ORIGINAL ARTICLE

Effects of Estradiol and Progesterone on Gonadotropin LHβ- and FSHβ-subunit Promoter Activities in Gonadotroph LβT2 cells

Haruhiko Kanasaki, Indri N. Purwana, Tselmeg Mijiddorj, Unurjargar Sukhbaatar, Aki Oride, Kohji Miyazaki

Department of Obstetrics and Gynecology, Shimane University School of Medicine, Izumo, Japan

Correspondence to: Haruhiko Kanasaki, MD., PhD.
Dept. of Obstetrics and Gynecology, Shimane University, School of Medicine
89-1 Enya Cho, Izumo City 693-8501, Shimane Prefecture, Japan.
tel: +81-853-20-2268; fax: +81-853-20-2264;
e-mail: kanasaki@med.shimane-u.ac.jp

Submitted: 2012-08-22 Accepted: 2012-09-29 Published online: 2012-11-15

Key words: **gonadotropin; LH; FSH; estradiol; progesterone**

Neuroendocrinol Lett 2012; **33**(6):608–613 **PMID:** 23160221 NEL330612A03

Abstract

OBJECTIVES: Sex steroid hormones play roles in the regulation of pituitary hormone synthesis and secretion. Here we investigated the role of estradiol (E2) and progesterone (P4) on pituitary gonadotropin luteinizing hormone (LH)β- and follicle stimulating hormone (FSH)β-transcriptional activity in a single colony of gonadotroph LβT2 cells. METHODS: Pituitary gonadotroph cell line, LβT2 cells were used in this study. Cells were transfected with LHβ- or FSHβ-subunit promoter region-linked luciferase vector, and stimulated with gonadotropin-releasing hormone (GnRH) in the presence or absence of sex steroids. Transcriptional activity for LHβ- and FSHβ-subunit were determined by luciferase assay. Effects of sex steroids on cell proliferation was also determined by measurement of 5-bromoe-2'-deoxyuridine (BrdU) incorporation.

RESULTS: The basal promoter activity of the LHβ subunit was not modulated by 10 nM E2, but gonadotropin releasing hormone (GnRH)-induced LHβ promoter activity was significantly increased by the same concentration of E2. Similarly, although the basal FSHβ promoter was not modulated by 10 nM E2, GnRH-induced FSHβ promoters were significantly potentiated in the presence of E2. One micromole E2 modulated neither basal nor GnRH-induced LHβ and FSHβ promoters. On the other hand, basal LHβ promoter activity was enhanced by 1 μM P4, but the stimulatory response of GnRH on LHβ promoters was significantly inhibited in the presence of 1 μM P4. Similar to LHβ promoters, the basal activity of the FSHβ promoter was increased by 1 μM P4; however, the response to GnRH was not modulated in the presence of P4. Ten micromoles P4 modified neither basal nor GnRH-induced promoter activity for LHβ and FSHβ. E2 had no antagonistic effect on P4-induced basal promoter activities of LHβ or FSHβ. A cell proliferation assay showed that neither E2 nor P4 modulated the growth of LβT2 cells, even in the presence or absence of GnRH.

CONCLUSION: These observations suggest that both E2 and P4 uniquely modulate basal and GnRH-stimulated gonadotropin promoters without affecting cell growth.

INTRODUCTION

Sex steroids such as estrogen (E2) and progesterone (P4) regulate or modulate the synthesis and secretion of several pituitary hormones and play an important role in reproductive functions. The pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are regulated mainly by hypothalamic gonadotropin-releasing hormone (GnRH) but also by sex steroid hormones (Gharib *et al.* 1990).

It is well documented that the reduction of sex steroids by ovariectomy increases levels of both gonadotropin secretion and gene expression (Papavasiliou *et al.* 1986; Gharib *et al.* 1987). In other instances, however, steroids can positively regulate gonadotropin synthesis and secretion. Estrogen increases LHβ mRNA synthesis *in vitro* in pituitary fragments (Crowley *et al.* 1985). The complexity of E2 effects on gonadotropin secretion has been well reviewed (Finkelstein *et al.* 1991). These effects can be both stimulatory and inhibitory in vivo depending on study design, the presence or absence of GnRH, and prevailing conditions. In experiments using pituitary fragments of female rat, E2 treatment resulted in a transient increase in LHβ expression, but had no effect on FSHβ mRNA expression (Shupnik *et al.* 1989). On the other hand, Phillips *et al.* demonstrated that E2 and P4 inhibited FSH synthesis and secretion in ovine pituitary cultures (Phillips *et al.* 1988). Gene expression for the LHβ and FSHβ subunits are also known to change throughout the rat estrus cycle (Ortolano *et al.* 1988; Zmeili *et al.* 1986).

Although the effects of P4 on gonadotropins have not been elucidated to the same degree as those of E2, P4 activity is known to be mediated largely at hypothalamic levels in association with GnRH. During the luteal phase when circulating progesterone concentration is high, LH pulse frequency slows markedly (Soules *et al.* 1984). P4 has also been reported to augment the stimulatory or inhibitory effect of E2 on gonadotropin secretion in different parts of the menstrual cycles (Odell & Swerdloff 1968; Goodman *et al.* 1981).

LβT2 cells are an immortalized murine pituitary gonadotroph-derived cell model that express the α-, LHβ-, and FSHβ-subunits as well as the GnRH receptor and synthesize and release LH and FSH in response to GnRH. We previously examined the mechanisms of GnRH pulse frequency-dependent specific regulation of gonadotropin subunit gene expression (Kanasaki *et al.* 2005, 2009; Purwana *et al.* 2010, 2011). Although all of these studies used cultured LβT2 cells, we did not previously examine the effects of sex steroid hormones in these cells. Because pituitary glands are responsive to sex steroids, including E2 and P4, it is important to determine their influence on gonadotropin subunit expression.

The present study examined the effects of sex steroid hormones on basal and GnRH-induced transcriptional activity of gonadotropin LHβ and FSHβ subunits in LβT2 cells.

MATERIALS AND METHODS

Materials

The following chemicals and reagents were obtained from the indicated sources: Fetal bovine serum and trypsin (GIBCO, Invitrogen, Carlsbad, CA); Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, GnRH, water-soluble β-estradiol, and water-soluble progesterone (Sigma Chemical Co., St. Louis, MO).

Cell culture

LβT2 cells were plated in 35-mm tissue culture dishes and incubated with high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C under a humidified atmosphere of 5% CO_2 in air. After 24 h, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin and incubated without (control) or with the test reagents for the indicated periods of time.

Reporter Plasmid Construct and Luciferase Assay

The reporter constructs were generated by fusing −797/+5 of the rat LHβ gene (LHβ-Luc) or −2000/+698 of the rat FSHβ gene (FSHβ-Luc) with firefly luciferase (Luc) cDNA in pXP2, as previously described (Kanasaki *et al.* 2005). Cells were transiently transfected by electroporation with either 2.0 μg/well of gonadotropin subunit-Luc or 0.1 μg/well of a PRL-TK vector containing Renilla luciferase. Cells were then plated in 35-mm culture dishes. After incubation with test reagents, cells were washed with ice-cold PBS and lysed with passive lysis buffer (Promega, Madison, WI/USA). After centrifugation at 15,000 rpm at 4 °C, firefly luciferase and Renilla luciferase activity were measured in the supernatant with the Dual-Luciferase Reporter Assay System (Promega) using a luminometer (TD-20/20) (Promega) according to the manufacturer's protocol. Luciferase activity was normalized for Renilla luciferase activity to correct for transfection efficiency, and results were expressed as fold increase compared with the unstimulated control. All experiments were independently performed three times, each in triplicate.

Cell proliferation assay

Cell proliferation was quantitated based on the measurement of 5-bromoe-2'-deoxyuridine (BrdU) incorporation during DNA synthesis using a commercial Cell Proliferation Biotrak ELISA System (Amersham Bioscience, Piscataway, NJ). A total of 10^5 LβT2 cells were cultured in 96-well plates with or without 10 nM E2 or 1 μM P4 in DMEM for 48 h. GnRH was added with the sex steroids. Media was changed 24 h before harvest to BrdU containing DMEM with or without the same amount of E2 and P4. The rate of cell proliferation was determined according to the manufacture's protocol using an ELISA plate reader at 450 nm.

Statistical analysis

All experiments were independently repeated at least three times. Each experiment was performed using triplicate samples in each experimental group. Data are expressed as mean ± SEM values. Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple range test. $p<0.05$ was considered significant.

RESULTS

Effect of E2 on gonadotropin promoter activities

The basal activity of the LHβ-promoter was not modulated in the presence of 10 nM or 1 μM E2 (Figure 1A). Similarly, the basal activity of the FSHβ-promoter was not modified in the presence of 10 nM or 1 μM E2 (Figure 1B). In contrast, GnRH-stimulated LHβ promoter activity was potentiated by 10 nM E2, but not by higher concentration of E2 (Figure 1C). Similar to LHβ, the FSHβ promoter activated by GnRH was enhanced in the presence of 10 nM E2 (Figure 1D).

Effects of P4 on gonadotropin promoter activities

Next, the effect of P4 on gonadotropin promoter activity was determined. Basal LHβ-promoter activity was increased 2.30±0.21-fold by 1 μM P4 (Figure 2A). Similarly, the basal promoter activity of FSHβ was increased 1.90±0.27-fold by 1 μM P4 (Figure 2B). On the other hand, GnRH-induced LHβ promoters were significantly reduced in the presence of 1 μM P4, reduced by 4.56±1.60-fold to 2.65±0.82-fold (Figure 2C). No GnRH-stimulated FSHβ transcriptional activity was modulated in the presence of P4 (Figure 2D).

Effect of E2 on P4 increased basal promoter activity for LHβ and FSHβ

P4, but not E2 increased the basal activity for both LHβ and FSHβ subunits. Incubation of the cells with both E2 and P4 did not modify the increased effect of P4 on LHβ and FSHβ basal promoter activities (Figure 3A and B).

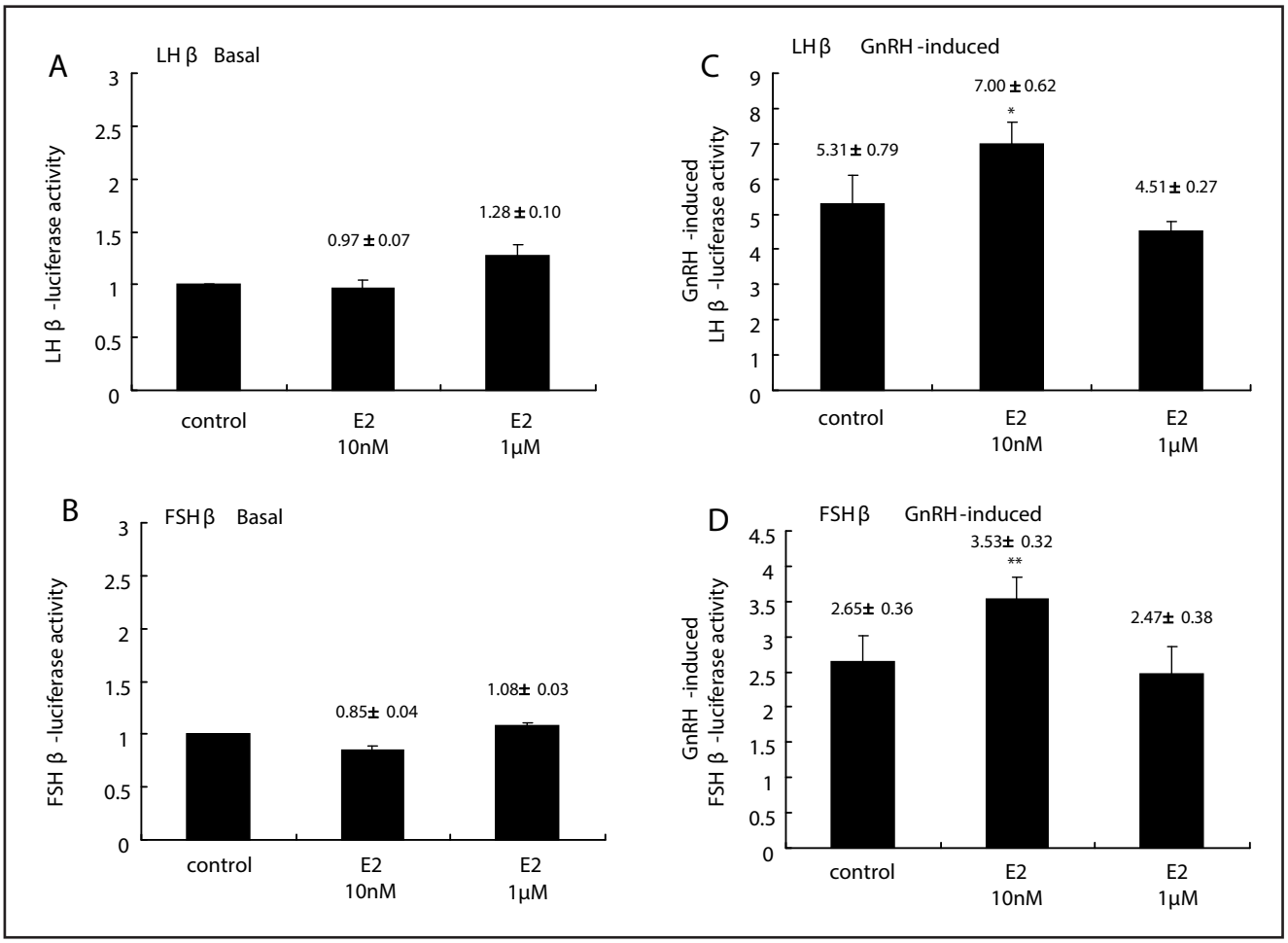

Fig. 1. Effect of E2 on basal and GnRH-stimulated promoter activity for LHβ and FSHβ. LβT2 cells were co-transfected with 0.1 μg PRL-TK vector and 2.0 μg LHβ- or FSHβ- luciferase promoter. After 48 h of culture with or without (control) the indicated concentrations of E2, cells were treated with (Figure 1C and 1D) or without (Figure 1A and 1B) 10 nM GnRH for 6 h. A luciferase assay was performed to measure gonadotropin promoter activity, which was normalized to PRL-TK activity and expressed as fold activation over unstimulated controls. Data are expressed as mean ± SEM values (three independent experiments were performed using triplicate samples). *$p<0.05$; **$p<0.01$ vs. control.

Effect of E2 and P4 on cell proliferation

Next, we examined cell growth with or without sex steroids by incubating cells with 1 μM P4 and 10 nM E2, respectively, for 48 h. Cells with sex steroids grew similarly to non-treated cells. In addition, the effect of E2 and P4 with GnRH on cell proliferation was unchanged compared with controls treated with GnRH only (Figure 4).

DISCUSSION

We previously examined specific regulation of gonadotropin LHβ and FSHβ expression by applying different frequencies of GnRH pulse stimulation (Kanasaki *et al.* 2005, 2009, 2011). It is generally agreed that a rapid GnRH pulse frequency increases LH secretion, while a slower frequency increases FSH secretion (Wildt *et al.* 1981). Indeed, a higher frequency of GnRH predominantly increases LHβ subunits, while a lower frequency of GnRH increases the FSHβ subunit in single gonadotroph LβT2 cells (Kanasaki *et al.* 2005). Most previous studies that used LβT2 cells to examine the differential regulation of GnRH pulse frequency-dependent gonadotropin subunit gene expression were performed without sex steroids. Because pituitary glands are constantly exposed to sex steroids such as E2 and P4, the present study aimed to investigate the direct effect of E2 and P4 on the transcriptional activities of gonadotropin subunit promoter in LβT2 cells. Here, the effects of these sex steroids on basal promoter activity and the GnRH-stimulated increase in gonadotropin promoters were examined.

Previous reports have demonstrated that the effects of E2 on gonadotropin gene expression were both stimulatory and inhibitory. Treatment of pituitary cells from infantile female rat with E2 transiently stimulated both LHβ and FSHβ mRNA expression (Wilson & Handa 1998). E2 has also been reported to stimulate the basal promoter activity in LHβ in LβT2 cells (Kowase *et al.* 2007). While, Phillips *et al.* reported that both E2 and

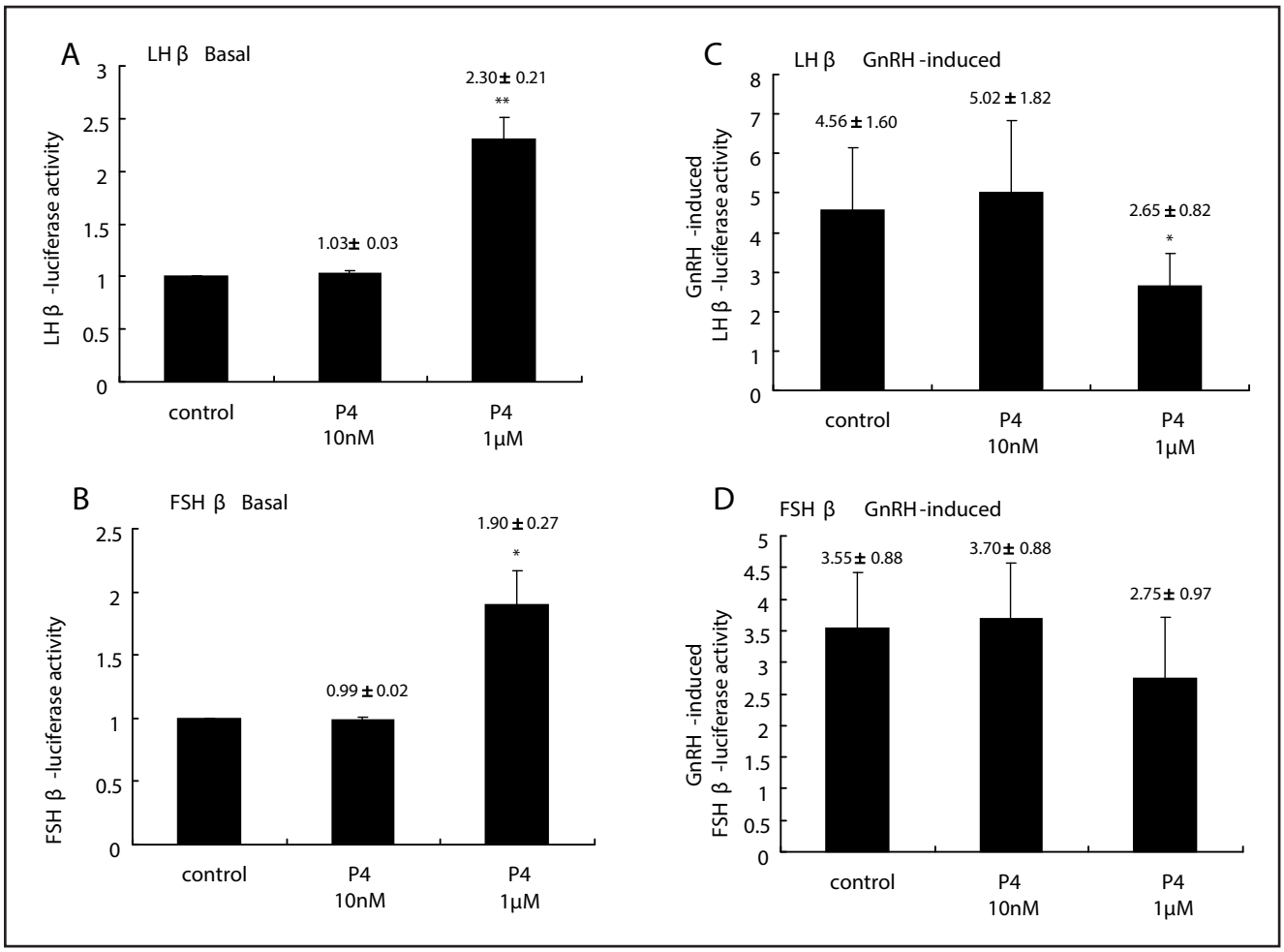

Fig. 2. Effect of P4 on basal and GnRH-stimulated promoter activity for LHβ and FSHβ. LβT2 cells were co-transfected with 0.1 μg PRL-TK vector and 2.0 μg LHβ- or FSHβ- luciferase promoter. After 48 h of culture with or without (control) the indicated concentrations of P4, cells were treated with (Figure 1C and 1D) or without (Figure 1A and 1B) 10 nM GnRH for 6 h. A luciferase assay was performed to measure gonadotropin promoter activity, which was normalized to PRL-TK activity and expressed as fold activation over unstimulated controls. Data are expressed as mean ± SEM values (three independent experiments were performed using triplicate samples). *$p<0.05$; **$p<0.01$ vs. control.

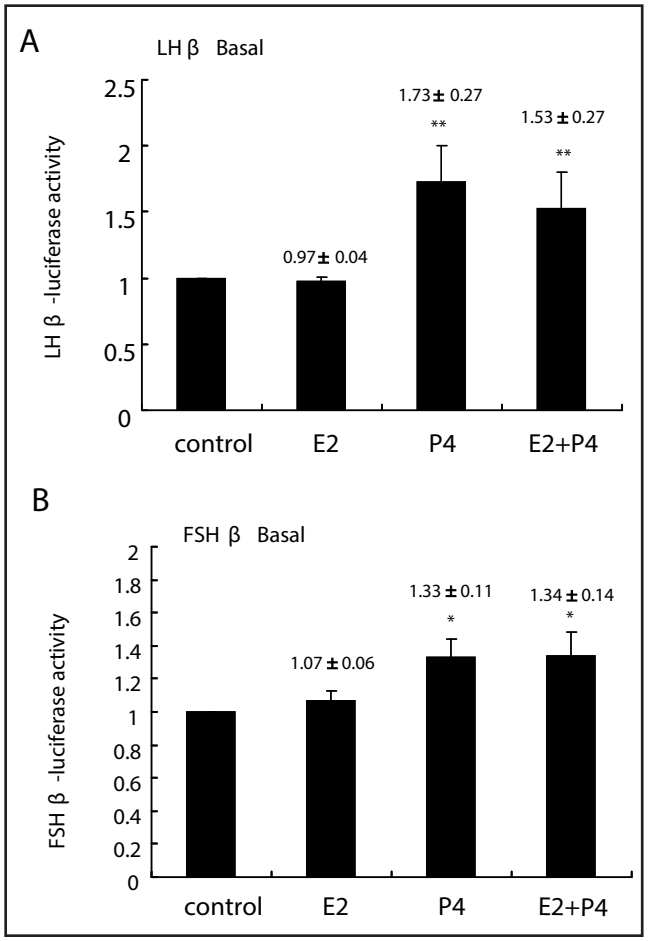

Fig. 3. Effect of E2 on P3 increased basal promoter activity for LHβ and FSHβ. LβT2 cells were co-transfected with 0.1 μg PRL-TK vector and 2.0 μg LHβ- or FSHβ- luciferase promoter. Cells were cultured in the presence of E2, P4, and both E2 and P4 for 48 h. Then, a luciferase assay was performed to measure gonadotropin promoter activity, which was normalized to PRL-TK activity and expressed as fold activation over unstimulated controls. Data are expressed as mean ± SEM values (three independent experiments were performed using triplicate samples). *p<0.05; **p<0.01 vs. control.

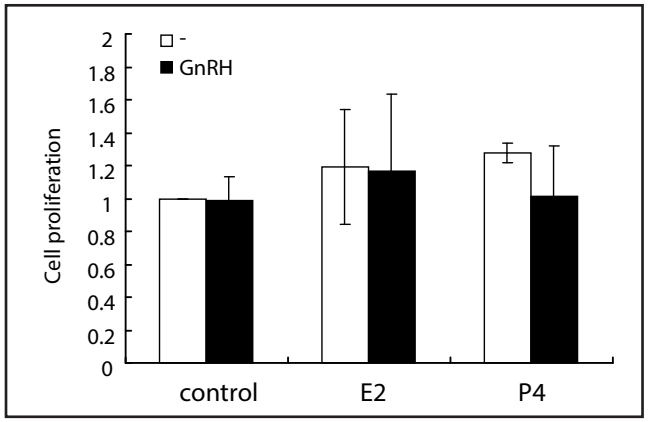

Fig. 4. Effect of sex steroids on LβT2 cell proliferation. LβT2 cells were maintained for 48 h in DMEM medium without (control) or with E2 (10 nM) and P4 (1 μM) in the presence (■) or absence (□) of 10 nM GnRH. Cells were also cultured together with E2 and P4. DNA synthesis was determined as described in Materials and Methods. The proliferation rate in controls without GnRH was taken as 1.0, and relative changes are shown.

P4 inhibited the transcriptional activity of the FSHβ promoter in ovine pituitary cultures (Phillips *et al.* 1988) and Thackray, VG *et al.* demonstrated the inhibitory effect of P4 on the basal promoter activity of LHβ using LβT2 cells (Thackray *et al.* 2009). Thus, experimental results for the role of sex steroids on gonadotropin subunit transcriptional activity varied according to species or study design.

In the present experiments using a single gonadotroph cell line, LβT2, the basal activities of the LHβ and FSHβ promoters were unchanged by 10 nM E2 (Figure 1A and 1B). In contrast, 1 μM P4 significantly increased the basal promoter activities of both LHβ and FSHβ (Figure 2A and 2B). Both sex steroids were presented over 48 h with luciferase vectors for LHβ and FSHβ, and only higher concentrations of P4 modulated the basal activity for both gonadotropin gene promoters. Considering that P4 did not alter the proliferation rate of LβT2 cells during P4 stimulation (Figure 4), the basal transcriptional activities of both LHβ and FSHβ promoters were likely regulated by only P4.

In the previous study which demonstrated the inhibitory effect of P4 on the LHβ subunit promoter in LβT2 cells, cells were treated with P4 only for 6 h, and transcriptional activity was then assayed (Thackray *et al.* 2009). The effect of sex steroids might vary according to duration of exposure. In addition, the stimulatory effect of P4 on the basal transcriptional activity of FSHβ and LHβ subunits was not modulated in the presence of E2. These observations suggest that E2 does not exert the same antagonistic effect as that of P4 on gonadotropin transcriptional activity.

On the other hand, the stimulatory effect of GnRH on both LHβ and FSHβ transcriptional activity was potentiated in the presence of 10 nM E2, although the same concentration of E2 did not modulate the basal gonadotropin promoter activity (Figure 1C and D). P4, which increases the basal promoters of LHβ and FSHβ, however, inhibited GnRH-induced LHβ promoter activity (Figure 2C). Activation of FSHβ promoters by GnRH was not modulated by pre-treatment with P4 (Figure 2D). Previous reports have shown E2 to potentiate GnRH induction of LHβ indirectly by enhancing repressor binding at −381 and −182 of the rat LHβ promoter using LβT2 cells [20]. These observations were similar to our results on LHβ. In contrast, inhibition of GnRH-induced LHβ gene expression by P4 treatment using LβT2 cells has also been described (Thackray *et al.* 2009).

Little is known about the effect of sex steroids on FSH, especially in single gonadotrophs, but in this experiment, we know that E2 potentiates GnRH-induced FSHβ transcriptional activity; however, P4 does not modulate the GnRH-induced FSHβ promoters although P4 reduced the LHβ promoter activity induced by GnRH. Considering the observation that neither E2 nor P4 modulated cell proliferation with GnRH (Figure 4), the effects of E2 on GnRH-induced

LH and FSH promoters and the effect of P4 on the GnRH-induced LHβ promoter were not concomitant with alteration of cell numbers.

E2 and P4 regulate gonadotropin gene expression by negative and positive feedback to the hypothalamus and to the anterior pituitary gland. The present study was designed to elucidate the direct action of sex steroids in a single model of pituitary gonadotrophs. It is true that the action of E2 and/or P4 on gonadotropin gene expression is indeed controversial. Results differ according to design; for example, *in vivo* experiments and studies using primary cultures of anterior pituitary cells or a single cell model of gonadotrophs. In *In vivo* experiments, the administration of sex steroids affects the number of hypothalamic factors including GnRH and dopamine (DeMaria *et al.* 2000) and ultimately modulates gonadotropin gene expression. In addition, experiments using cultured anterior pituitary cells which contain several types of pituitary hormone-secreting cells have been found to be influenced by paracrine factors. Thus, the present study showed the simple action of 48 h of ovarian steroids treatment on LHβ and FSHβ transcriptional activities as well as on gonadotroph cell proliferation.

In summary, we showed that E2 increased GnRH-induced LHβ and FSHβ transcriptional activities, although the basal activities of LHβ and FSHβ promoters were unchanged. High concentrations of P4 increased the basal promoter activities of both LHβ and FSHβ, however, GnRH-induced LHβ promoters were inhibited by the same concentration of P4. GnRH-induced FSHβ promoters were unchanged by P4. Moreover, no sex steroids modified gonadotroph proliferation with or without GnRH.

ACKNOWLEDGEMENTS

This work was supported by Grants in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (grant numbers 20791144); and Grants from Yamaguchi Endocrine Research Foundation.

Conflict of interests

The authors declare that they have no conflict of interests.

REFERENCES

1 Crowley WF, Jr., Filicori M, Spratt DI, Santoro NF (1985). The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. Recent Prog Horm Res. **41**: 473–531.
2 DeMaria JE, Livingstone JD, Freeman ME (2000). Ovarian steroids influence the activity of neuroendocrine dopaminergic neurons. Brain Res. **879**: 139–147.
3 Finkelstein JS, O'Dea LS, Whitcomb RW, Crowley WF, Jr. (1991). Sex steroid control of gonadotropin secretion in the human male. II. Effects of estradiol administration in normal and gonadotropin-releasing hormone-deficient men. J Clin Endocrinol Metab. **73**: 621–628.
4 Gharib SD, Wierman ME, Badger TM, Chin WW (1987). Sex steroid hormone regulation of follicle-stimulating hormone subunit messenger ribonucleic acid (mRNA) levels in the rat. J Clin Invest. **80**: 294–299.
5 Gharib SD, Wierman ME, Shupnik MA, Chin WW (1990). Molecular biology of the pituitary gonadotropins. Endocr Rev. **11**: 177–199.
6 Goodman RL, Bittman EL, Foster DL, Karsch FJ (1981). The endocrine basis of the synergistic suppression of luteinizing hormone by estradiol and progesterone. Endocrinology. **109**: 1414–1417.
7 Kanasaki H, Bedecarrats GY, Kam KY, Xu S, Kaiser UB (2005). Gonadotropin-releasing hormone pulse frequency-dependent activation of extracellular signal-regulated kinase pathways in perifused LbetaT2 cells. Endocrinology. **146**: 5503–5513.
8 Kanasaki H, Mutiara S, Oride A, Purwana IN, Miyazaki K (2009). Pulse frequency-dependent gonadotropin gene expression by adenylate cyclase-activating polypeptide 1 in perifused mouse pituitary gonadotroph LbetaT2 cells. Biol Reprod. **81**: 465–472.
9 Kanasaki H, Purwana IN, Mijiddorj T, Oride A, Miyazaki K (2011). Possible involvement of PACAP and PACAP type 1 receptor in GnRH-induced FSH beta-subunit gene expression. Regul Pept. **167**: 227–232.
10 Kowase T, Walsh HE, Darling DS, Shupnik MA (2007). Estrogen enhances gonadotropin-releasing hormone-stimulated transcription of the luteinizing hormone subunit promoters via altered expression of stimulatory and suppressive transcription factors. Endocrinology. **148**: 6083–6091.
11 Odell WD, Swerdloff RS (1968). Progestogen-induced luteinizing and follicle-stimulating hormone surge in postmenopausal women: a simulated ovulatory peak. Proc Natl Acad Sci U S A. **61**: 529–536.
12 Ortolano GA, Haisenleder DJ, Dalkin AC, Iliff-Sizemore SA, Landefeld TD, Maurer RA, Marshall JC (1988). Follicle-stimulating hormone beta subunit messenger ribonucleic acid concentrations during the rat estrous cycle. Endocrinology. **123**: 2149–2151.
13 Papavasiliou SS, Zmeili S, Herbon L, Duncan-Weldon J, Marshall JC, Landefeld TD (1986). Alpha and luteinizing hormone beta messenger ribonucleic acid (RNA) of male and female rats after castration: quantitation using an optimized RNA dot blot hybridization assay. Endocrinology. **119**: 691–698.
14 Phillips CL, Lin LW, Wu JC, Guzman K, Milsted A, Miller WL (1988). 17 Beta-estradiol and progesterone inhibit transcription of the genes encoding the subunits of ovine follicle-stimulating hormone. Mol Endocrinol. **2**: 641–649.
15 Purwana IN, Kanasaki H, Mijiddorj T, Oride A, Miyazaki K (2011). Induction of dual-specificity phosphatase 1 (DUSP1) by pulsatile gonadotropin-releasing hormone stimulation: role for gonadotropin subunit expression in mouse pituitary LbetaT2 cells. Biol Reprod. **84**: 996–1004.
16 Purwana IN, Kanasaki H, Oride A, Mijiddorj T, Shintani N, Hashimoto H, Baba A, Miyazaki K (2010). GnRH-induced PACAP and PAC1 receptor expression in pituitary gonadotrophs: a possible role in the regulation of gonadotropin subunit gene expression. Peptides. **31**: 1748–1755.
17 Shupnik MA, Gharib SD, Chin WW (1989). Divergent effects of estradiol on gonadotropin gene transcription in pituitary fragments. Mol Endocrinol. **3**: 474–480.
18 Soules MR, Steiner RA, Clifton DK, Cohen NL, Aksel S, Bremner WJ (1984). Progesterone modulation of pulsatile luteinizing hormone secretion in normal women. J Clin Endocrinol Metab. **58**: 378–383.
19 Thackray VG, Hunnicutt JL, Memon AK, Ghochani Y, Mellon PL (2009). Progesterone Inhibits basal and gonadotropin-releasing hormone induction of luteinizing hormone beta-subunit gene expression. Endocrinology. **150**: 2395–2403.
20 Wildt L, Hausler A, Marshall G, Hutchison JS, Plant TM, Belchetz PE, Knobil E (1981). Frequency and amplitude of gonadotropin-releasing hormone stimulation and gonadotropin secretion in the rhesus monkey. Endocrinology. **109**: 376–385.
21 Wilson ME, Handa RJ (1998). Direct actions of gonadal steroid hormones on FSH secretion and expression in the infantile female rat. J Steroid Biochem Mol Biol. **66**: 71–78.
22 Zmeili SM, Papavasiliou SS, Thorner MO, Evans WS, Marshall JC, Landefeld TD (1986). Alpha and luteinizing hormone beta subunit messenger ribonucleic acids during the rat estrous cycle. Endocrinology. **119**: 1867–1869.