate or repress gene transcription.

In addition to the transcription factors already mentioned, a number of orphan nuclear receptors as well as GATA transcription factors have been shown to regulate gonadotropin

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gene expression. By definition, the orphan receptors lack an identified ligand. Of the orphan receptors, steroidogenic factor 1 (SF-1; NR5A1) is the best studied in both human and animal models. Steroidogenic factor 1 is present in the pituitary gland as well as in neurons in the mediobasal hypothalamus and in steroidogenic cells of the adrenal gland, ovary, and testis. Within the anterior pituitary, expression is restricted to the gonadotrope subpopulation and, thus, contributes to cell-specific gene expression in this tissue.[17-19] The SF-1 binds as a monomer to a consensus sequence, which resembles a nuclear receptor half-site. Functionally important SF-1 response elements have been characterized in the α-subunit, LHβ-subunit, and GnRH-receptor gene promoters.[20-25] Confirming the importance of SF-1 to gonadotrope function, pituitary-specific SF-1 null animals have been reported to have markedly decreased LH and FSH expression with the expected lack of sexual maturation.[26]

Although less well-characterized, our group and others have demonstrated the ability of a closely related orphan receptor, liver receptor homologue 1 (LRH-1; NR5A2), to increase basal gonadotropin and GnRH-R promoter activity using transient transfection and small interfering RNA (siRNA) knockdown approaches.[27,28] As suggested by its name, LRH-1 is expressed at high levels in the liver, where it acts as a key regulator of bile acid metabolism and cholesterol hemostasis.[29] More recent work has demonstrated functional expression in the ovary, testes, adrenal gland, adipocytes, and intestines, demonstrating a more widespread role for this transcription factor.[30-33] Consistent with their close sequence homology, LRH-1 and SF-1 have been shown to increase promoter activity via the same cis-element in a number of genes, including the gonadotropin LHβ gene.[10,28,33-35] Although its physiologic relevance has been questioned recently by the lack of phenotype in a gonadotropic-specific knockout model, endogenous LRH-1 clearly regulates both basal and GnRH-stimulated LHβ and FSHβ promoter activity in a gonadotrope cell line.[27]

A second family of orphan nuclear hormone receptors, chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI; NR2F1) and chicken ovalbumin upstream promoter-transcription factor II (COUP TFII; NR2F2), has also been implicated in pituitary function. During embryogenesis, the COUP-TFs play a critical role in organogenesis and cell-specific differentiation, including development of the testicular Leydig cells.[36-40] In the adult, COUP-TFI and COUP-TFII exhibit both overlapping and distinct expression. Within the anterior pituitary gland, COUP-TFI has been detected in the gonadotrope and somatotrope cells, with COUP-TFII expression in the gonadotropes and lactotropes.[41,42] Although they most commonly inhibit promoter activity, they may also stimulate gene expression depending on promoter and cellular context.[43-45] Supporting the functional importance of these factors in the gonadotrope, we have reported that siRNA-mediated decreases in endogenous COUP-TFI or COUP-TFII expression blunt α-subunit, LHβ-, FSHβ-, and GnRHR messenger RNA (mRNA) levels in the LβT2 cell line.[42]

Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1; NR0B1) and small heterodimer partner (SHP; NR0B2) are 2 closely related atypical orphan members of the nuclear receptor family. Although DAX-1 and SHP have classically been thought to inhibit SF-1 and LRH-1 transcriptional activity, respectively, evidence now suggests that the distinction may not be as clear cut. In place of the classic nuclear receptor DNA-binding domain, DAX-1 and SHP contain a unique N-terminal region with an LXXLL-binding motif often found in coregulators.[46,47] As suggested by its name, DAX-1 mutations result in adrenal hypoplasia and hypogonadotropic hypogonadism in humans.[48] In the adult, DAX-1 colocalizes with SF-1 expression in the anterior pituitary, gonads, adrenal gland, and gonads consistent with a central role in the regulation of hormone biosynthesis.[49] Within the gonadotrope, DAX-1 has been shown to inhibit SF-1-mediated transcriptional activation of the LHβ-subunit gene.[21] The DAX-1 has been shown to have physiological relevance to pituitary function based on the characterization of 2 human kindreds with mutation in the DAX-1 gene.[50]

Small heterodimer partner has been best studied for its role in maintaining cholesterol and glucose homeostasis.[51] Nevertheless, we now report that SHP mRNA expression can be detected in the anterior pituitary and in gonadotrope cell lines, suggesting functional importance in these cells. As SHP inhibits SF-1 and LRH-1 expression in the testes, and SF-1 is clearly critical for gonadotrope development and adult function, we were interested to further investigate its expression pattern in the pituitary following hormonal treatment.[52]

Members of the GATA family of transcription factors are known to regulate adult reproductive function at all levels of the hypothalamic–pituitary–gonadal axis.[53] There are 6 highly conserved GATA factors in vertebrates. Within the reproductive system, GATA4 is known to regulate GnRH expression, while GATA4 and GATA6 play important roles in gonadal steroidogenesis. GATA2 exerts critical differentiation functions in the developing pituitary gland through reciprocal interactions with the transcription factor Pit1, with GATA2 directing cell fate toward the gonadotrope lineage and away from the thyrotrope lineage.[54] Both GATA2 and GATA4 protein have been demonstrated in adult mouse pituitary glands and shown to stimulate expression of gonadotropin subunit and GnRHR genes, both alone and in synergy with SF-1. Conversely, loss of endogenous GATA2 expression in LβT2 gonadotrope cells is associated with a decrease in LHβ mRNA expression.[55-57] Pituitary-specific knockout of GATA2 expression has been shown to result in a decreased number of gonadotropes, as well as decreased circulating FSH levels basally and in response to castration. Although adult males remain fertile, this was likely attributable to the markedly elevated compensatory increase in GATA3 expression in these animals, again suggesting a critical role for GATAs in gonadotrope function.[58]

In a preliminary set of experiments, we observed alterations in pituitary orphan nuclear receptor and GATA factor gene

expression across the estrous cycle, suggesting physiologically important modulation by GnRH and/or gonadal steroids. In order to further investigate these findings, we analyzed the effect of GnRH, dihydrotestosterone (DHT), estradiol, or progesterone on pituitary transcription factor mRNA levels in both cultured primary pituitary cells and in the gonadotrope-derived cell line, LβT2. To our knowledge, this is the first report of a systematic analysis of hormonal regulation of gonadotrope transcription factors.

Materials and Methods

Hormonal Reagents

Dispersed primary pituitary cells or immortalized cells were treated with a GnRH analog (L4897; Sigma-Aldrich, St Louis, Missouri), DHT (5α-androstan-17β-ol-3-one, A8380; Sigma-Aldrich Inc), estradiol (17β-estradiol, 3301; Calbiochem, EMD Millipore, Billerica, Massachusetts), or progesterone (P6149: Sigma-Aldrich) for the doses and durations indicated in the figure legends.

Animal and Pituitary Tissue Collection

Male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts) at 57 to 70 days of age. Rats were housed in the University of Texas Southwestern Medical Center Animal Resource Center on a 14L:10D light cycle. Estrous cycle stage was determined in the female animals by vaginal smear for a minimum of 2 cycles. After brief CO_2 exposure, animals were decapitated and the anterior pituitaries were collected.

Anterior pituitaries from the female animals were snap frozen and stored at 80°C prior to RNA preparation (N = 4). Pituitaries from the male animals were dispersed and cultured as described subsequently. All animal procedures were performed in accordance with guidelines established by the UT Southwestern Institutional Animal Care and Use Committee.

Primary Pituitary Dispersion and Culture

The anterior pituitaries were washed twice in Hanks balanced salt solution (HBSS) supplemented with 0.1% fetal bovine serum (FBS) and 15 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The gland was cut into small blocks and suspended in 10 mL of dissociation medium consisting of HBSS with 0.3% trypsin, 2 mg/mL DNase, 15 mmol/L HEPES, and 1.2 mmol/L EDTA. The tissue and enzymatic mixture was incubated in a shaking water bath at 30°C for 20 minutes. The tissue blocks were gently triturated approximately 20 times with a disposable Pasteur pipette. The cell suspension was transferred to a new conical tube containing an equal volume of Dulbecco modified eagle medium (DMEM; Invitrogen, Carlsbad, California) with high glucose and 10% FBS, 23 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 1× penicillin/streptomycin (DMEM culture medium). The undissociated tissue was allowed to settle to the bottom of the tube, followed by repeat enzymatic dissociation and trituration as described previously. The gland was fully dispersed after 3 dissociation periods. The individual cell suspensions obtained from each dissociation were centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the cells resuspended in DMEM culture medium.

The dispersed primary cells were counted and plated in 48-well tissue culture plates at a density of 250 000 cells/well. After overnight culture, cells were switched to Optimem culture medium (Invitrogen) and treated with the hormones or appropriate vehicles at the concentration and duration indicated in the figure legends. Results were obtained from a minimum of 3 independent experiments with each point tested in duplicate.

Culture of Immortalized Cell Line

Immortalized gonadotrope-derived LβT2 cells were generously provided by Dr P.L. Mellon (University of California, San Diego, California). Cells were maintained in high glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1 mmol/L sodium pyruvate. Cells were split into 12-well plates (2.5-3 × 10^5 cells/well) and cultured overnight. Cells were then washed with PBS followed by culture in Optimem and treatment with vehicle and hormones as indicated. Results were obtained from a minimum of 3 independent experiments with each point tested in duplicate.

RNA Extraction and Reverse Transcription

Total RNA was prepared from primary pituitary cells or from cultured LβT2 cells using TRI Reagent (Ambion, Austin, Texas) according to the manufacturer's instructions. The concentration of total RNA was measured on a Bio-Rad SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, California). Total RNA samples were DNase treated using the Turbo DNA-free kit (Ambion). DNase-treated total RNA of 2 μg was reverse transcribed at 42°C for 50 minutes in the presence of 500 ng of random hexamers and 20 units Superscript II reverse transcriptase in 1× first strand buffer containing 0.5 mmol/L deoxynucleotide triphosphate and 40 units RNase Out (Invitrogen). A parallel reaction lacking the reverse transcriptase was prepared as a negative control.

Polymerase Chain Reactions

Quantitative real-time polymerase chain reaction (qPCR) was performed with the above-mentioned reverse transcribed complementary DNA in a 384-well plate on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, California) using Taqman Universal PCR Master Mix and gene-specific Taqman Gene Expression Assays (Table 1) with universal cycling conditions. Expression of LRH-1 mRNA was detected using the SYBR green method with SYBR Green PCR Master Mix and the primer pair: forward: 5′-CCAGACCCTGTTCTCC ATTGTT; reverse: 5′-CATTTGGTCATCAACC-TTCAGTTC.

Table 1. Real-Time PCR Primer Sequences.

	Species	NR Gene	Assay ID (ABI[a])
TaqMan	Mouse	SF-1, Nr5a1	Mm00446826_m1
		COUP-TFI, Nr2f1	Mm00657937_m1
		COUP-TFII, Nr2f2	Mm00772789_m1
		DAX-1, Nr0b1	Mm00431729_m1
		SHP, Nr0b2	Mm00442278_m1
		GATA2	Mm00492300_m1
		GATA4	Mm00484689_m1
	Rat	SF-1, Nr5a1	Rn00584298_m1
		COUP-TFI, Nr2f1	Rn01489978_m1
		COUP-TFII, Nr2f2	Rn00756179_m1
		DAX-1	Rn00584062_m1
		SHP	Rn00589173_m1
		GATA2	Rn00583735_m1
	Mouse/rat	18s rRNA, vic-MGB	4319413E
SYBR Green	Mouse/rat	LRH-1F	CCA GAC CCT GTT CTC CAT TGT T
		LRH-1R	CAT TTG GTC ATC AAC CTT CAG TTC

Abbreviation: PCR, polymerase chain reaction.
[a]Applied Biosystems, Inc (ABI, Carlsbad, California).

Each reaction was run in 15 μL total volume in triplicate. Target gene expression levels were calculated using the Comparative C_T method as described in Applied Biosystems User Bulletin No. 2 in which the average C_T of the target gene is corrected against the average 18S C_T for each sample. Of note, the basal CT values for all of the transcripts were between 20 and 28 in both of the cell lines, with the exception of 30 to 32 cycles for SHP. As expected, the CT for SF-1 was much lower in the gonadotrope cell line than in the mixed population primary cells (22 vs 26), consistent with its known restriction to the gonadotrope subpopulation within the pituitary.

Statistical Analysis

For graphical representation and statistical analysis, one of the vehicle-treated wells was arbitrarily set to a value of 1, allowing for calculation of the variation in expression levels in both control and hormone-treated cells. For Figure 1, one of the samples obtained at 0900 on proestrus (P0900) was arbitrarily designated the control and set as 1. Statistical analysis was performed using the SigmaStat Software package (SPSS Science, Chicago, Illinois). Data were analyzed for normality followed by analysis of variance. The Tukey test or Student-Newman-Keuls test was used for post hoc comparison, except for experiments with different sample sizes, in which case the Dunn test was employed. Statistical significance was set at $P < .05$.

Results

Changes in Transcription Factor Expression Across the Rat Estrous Cycle

We first asked whether there was evidence for hormonal regulation of the orphan receptors and GATA factors known to be important for gonadotropin biosynthesis. We reasoned that expression of these factors should be altered across the female reproductive cycle if, in fact, hormonal regulation was occurring in vivo.

As shown in Figure 1, steady-state SF-1 mRNA levels fluctuated significantly across the female rat estrous cycle with a nadir late on proestrus. In contrast, transcript levels of the closely related factor, LRH-1, did not vary between time points. A similar divergence in regulatory patterns was observed for the homologous nuclear receptors, COUP-TFI and COUP-TFII, in which only the latter factor exhibited cycle-related changes in expression. The inhibitory factors DAX-1 and SHP demonstrated parallel fluctuations in expression pattern, with a particularly marked drop in SHP transcripts on the mornings of estrus and diestrus. GATA2 mRNA levels varied significantly with a peak on the morning of metestrus. We have previously demonstrated the presence of GATA4 RNA and protein in primary pituitary cells from the mouse and from the mouse gonadotrope-derived LBT2 cell line using conventional RT-PCR. Unfortunately, we have been unable to generate a successful qPCR primer set for the rat and, as a result, could not evaluate quantitative changes in this transcript. As a whole, these results strongly indicate that pituitary transcription factor gene expression is regulated by GnRH and/or gonadal steroid levels, which vary across the reproductive cycle.

Regulation of Orphan Nuclear Receptor and GATA mRNA Levels by GnRH and Gonadal Steroids in Cultured Primary Pituitary Cells

Gonadotrope function reflects a complex response to hypothalamic and gonadal hormones. In order to isolate the effects of GnRH and the gonadal steroids, we next looked at the effects

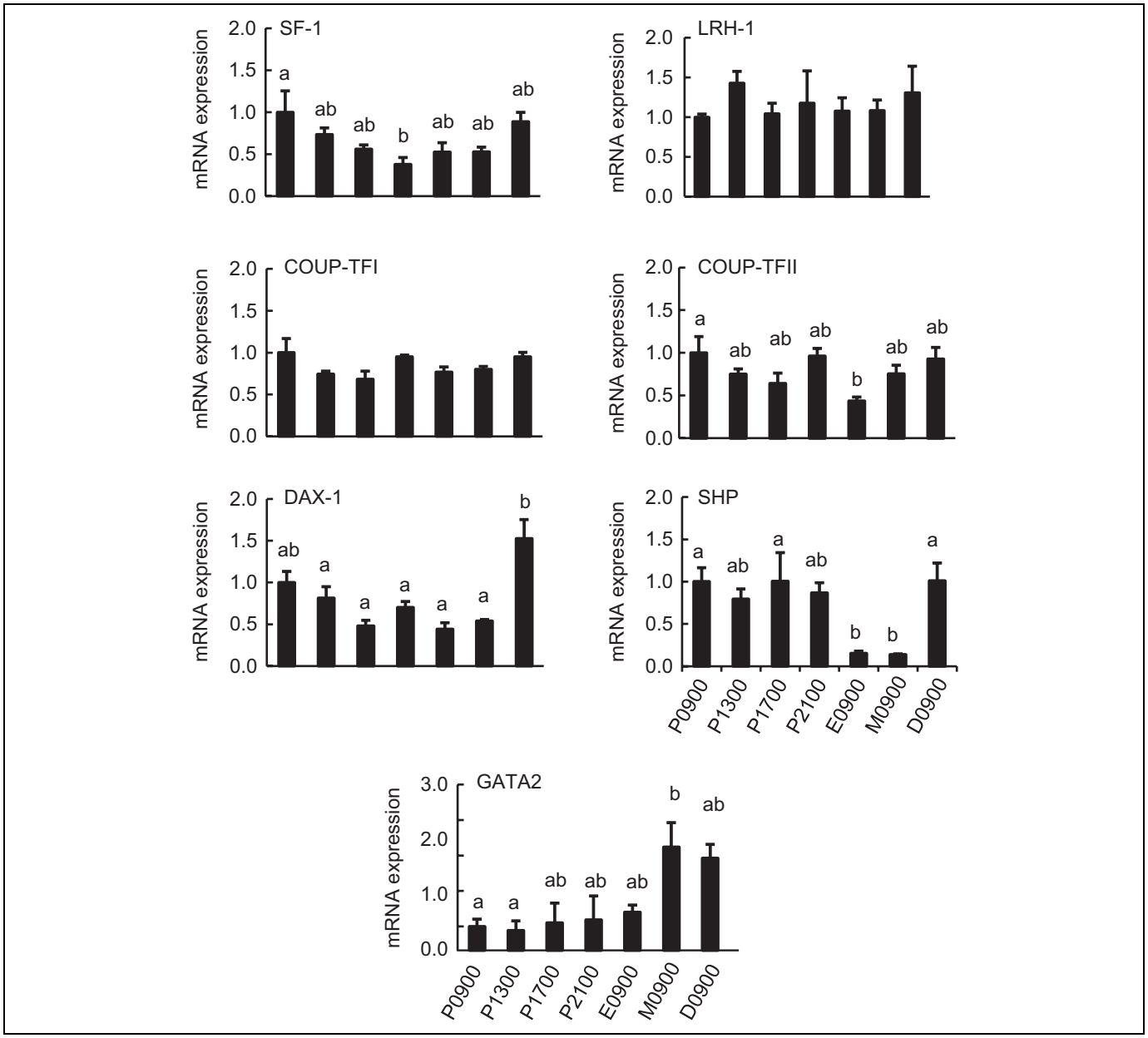

Figure 1. Expression of selected transcription factors across the female rat reproductive cycle. Stage of estrous cycle was determined by monitoring vaginal smears for a minimum of 2 cycles. Steady-state messenger RNA (mRNA) levels were determined using quantitative real-time polymerase chain reaction (qPCR). Numbers after the cycle day indicate the time of day the pituitaries were harvested. Bars with different letters vary significantly for each transcription factor ($P < .05$). PE indicates proestrus; E, estrus; ME, metestrus; DE, diestrus; SF-1, steroidogenic factor-1 (NF5A1); LRH-1, liver receptor homologue 1 (NF5A2); COUP TFI/II, chicken ovalbumin upstream promoter-transcription factors I and II (NR2F1 and NR2F2): DAX-1, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (NR0B1); SHP, small heterodimer partner (NR0B2).

of these hormonal factors on dispersed rat primary anterior pituitary cells in static culture. We decided to utilize cells from male animals for subsequent experiments as hormonal sensitivity changes in pituitary cells across the estrous cycle and, therefore, we were concerned that the use of female cells would introduce another variable into the analysis.

As shown in Figure 2, GnRH treatment (100 nmol/L × 6 hours) markedly decreased SF-1 and SHP transcript levels by approximately 80% and 60%, respectively ($P < .05$). No statistical changes were observed in the other transcription factors analyzed.

Analysis was then performed on cells treated for 6 or 24 hours with vehicle, 1, 10, or 100 nmol/L of the nonaromatizable androgen, DHT (Figure 3). The DHT generated a dose-dependent decrease in SF-1 and SHP mRNA levels following 24 hours of treatment with blunting of GATA2 expression at both the 6- and 24-hour time points ($P < .05$). COUP-TFI and COUP-TFII showed a biphasic dose–response with 24 hours

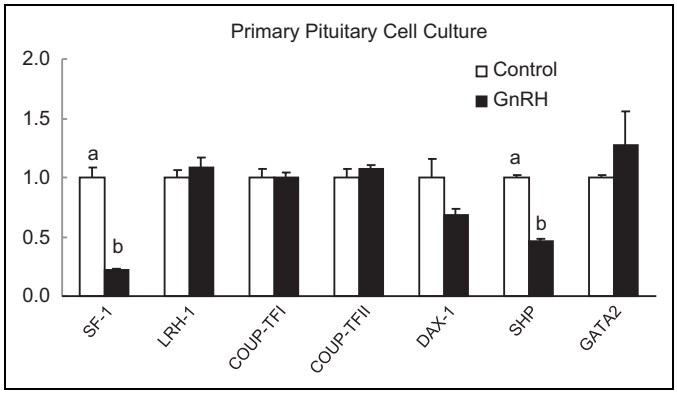

Figure 2. GnRH regulates transcription factor gene expression in dispersed rat primary pituitary cells in vitro. After dispersion and overnight culture, anterior pituitary cells were treated with GnRH (100 nmol/L × 6 hours) or vehicle. Cells were harvested to obtain total RNA followed by analysis with quantitative real-time polymerase chain reaction (qPCR). Bars with different letters indicate a significant difference between the control group and the GnRH-treated group ($P < .05$). GnRH indicates gonadotropin-releasing hormone.

of treatment with a maximum at the 10 nmol/L dose, but this change only reached statistical significance for COUP-TFI.

The ovarian steroid, estradiol (E2), was likewise tested for effects at 6 or 24 hours of treatment using the same range of concentrations. As shown in Figure 4, estradiol significantly increased SF-1 transcript number following 24-hour treatment with the lowest dose of E2 tested (1 nmol/L); however, higher doses were not effective. Interestingly, a similar pattern was observed for COUP-TFI and COUP-TFII, although the response did not reach statistical significance for the COUP-TFs. In contrast, estradiol blunted GATA2 and DAX-1 mRNA expression in a clear dose–response manner following 6 hours of treatment, although it was without effect at the 24-hour time point.

It has been well established that PRs are expressed at very low levels in most tissues unless induced by estrogen prior to progesterone treatment. We, therefore, treated the dispersed pituitary cells with 1 nmol/L of estradiol prior to the addition of 100 nmol/L of progesterone for 6 or 24 hours (Figure 5). This dose and duration of estradiol treatment have been shown to induce PR expression (data not shown). Although statistically significant, progesterone induced a very modest decrease in SF-1 and COUP-TFI expression and a 1.3-fold increase in GATA2 levels with 24 hours of treatment ($P < .05$).

Effects of GnRH or Gonadal Steroid Treatment on Orphan Nuclear Receptor and GATA Factor mRNA levels in an Immortalized Gonadotrope Cell Line

In addition to gonadotropes, the anterior pituitary contains 4 additional endocrine cell types, which secrete prolactin (lactotropes), thyroid-stimulating hormone (thyrotropes), growth hormone (somatotropes), and adrenal corticotropin-releasing hormone (corticotropes). The pituitary also contains a large number of folliculostellate cells that secrete multiple factors that act locally, including follistatin, which modulates the effects of activin on follicle-stimulating hormone (FSH) biosynthesis. Detailed expression patterns for the gonadal steroid receptors, orphan nuclear receptors, and GATA family members have not been fully defined in each of these anterior pituitary cell populations. Nevertheless, it is possible that a number of the responses observed in the primary cell experiments were due, at least in part, to effects on nongonadotrope cells.

In order to isolate the gonadotrope response, we next evaluated responses in an immortalized mouse gonadotrope-derived cell line, LβT2. These cells are believed to represent mature gonadotropes in that they express the gonadotropin α, LHβ, and FSHβ subunits as well as functional GnRH receptors. We and others have demonstrated expression of ERα, PR-B, and AR in this cell line.[14,59-62]

The LβT2 cells were treated with 100 nmol/L GnRH for 6 or 24 hours (Figure 6). A substantially greater number of transcripts were modulated by GnRH in the LβT2 cells compared with the response in the mixed cell population. The GnRH decreased SF-1 transcript number by approximately 50% and increased LRH-1 transcripts by the same magnitude following 6 hours of treatment. Levels of COUP-TF1 and COUP-TFII mRNA were also inhibited at the 6-hour time point, a response which persisted following 24 hours for COUP-TFI only. Interestingly, GnRH decreased DAX-1 expression but increased SHP expression. Although GATA2 transcripts trended up after 6 hours of treatment in primary pituitary cells, a significant decrease was observed at this time point in the cell line. The levels of GATA4 mRNA, which we were not able to evaluate in the primary cell cultures, were not observed to change in the cell line.

The impact of DHT on transcription factor expression was less pronounced in the gonadotrope cell line relative to findings in the mixed primary pituitary cell population (Figure 7 vs Figure 3) with no change in SF-1, LRH-1, COUP-TFI, or SHP observed in the cell line. Furthermore, in contrast to the DHT-mediated decrease in GATA2 in primary cells, both GATA2 and GATA4 transcript number increased significantly in LβT2 cells, albeit to a very small degree.

The LβT2 cells were then treated with vehicle or 100 nmol/L estradiol for 6 or 24 hours (Figure 8). As seen in primary pituitary cells, estradiol again decreased DAX-1 mRNA levels. In contrast, no changes in SF-1 or GATA2 expression were observed in the LβT2 cell line; however, GATA4 expression increased at both time points analyzed. Estradiol decreased both COUP-TFI and SHP mRNA levels, an effect not observed in primary cell cultures.

Figure 9 demonstrates the effect of 100 nmol/L progesterone treatment on transcription factor expression in the gonadotrope cell line. In contrast to primary pituitary cells, PR expression is not upregulated by estrogen in the LβT2 cell line and therefore cells were not pretreated (Turgeon and Waring[62] and data not shown). Progesterone did not statistically alter SF-1 or LRH-1 mRNA levels in LβT2 cells, which differed from the small decrease observed in the mixed primary pituitary cell population. Once again, progesterone did not alter DAX-1 expression.

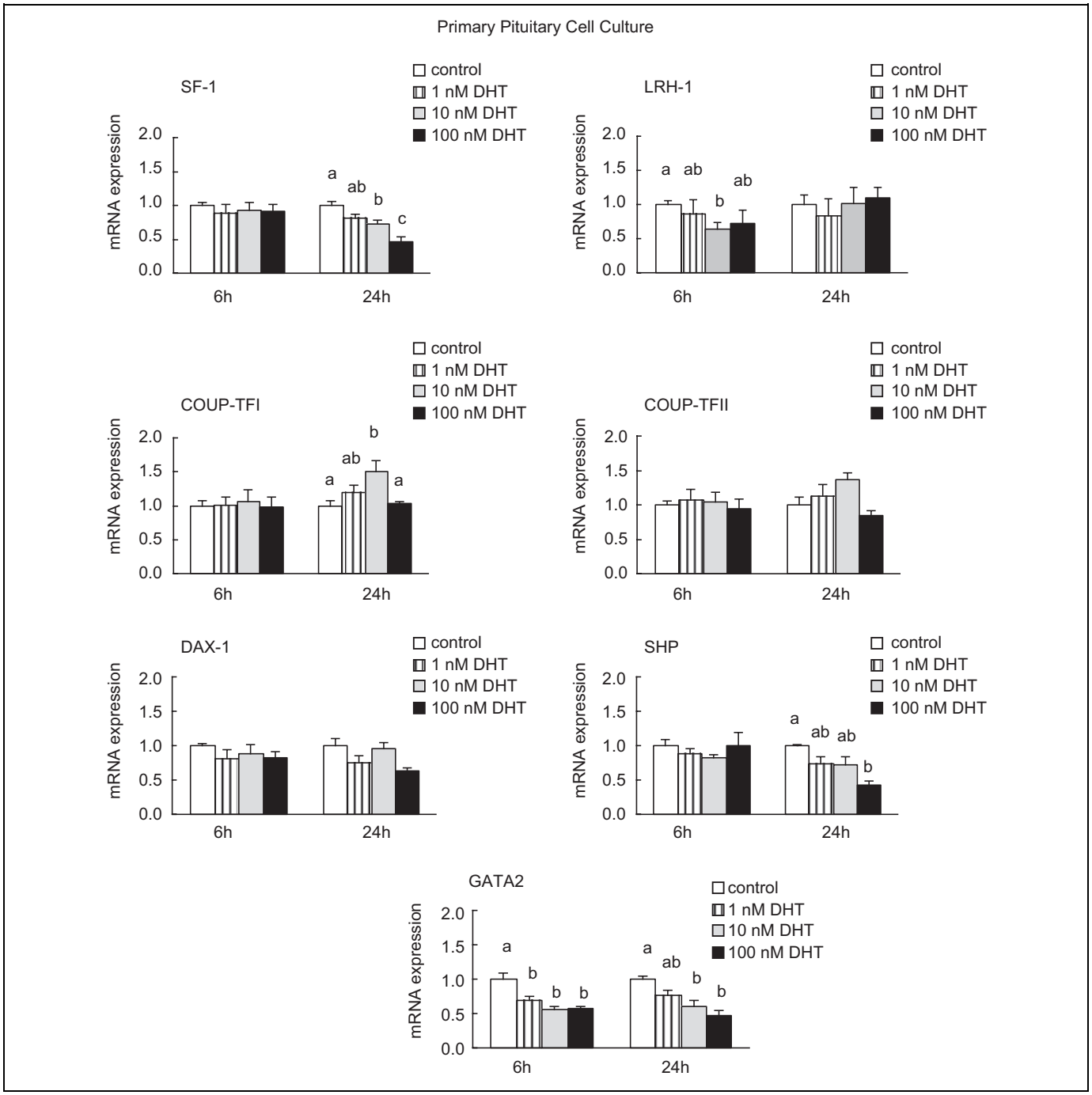

Figure 3. Expression of transcription factor messenger RNA (mRNA) in rat primary pituitary cells treated with dihydrotestosterone (DHT). Dispersed anterior pituitary cells were treated with vehicle (control) or with DHT at concentrations of 1, 10, or 100 nmol/L for 6 or 24 hours, as indicated. RNA was extracted and gene expression analyzed by quantitative real-time polymerase chain reaction (qPCR). Bars with different letters differ significantly within the same treatment group ($P < .05$).

As observed in the primary cells, GATA2 transcripts were increased significantly following 24 hours of progesterone treatment, but not by 6 hours of treatment. Conversely, GATA4 mRNA levels were increased with the shorter treatment duration. Progesterone treatment for 24 hours increased COUP-TF1 transcripts at 24 hours in LβT2 cells, in contrast to the slight decrease observed in cultured primary pituitary cells. Furthermore, an increase in COUP-TFII and SHP mRNA levels was observed in this cell line but not in primary cells.

Discussion

In this article, we report the effects of GnRH, DHT, estradiol (E2), and progesterone (P4) on steady-state mRNA levels of

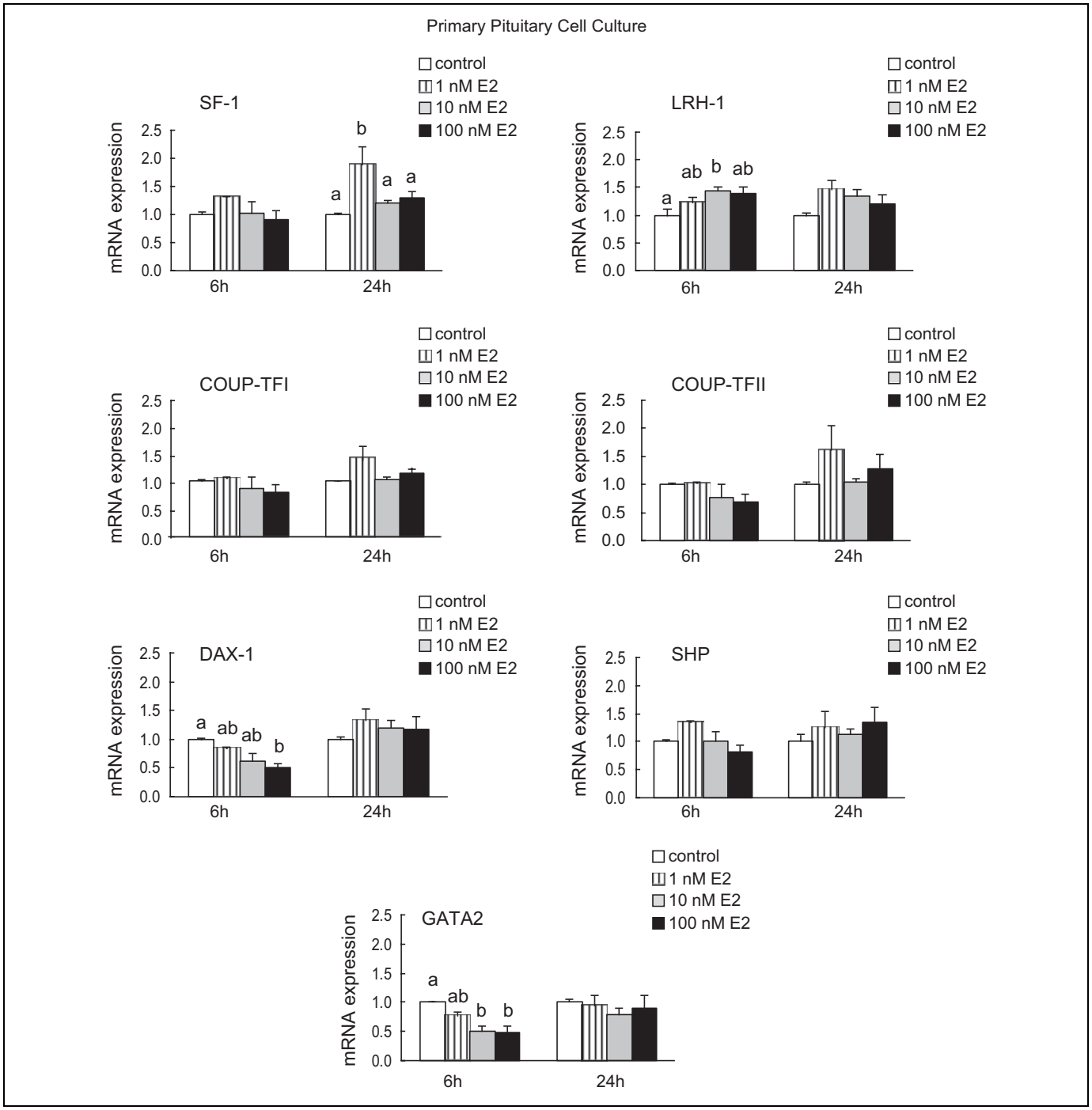

Figure 4. Effect of estradiol (E2) on orphan nuclear receptor and GATA messenger RNA (mRNA) levels in cultured primary pituitary cells. Dispersed cells were treated with 1, 10, or 100 nmol/L estradiol or vehicle (control) for 6 or 24 hours followed by total RNA extraction and analysis of gene expression by quantitative real-time polymerase chain reaction (qPCR). Bars with different letters differ significantly within the same treatment group ($P < .05$).

a range of transcription factors with known importance for gene expression in the anterior pituitary gonadotrope. Although a role for SHP in the pituitary has not been described, we now demonstrate SHP mRNA expression in both primary pituitary cells and a gonadotrope cell line, suggesting possible functional significance in these cells. These results are provided in overview form in Table 2 in which the presence of a change is indicated without reference to dose or time course.

Although a large amount of information has been learned regarding the molecular regulation of gonadotropin gene expression, a number of critical questions remain. For instance, why do gonadotropes express transcription factors

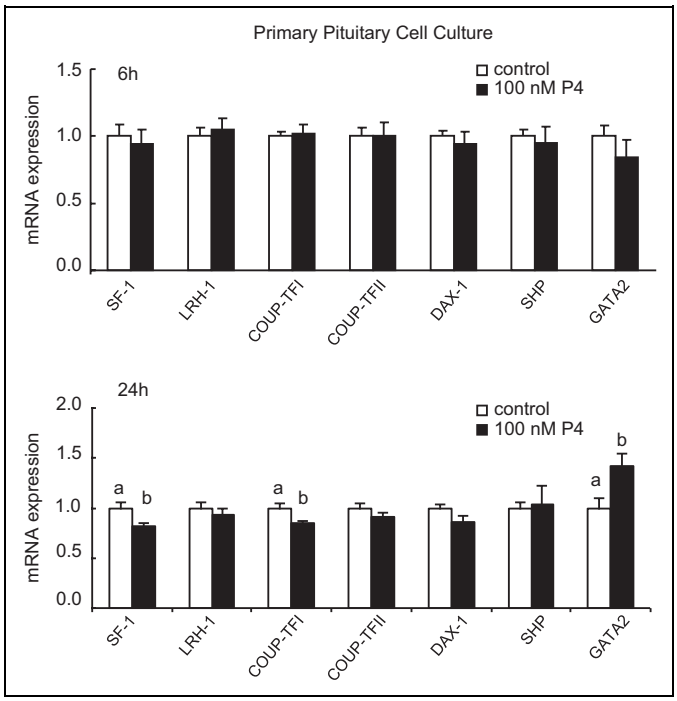

Figure 5. Progesterone (P4) exerts minimal effects on expression of orphan nuclear receptor and GATA messenger RNA (mRNA) levels in anterior pituitary cells. Dispersed cells were treated with 100 nmol/L progesterone or vehicle for 6 or 24 hours followed by total RNA extraction and analysis of gene expression by quantitative real-time polymerase chain reaction (qPCR). Bars with different letters indicate a significant difference between the control group and the progesterone-treated group ($P < .05$).

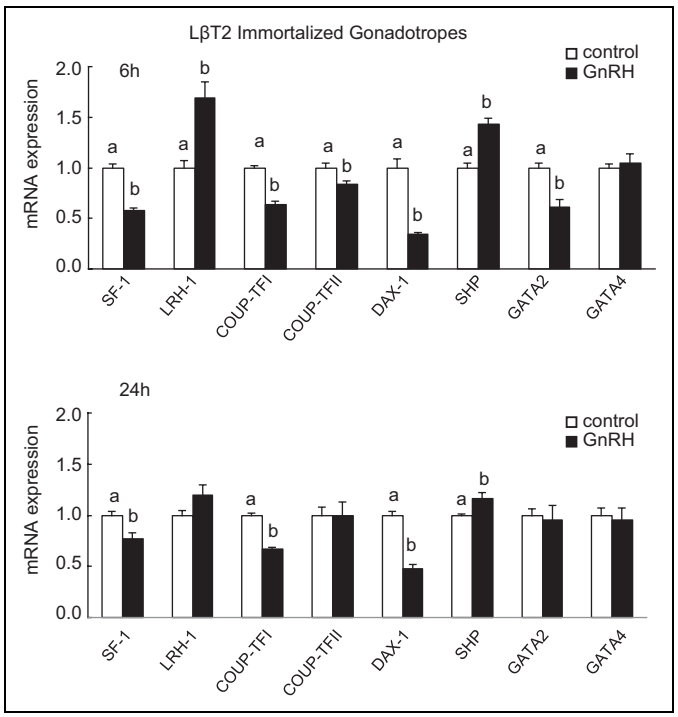

Figure 6. GnRH regulates GATA and orphan nuclear receptor gene expression in LβT2 gonadotrope cells. Cells were treated with 100 nmol/L GnRH for 6 or 24 hours, harvested for RNA, and gene expression analyzed by quantitative real-time polymerase chain reaction (qPCR). Bars with different letters indicate a significant difference between the control group and the GnRH-treated group ($P < .05$). GnRH indicates gonadotropin-releasing hormone.

which appear to be redundant in that they are closely related at the protein level and/or bind to similar DNA-regulatory elements? We hypothesized that differential hormonal regulation of these apparently redundant factors allows for greater fine-tuning of gonadotropin expression.

Transcriptional regulation of the rat LHβ gene provides a good illustration of these concepts. The proximal LHβ gene promoter contains 2 compound SF-1/Egr-1 DNA-regulatory regions that flank a site for the homeobox transcription factor, Pitx1, which may also act as a GATA-responsive regulatory site.[55,63-65] Work in our laboratory has demonstrated that LRH-1 and COUP-TFs can also exert transcriptional effects via nucleotides that overlap the SF-1 cis-elements.[28,42] Interestingly, although SF-1 and LRH-1 bind to the same cis-elements, distinct function may be achieved, at least in part, via divergent functional interactions with COUP-TF. Although the exact molecular mechanisms are unknown, COUP-TFs inhibit SF-1-mediated stimulation of LHβ promoter activity, but augment LRH-1 effects on this gene.[42]

GnRH-mediated regulation of the LHβ gene promoter has long been attributed to induction of early growth response protein 1 (Egr-1) with subsequent formation of an SF-1–Egr-1–Sp1 complex, with the Sp1 sites located a few hundred nucleotide base pairs upstream of the SF1–Egr-1 region.[3] We observed a GnRH-mediated decrease in SF-1 expression in both primary pituitary cells and the LβT2 cell line (Figures 2 and 6). Furthermore, GnRH treatment blunted COUP-TFI/II mRNA levels, at least in the gonadotrope cell line. These data suggest a secondary pathway by which GnRH could modulate LHβ gene transcription, that is, via modulation of the relative expression levels of stimulatory and inhibitory factors acting at common DNA regulatory sites.

Precise hormonal regulation of LHβ gene expression may also be achieved via differential hormonal regulation of closely related transcription factors. Perhaps most notably, GnRH treatment divergently regulated SF-1 and LRH-1 mRNA levels, decreasing SF-1 expression in both primary pituitary and LβT2 cells but either not altering or actually increasing LRH-1 expression in these 2 cell models, respectively (Figure 6). Weck et al have reported a hormonally mediated switch between SF-1 and LRH-1 binding to the inhibin promoter.[10] It would be of interest to determine whether a similar substitution is occurring on the gonadotropin subunit or GnRH-receptor gene promoters. Divergent GnRH effects were also observed for the GATA factors in which GnRH decreased GATA2 mRNA levels but did not alter GATA4 expression. Parallel effects have been observed at the GATA protein level, further supporting differential expression as a means for mediating hormonal responses.

The GnRH may have been expected to increase SF-1 mRNA levels as both factors increase gonadotropin expression. As one

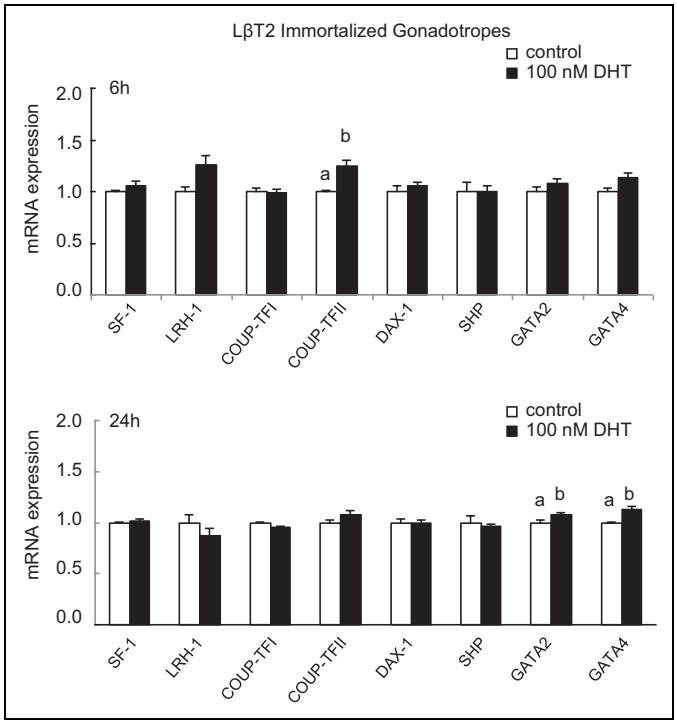

Figure 7. Effect of dihydrotestosterone (DHT) treatment on the expression of transcription factors in LβT2 gonadotrope cells. Cells were treated with estradiol (100 nmol/L × 6 or 24 hours), harvested for RNA, and gene expression analyzed by quantitative real-time polymerase chain reaction (qPCR). Bars with different letters indicate a significant difference between the control group and the gonadotropin-releasing hormone (GnRH)-treated group ($P < .05$).

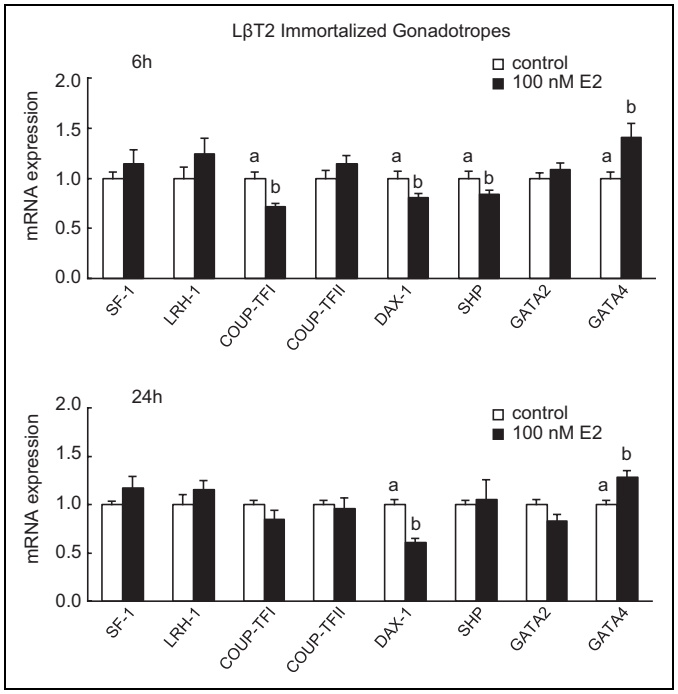

Figure 8. Estradiol (E2) alters expression of multiple transcription factors in the LβT2 cell line. Cells were treated with vehicle or estradiol (100 nmol/L × 6 or 24 hours), harvested for RNA, and gene expression analyzed by quantitative real-time polymerase chain reaction (qPCR). Bars with different letters indicate a significant difference between the control group and the estradiol-treated group ($P < .05$).

possibility, it may be that the results are due to the fact that cells were treated with continuous rather than pulsatile GnRH. Of note, continuous GnRH treatment has also been shown to decrease SF-1 mRNA levels in the αT3-1 gonadotrope cell line.[7] Also in support of this possibility, in vivo model systems using male rats have shown a transient increase in SF-1 transcription rates with fast pulse GnRH treatment, as might be predicted with the concomitant increase in gonadotropin levels.[1] Nevertheless, perifusion of female rat primary pituitary cells with pulsatile GnRH was reported to blunt SF-1 mRNA levels.[66] These apparently conflicting results could perhaps be due to differential exposure to sex steroids or other hormonal factors. It may also be that the loss of SF-1 stimulation is counteracted by the marked increase in stimulatory LRH-1 expression or decrease in inhibitory DAX-1 expression, at least as observed in the LβT2 cells.

Ovarian-derived estrogen exerts feedback effects at the level of the hypothalamus and the pituitary to modulate gonadotropin biosynthesis and secretion. In the pituitary, estrogen exerts a gonadotropin-specific effect, increasing LHβ gene transcription but not glycoprotein α- or FSHβ-subunit expression.[67] Estrogen may induce LHβ gene expression, in part, via "tethering" of ligand-bound ERα to SF-1 and Ptx1.[68] Estradiol has been shown previously to enhance Egr-1 gene expression and we now show stimulation of both SF-1 and LRH-1 mRNA levels in primary pituitary cells (Figure 7).[69]

Our results also demonstrated an estradiol-mediated repression of the inhibitory nuclear receptor, DAX-1, in both cell models tested. The DAX-1 has been shown to blunt SF-1-mediated increases in LHβ gene promoter activity in gonadotropes and to repress transcriptional cooperation between GATA-4 and SF-1 in Sertoli cells.[21,70] Estradiol was also observed to decrease expression of the related orphan nuclear receptor, SHP, in the LβT2 cells, although this response was not detected in primary cells. The function of SHP in the pituitary has not been studied, but it is known that SHP inhibits expression of steroidogenic genes by directly repressing transcriptional activity of LRH-1 and SF-1, as well as by inhibiting expression of both SHP and DAX-1.[52] Therefore, the data presented here suggest that E2 may increase LHβ expression via blunted expression of these inhibitory orphan nuclear receptors in addition to the effects of ERα protein–protein interactions with SF-1 or Pitx1. Furthermore, the decrease in expression of these orphan receptors may augment estrogen action as both DAX-1 and SHP inhibit ERα transcriptional activity.[71,72]

Dihydrotestosterone and progesterone have been shown to stimulate FSHβ gene promoter activity via direct binding of their respective nuclear receptors to promoter sequences.[15] In contrast, induction of LHβ transcription by these steroids is mediated via tethering to DNA-bound transcription factors, specifically via binding to SF-1 or Sp1 in the case of the AR.[11,12] As shown in Figure 3, DHT treatment

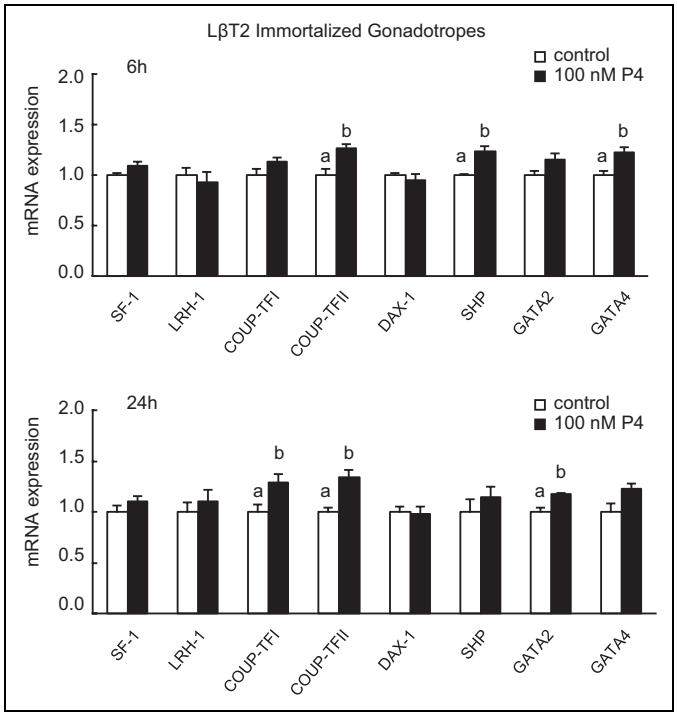

Figure 9. Progesterone (P4) effects on the expression of transcription factors in LβT2 gonadotrope cells. Cells were treated with 100 nmol/L progesterone for 6 or 24 hours, harvested for RNA, and gene expression analyzed by quantitative real-time polymerase chain reaction (qPCR). Bars with different letters indicate a significant difference between the control group and the progesterone-treated group ($P < .05$).

Table 2. GnRH- and Steroid-Induced Changes in mRNA Expression.

	Expressed in Gonadotropes?	GnRH	DHT	E2	P4
Primary pit					
SF-1	Yes[17]	↓↓	↓↓	↑↑	↓
LRH-1	Yes[31,28]	~	↓	↑	~
COUP-TFI	Yes[42]	~	↑	~	↓
COUP-TFII	Yes[42]	~	~	~	~
DAX-1	Yes[48,a]	~	~	↓↓	~
SHP	Current[a]	↓↓	↓↓	~	~
GATA2	Yes[55,54]	~	↓↓	↓↓	↑
GATA4	Yes[55]	ND	ND	ND	ND
LβT2 cells					
SF-1	Yes[21]	↓	~	~	~
LRH-1	Yes[28]	↑↑	~	~	~
COUP-TFI	Yes[42]	↓	~	↓	↑
COUP-TFII	Yes[42]	↓	↑	~	↑
DAX-1	Current[a]	↓↓	~	↓	~
SHP	Current[a]	↑	~	↓	↑
GATA2	Yes[55]	↓	↑	~	↑
GATA4	Yes[55]	~	↑	↑	↑

Abbreviations: SF-1, steroidogenic factor 1 (NF5A1); LRH-1, liver receptor homologue 1 (NF5A2); COUP-TFI/II, chicken ovalbumin upstream promoter-transcription factors I and II (NR2F1 and NR2F2); DAX-1, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (NR0B1); SHP, small heterodimer partner (NR0B2); ↓, decrease less than 50%; ↓↓, decrease by at least 50%; ↑, increase less than 50%; ↑↑, increase at least 50%; ND, not tested; GnRH, gonadotropin-releasing hormone; DHT, dihydrotestosterone; mRNA, messenger RNA; pit, pituitary.
[a]Expression shown at mRNA level only.

significantly decreased SF-1 and GATA2 transcript levels and increased COUP-TFI expression in primary pituitary cells. Data exist to support a role for each of these transcription factors in regulation of the gonadotropin and GnRH-receptor genes.[20,22,23,42,55-57] Thus, although androgens may increase LHβ and FSHβ promoter activity via the AR, concomitant changes in additional transcription factors may modulate this response.

For these studies, we theorized that stimulatory transcription factors and inhibitory transcription factors would be inversely regulated in response to hormonal treatment as a means of magnifying hormonal effects on gonadotropin expression. Nevertheless, this pattern was not uniformly observed. For example, the α, LHβ, and GnRH receptor genes are all known to be stimulated by SF-1 whose effects are inhibited by DAX-1. Nevertheless, GnRH was found to inhibit both SF-1 and DAX-1 mRNA levels in LβT2 gonadotropes (Figure 6).

These studies also revealed differences in the response of dispersed primary pituitary cells compared with the immortalized mouse gonadotrope cell line. A number of potential explanations exist and their importance may differ for each factor. The difference in species may be a confounder, as could the process of immortalization or the presence of multiple cell types in the primary pituitary dispersion. As a general rule, GnRH effects were more frequently observed in the LβT2 cell line, perhaps because only the gonadotrope subpopulation expresses GnRHR in the pituitary cell cultures while all of the immortalized cells are GnRH responsive. Therefore, one could postulate that a small GnRH response in the gonadotrope subpopulation could be obscured by unregulated expression in the other cell types. For example, both COUP-TFI and COUP-TFII expression decreased slightly in response to GnRH in the pure gonadotrope cell line but did not change in the primary pituitary cells. This observation may be explained by the presence of high numbers of COUP-TFII expressing lactotropes, which lack GnRH receptors in the mixed cell population.[41] Conversely, hormonally induced changes in expression pattern in primary pituitary cells but not in the gonadotrope cell line may reflect effects on nongonadotrope cell populations. At present, it is not possible to determine which of these mechanisms is operating as cell-specific expression of the majority of these factors within the anterior pituitary is unknown.

Although the LβT2 cell line has been found to express AR, ERα, and PR proteins, the functional activity of these receptors has been questioned with investigators commonly overexpressing the relevant receptor when analyzing transcriptional regulation in these cells.[60,73,74] Nevertheless, although the estrogen response has been reported to be unstable in this cell line, we observed small but statistically significant changes in

mRNA expression of a number of transcription factors following estradiol treatment (Figure 8).[73] Furthermore, responses were not substantially different in cells transfected with the AR or PR expressing plasmids prior to hormonal treatment (data not shown), arguing against a lack of functional or adequate receptor number in this cell line as a cause for discrepancies between the primary and immortalized cells.

Although statistically significant, a number of the observed responses were relatively small. Where possible, we tested additional transcripts that have been previously shown to be hormonally regulated in pituitary cells at the steady-state mRNA level. For the primary pituitary cell experiments, the effectiveness of the GnRH treatment was confirmed by a 5-fold increase in GnRHR mRNA levels at 6 hours, while DHT treatment increased FSHβ-subunit transcripts by 2- to 3-fold, and E2 increased PR-B mRNA levels approximately 3-fold (data not shown). Our laboratory is also interested in regulation of the autocrine–paracrine factor pituitary adenylate-cyclase polypeptide (PACAP, Adcyap1) and we have reported the ability of P4 to augment GnRH-mediated stimulation on PACAP mRNA levels by approximately 3-fold using a similar paradigm.[75] In terms of the LβT2 cells, the majority of prior studies have used these cells to analyze for transcriptional activation using luciferase expression rather than mRNA levels as an end point, with the exception of a response to GnRH. It was therefore not possible to identify an obvious positive mRNA control for steroid responsiveness. Nevertheless, as expected, GnRH increased FSHβ mRNA levels by approximately 10-fold and PACAP mRNA levels by as much as 100-fold in our experiments (data not shown).

Also of interest, the majority of the investigated transcription factors assessed in these studies are expressed at high levels in other steroidogenic tissues, including the gonads. For example, ovarian steroidogenesis has been linked to expression of SF-1, COUP-TFII, and DAX-1 and several gonadal genes are known to respond to interactions between SF-1 and GATA factors.[76-78] Although speculative, it is possible that the hormonal responses which were observed in pituitary cells may shed light on regulation of the evaluated transcription factors in other reproductive and nonreproductive tissues.

Of note, as these studies were limited to measurement of mRNA levels, it will be important in the future to correlate changes in transcript number with alterations in protein expression. It will also be of interest to look at the combinatorial effects of GnRH and steroids in future experiments, as well as the importance of continuous versus pulsatile GnRH stimulation on regulation of transcription factor expression. It should also be noted that a number of additional hormonal factors beyond GnRH and gonadal steroids are well known to have critical effects on gonadotropin gene expression. For example, bioavailable activin is known to vary across the estrous cycle and may alter transcription factor expression, acting both alone and in synergy with other hormonal modulators. We believe that the data presented here provide a firm foundation and justification for pursuing an array of future studies.

In conclusion, the data presented here suggest that GnRH and gonadal steroids alter pituitary expression of orphan nuclear receptor and GATA mRNA levels. Prior studies have demonstrated the ability of GnRH to alter gonadotropin biosynthesis via increases in immediate early gene expression, while gonadal steroids exert effects via interaction with their respective nuclear steroid receptor. We now propose that hormonally mediated changes in transcription factor expression may provide an additional mechanism for mediating hormonal effects on gonadotrope function beyond these more classically appreciated pathways.

Authors' Note

All work was performed at the University of Texas Southwestern Medical Center. Weiming Zheng and Constance M. Grafer contributed equally to this work. The sponsors did not have a role in the study design, analysis or interpretation of the data, or in the decision to submit the article for publication.

Declaration of Conflicting Interests

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