Pregnenolone sulfate regulates prolactin production in the rat pituitary

Eun-Jin Kang¹, So-Hye Hong¹, Jae-Eon Lee¹, Seung Chul Kim², Hoe-Saeng Yang³, Pyong in Yi⁴, Sang-Myeong Lee⁵ and Beum-Soo An¹

¹Department of Biomaterials Science, College of Natural Resources and Life Science/Life and Industry Convergence Research Institute, Pusan National University, Milyang, Republic of Korea

²Department of Obstetrics and Gynecology, Biomedical Research Institute, Pusan National University School of Medicine, Milyang, Republic of Korea

³Department of Obstetrics and Gynecology, Medical College, Dongguk University, Seoul, Republic of Korea

⁴Department of Bioenvironmental Energy, College of Natural Resources and Life Science, Pusan National University, Milyang, Republic of Korea

⁵College of Environmental and Bioresource Sciences, Chonbuk National University, Jeonju, Republic of Korea

Correspondence should be addressed to B-S An

Email
anbs@pusan.ac.kr

Abstract

Pregnenolone sulfate (PS) is a neuroactive steroid hormone produced in the brain. In this study, the effects of PS on synthesis and secretion of rat pituitary prolactin (PRL) were examined. To accomplish this, GH3 rat pituitary adenoma cells were treated with PS, which showed significantly increased mRNA and protein levels of PRL compared with the control. The mechanism of action responsible for the effects of PS on PRL synthesis and secretion was investigated by pretreating cells with inhibitors of traditional PRL- or the PS-related signaling pathway. PS-stimulated PRL transcription was significantly reduced by inhibitors of PKA, PKC and MAPK, but unchanged by GABAAR and NMDAR inhibitors. Western blotting analysis revealed that the total ERK1/2 level was upregulated in a time-dependent manner following PS treatment. An approximate 10% increase in GH3 cell proliferation was also observed in response to PS relative to the control. In the animal study, levels of PRL in the pituitary and in serum were elevated by PS. PS-stimulated PRL synthesis was also found to be associated with decreased expression of PRL target genes such as GNRH1, FSHB and LHB. These findings show that PS upregulates PRL synthesis and secretion *in vivo* and *in vitro* via MAPK signaling, suggesting that it has the potential for use as a therapeutic hormone to treat PRL-related disorders such as hypoprolactinemia and low milk supply.

Key Words

- ▶ pregnenolone sulfate
- ▶ neurosteroid
- ▶ pituitary prolactin

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Introduction

Prolactin (PRL) is a polypeptide hormone synthesized and secreted from specialized cells of the anterior pituitary gland, which is known for its multiple effects on the female mammary gland, including regulation of growth and development of the gland (mammogenesis),

synthesis of milk (lactogenesis) and maintenance of milk secretion (galactopoiesis) (Freeman *et al.* 2000). In addition, the range of PRL functions has greatly increased in females and males. An early review revealed that PRL had 85 functions (Cowie 1973), which has subsequently

been updated to over 300, including immune functions, reproduction, osmoregulation and behavior (Bole-Feysot *et al.* 1998).

PRL-inhibiting factors (PIFs) and PRL-releasing factors (PRFs) supplied by neurosecretory cells control synthesis and secretion of PRL from the pituitary (Arey *et al.* 1989, Lamberts & Macleod 1990). The PIF includes dopamine (DA), somatostatin and gamma-aminobutyric acid (GABA), whereas PRF includes thyrotropin-releasing hormone (TRH), neurotensin and oxytocin. Internal milieu such as suckling, stress and increased levels of ovarian steroids modulate PRF and PIF activities, which transduce PRL-regulating signals (Meites *et al.* 1963, Neill 1970, Terkel *et al.* 1972).

DA is the predominant hypothalamic factor involved in regulation of PRL synthesis and secretion. In addition, factors influencing the dopaminergic tone in lactotrophs have been investigated to illuminate the complex connection between PRL and DA. DA directly influences lactotrophs by binding to the D2 receptor (D2R) subclass expressed on their cell membranes (Lefkowitz & Labrie 1978). Other pituitary-derived factors also regulate PRL secretion, including transforming growth factor alpha (TGF- α), transforming growth factor beta (TGF- β), interleukins and galanin (Sarkar *et al.* 1998). In addition, the secretion and synthesis of PRL are modulated by calcium (Ca^{2+}) concentration. PRL secretion is sensitive to changes in extracellular Ca^{2+} concentration in normal rat pituitary cells *in vitro*. TRH stimulates PRL release via elevated intracellular free Ca^{2+} (Gershengorn 1986). Chelation of extracellular Ca^{2+} has been shown to reduce PRL secretion and synthesis in the GH3 pituitary cells. TRH-stimulated PRL secretion is also suppressed by reduction of Ca^{2+} and verapamil (Ca^{2+} channel blocker) (Gershengorn & Thaw 1985).

Transcription of PRL likely involves activation of protein kinases, which leads to phosphorylation of specific regulators such as PIF and PRF. Activation of cAMP-dependent protein kinase (PKA), protein kinase C (PKC), Ca^{2+} /calmodulin-dependent protein kinase type II, or the mitogen-activated protein kinase (MAPK) cascade has been shown to be sufficient to stimulate transcription of the PRL gene. The PRL promoter contains multiple binding sites for tissue-specific transcription factor including pituitary-specific positive transcription factor 1 (PIT-1), and PIT-1 binding sites may contribute to hormonally regulation and basal transcription of PRL. The finding that cAMP or phorbol ester treatment of

GH3 cells stimulates phosphorylation of PIT-1 is consistent with a role of PIT-1 in regulation of PRL transcription (Kapiloff *et al.* 1991).

Pregnenolone sulfate (PS) is an endogenous neuroactive steroid metabolized from pregnenolone (P5), which is a precursor of endogenous steroid hormones such as progesterone (P4) and estrogen (E_2). Neuroactive steroids synthesized directly in the brain are independent from peripheral sources and rapidly alter neuronal excitability by binding to their receptors (Baulieu 1998). PS is endogenously present in the brain and synthesized by glial cells, which has been shown to modulate neurotransmission in a variety of systems via both presynaptic and postsynaptic mechanisms (Bowlby 1993). PS is also known to have cognitive and memory-enhancing, antidepressant, anxiogenic and proconvulsant effects (Reddy 2010).

Although PS is defined as a neurosteroid hormone, its mechanisms and functions have not been well established. Neuroactive steroids including PS could affect the pituitary cells (Le Foll 1997). Therefore, this study examined the effects of PS on synthesis and secretion of PRL and also the PRL target genes in the rat pituitary.

Materials and methods

Cell culture and treatments

GH3 rat pituitary epithelial-like tumor cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Welgene, Seoul, Korea), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Welgene) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were seeded in DMEM culture medium. After 24 h the medium was replaced by phenol red-free DMEM (Sigma-Aldrich) supplemented with 10% charcoal/dextran-treated FBS (Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin for 24 h before treatment. All the chemicals for treatment were dissolved in ethanol, diluted with experimental medium, and added to the cell culture medium. The tumor cells were treated with E_2 (100 nM), P4 (10 μM), P5 (10 μM), PS (100 μM) or ethanol (EtOH) to obtain the vehicle control. The cells were also pretreated with muscimol (Mus; 10 μM), 3-[(R)-2-carboxypiperazin-4-yl]-prop-2-enyl-1-phosphonic acid (CPP; 10 μM), H89 (10 μM), staurosporine (Sta; 1 μM) or PD98059 (PD; 10 μM) 1 h before PS treatment to block PRL or PS signaling.

Experimental animals and treatments

Immature female Sprague–Dawley ($n=30$) rats were acquired from Samtako (Osan, Republic of Korea). The rats were housed at the Pusan National University Laboratory Animal Resources Center, which is accredited by the Korea FDA, according to the National Institutes of Health guidelines. The rats were housed in cages under a 12h light:12h darkness cycle and a constant temperature of $23\pm1^\circ\text{C}$. The Ethics Committee of Pusan National University (Busan, Republic of Korea) approved all experimental animal procedures (approval number; PNU-2014-0665). Rats were treated daily with PS (10mg/kg/day), E_2 (40 μg /kg/day), P4 (1mg/kg/day), P5 (10mg/kg/day) or corn oil (vehicle control) via subcutaneous injection (SC) from postnatal days (PNDs) 17 to 19. Dosage was adjusted according to changes in body weight (BW); BW, clinical signs and abnormal behaviors were recorded daily throughout the experimental period. On PND 20, all animals were killed using CO_2 gas, after which tissue and serum samples were collected.

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA was measured using a spectrophotometer. First-strand complementary DNA (cDNA) was prepared from total RNA (3 μg) by reverse transcription (RT) using M-MLV reverse transcriptase (Invitrogen) and random primers (9-mers; Takara Bio). Quantitative real-time PCR (Q-PCR) was performed using cDNA template (2 μL) and 2 $\times$ Power SYBR Green (6 μL ; TOYOBO Co Ltd, Katata, Ohtsu, Japan) containing

specific primers. Primer sequences for PRL, D2R, PIT-1, V-Ets Avian Erythroblastosis Virus E_2 6 Oncogene Homolog 1 (ETS-1), gonadotropin-releasing hormone (GNRH1), gonadotropin- α , follicle-stimulating hormone beta (FSHB), and luteinizing hormone beta (LHB) are shown in Table 1. Real-time PCR was conducted by subjecting the samples to 40 cycles of denaturation at 95°C for 15 s, followed by annealing and extension at 70°C for 60 s. Fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for fluorescence intensity of all samples was set manually. The reaction cycle at which the PCR products exceeded this fluorescence intensity threshold during the exponential phase of PCR amplification was considered to be the threshold cycle (CT). Expression of the target gene was quantified relative to that of β -actin, a ubiquitous housekeeping gene, based on the comparison of CTs at constant fluorescence.

Western blotting analysis

Protein samples of GH3 cells were extracted with cell lysis buffer (20mM Tris, 100mM NaCl, 0.5% NP-40, 0.5mM EDTA, 0.5% protease inhibitor cocktail). A total 30 μg of protein (15 μL of protein in serum, $n=3$) were separated by 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Daeillab Service Co, Ltd, Seoul, Korea). Membranes were subsequently blocked for 1 h with 5% skim milk (Difco, Spark, MD, USA) in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBS-T). The blocked membranes were incubated with antibodies specific for PRL, extracellular signal-regulated kinases

Table 1 Primer sequences for Q-PCR analyses.

Gene name	Primer	Sequence (5'-3')	Fragment (bp)
PRL	Forward	AGTCTGTTCTGGTGGCGACT	171
	Reverse	GAAGTGGGGCAGTCATTGAT	
D2R	Forward	CATTGTCTGGGTCTCTGCTCT	154
	Reverse	GACCAGCAGAGTGACGATGA	
PIT-1	Forward	TCAGTATCGCCGCTAAGGAT	151
	Reverse	CGTTTTCTCTCTGCCTTCG	
ETS-1	Forward	TTGCCATCAAGCAAGAAGTG	205
	Reverse	TTCCTCTTCCCCATCTCTCT	
GNRH1	Forward	AGCACTGGTCCTATGGGTTG	248
	Reverse	GTCACACTCGGATGTTGTGG	
Gonadotropin- α	Forward	CAGAAGATATGCGGCTGTCA	184
	Reverse	GTATGCCCTGGAGAAGCAAC	
FSHB	Forward	AAGTCGATCCAGCTTTGCAT	248
	Reverse	CAGCCAGGCAATCTTATGGT	
LHB	Forward	ATCACCTTCAACCACGAGCAT	229
	Reverse	GACCCACAGTCAGAGCTA	

1/2 (ERK1/2), phosphorylated ERK1/2 (p-ERK1/2), p38-MAPK (p38), and phosphorylated p38 (p-p38) overnight at 4°C as well as horse radish peroxidase (HRP)-conjugated anti-rabbit and anti-goat secondary antibodies (diluted 1:2000) in 5% skim milk with PBS-T for 1 h. Luminol reagent (Bio-Rad) was used to visualize antibody binding. Each blot was scanned using Gel Doc 1000, version 1.5 (Bio-Rad), and band intensities were normalized to β -actin levels or total protein by from Coomassie Brilliant Blue (Biosesang, Seongnam, Korea) staining.

MTT assay

GH3 cells (3×10^4 cells/well) were seeded on 96-well plates in 200 μ L DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cells were then allowed to attach to the bottom during 24 h of incubation, after which the seeding medium was removed and replaced with experimental medium (phenol red-free DMEM supplemented with 10% charcoal/dextran-treated FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin) for 24 h before treatment. Cells were treated with E_2 (100 nM), P4 (10 μ M), PS (100 μ M) or EtOH (vehicle control) for 24 h, after which 50 μ L 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) solution (2 mg/mL) was added to 200 μ L medium without phenol red, and the plates were incubated for 4 h at 37°C. DMSO was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the absorbance in the wells was measured at 570 nm with a reference wavelength of 650 nm.

Cell proliferation assay

5-bromo-2'-deoxyuridine (BrdU) assays were conducted using Cell Proliferation ELISA, BrdU kit (Roche) according to the manufacturer's recommendations. GH3 cells were cultured in the same condition with MTT assay. Cells were treated with E_2 (100 nM), PS (100 μ M) or EtOH (vehicle control) for 24 h, after which 10 μ L BrdU solution (100 μ M) were added to each well in 100 μ L phenol red-free medium, and the plates were incubated for 8 h at 37°C. Then the cells were fixed using 200 μ L FixDenat (Roche) for each well for 30 min, and incubated with BrdU antibody for 90 min at room temperature. After washing, the cells were incubated with 100 μ L substrate for 30 min and then 25 μ L 1 M H_2SO_4 were added. Absorbance was measured at 450 nm.

Statistical analyses

The results are presented as mean $\pm$ standard deviation (s.d.). Data were analyzed using one-way analysis of variance (ANOVA) (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL, USA). P values <0.05 were considered statistically significant.

Results

Regulation of PRL expression by PS in GH3 cells

To investigate the effects of PS on pituitary PRL regulation, GH3 cells were treated with PS or its metabolites, including E_2 , P4 and P5 for 24 h. The mRNA and protein levels of PRL were upregulated in response to PS by approximately two- to three-folds, whereas P4 and P5 reduced the PRL mRNA and protein

Figure 1

Regulation of PRL expression by PS in GH3 cells. Transcriptional (A) and translational (B) levels of PRL were evaluated after treatment of GH3 cells with E_2 , P4, P5 and PS. The mRNA expression of PRL after PS treatment was tested in a dose-dependent (C) and time-dependent (D) manner. (E) Protein production of PRL in growth medium secreted from GH3 cells was analyzed. The mRNA and protein levels were analyzed by Q-PCR and Western blotting assay. Data are expressed as the mean $\pm$ s.d. of triplicate experiments. The expression level of PRL was normalized to that of an internal control, β -actin. Protein in growth medium was normalized to total protein stained with Coomassie Brilliant Blue. * $P < 0.05$ relative to the control group. A full colour version of this figure is available at <http://dx.doi.org/JOE-16-0008>.

levels (Fig. 1A and B). These results suggest that PS upregulates PRL synthesis, which is different from its precursor (P5) and metabolites (E_2 and P4). Treatment with increasing concentrations (10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} and 2×10^{-4} M) of PS for 24 h resulted in significantly elevated PRL mRNA levels at concentrations of 10^{-4} and 2×10^{-4} M in a dose-dependent manner (Fig. 1C). In Fig. 1D, the basal expression of PRL mRNA was gradually decreased according to the duration of culture, which may be due to autocrine regulation of PRL itself. The mRNA expression was upregulated from 8 to 48 h when the cells were treated with PS, with the maximum effect occurring at 24 h compared with control (Fig. 1D). To confirm the regulation of PRL by PS, we further examined the amount of PRL in a medium of GH3 cells after PS treatment (Fig. 1E). The amount of PRL in the cell culture media was enhanced as the cell culture time goes as we expected. Similar to mRNA levels, the amount of PRL in the media was increased by PS compared with control.

Figure 2

Effects of signaling inhibitors on mRNA levels of PRL upregulated by PS. GH3 cells were treated with PS alone or with Mus (A), CPP (B), H89 (C), Sta (D) and PD (E). After treatment of GH3 cells with PS alone or with signaling inhibitors, mRNA levels of PRL were examined by Q-PCR. Data represent the mean $\pm$ s.d. of triplicate experiments. The mRNA expression level was normalized to that of an internal control gene, β -actin. * $P < 0.05$ compared with the control group; # $P < 0.05$ based on the comparison of combined treatment and treatment with PS alone.

Mechanisms of PRL regulation by PS in GH3 cells

GH3 cells were pretreated with the type-A GABA receptor ($GABA_A$ R) activator, Mus, or the *N*-methyl-D-aspartate receptor (NMDAR) inhibitor, CPP, for 1 h before PS administration to examine how PS regulates PRL levels. Neither Mus nor CPP significantly altered PRL mRNA levels (Fig. 2A and B). These results suggest that PRL transcription stimulated by PS in GH3 cells is not mediated by $GABA_A$ R- or NMDAR-related signaling pathways. To further explore PS signaling, we pretreated GH3 cells with PKA inhibitor (H89), PKC inhibitor (Sta) or MAPK inhibitor (PD) for 1 h before PS. Enhanced transcription of PRL in response to PS was significantly inhibited by H89, Sta and PD compared with PS alone (Fig. 2C, D and E). Total ERK1/2 or phosphorylated forms of ERK1/2 and p38 were examined by Western blotting. The total ERK1/2 level was elevated in a time-dependent manner, peaking at 10–30 min after PS treatment (Fig. 3A). However, no significant change in p-ERK1/2, p38 and p-p38 levels were observed. Evaluation of other

Figure 3

PS regulates PRL gene expression and cell proliferation by MAPK. (A) After treatment of GH3 cells with PS, protein levels of p-ERK1/2, ERK1/2, p-p38 and p38 were analyzed by Western blotting assay. The mRNA levels of PIT-1 (B), ETS-1 (C) and D2R (D) were analyzed by Q-PCR. Cell proliferation was analyzed by MTT (E) and BrdU (F) assays after treatment of GH3 cells with E_2 and PS. Data are expressed as mean $\pm$ s.d. The mRNA and protein expression levels were normalized to that of an internal control, β -actin. * $P < 0.05$ compared with the control group.

Figure 4
Regulation of PRL expression and secretion by PS in immature rats. Immature female rats were administrated E₂, P4 and PS for 3 days, after which pituitary mRNA (A) and serum protein (n=3) (B) levels of PRL were analyzed by Q-PCR and Western blotting assay. Data are expressed as mean $\pm$ s.d. The mRNA and protein expression levels were normalized to that of total protein. * $P < 0.05$ compared with the control group. A full colour version of this figure is available at <http://dx.doi.org/JOE-16-0008>.

well-known transcriptional regulators of PRL, including PIT-1, ETS-1 and D2R, was tested, which revealed that their expression levels were not significantly altered by PS (Fig. 3B, C and D).

Effect of PS on GH3 cell proliferation

To determine if PS modulates the proliferation of GH3 cells, we conducted MTT and BrdU assays. GH3 cells

were treated with PS for 24h, after which the rate of proliferation was measured. Cell proliferation was elevated by approximately 10% in response to PS relative to the control in both MTT and BrdU results (Fig. 3E and F), suggesting that not only PRL production but also proliferation of PRL-secreting cells are augmented by PS.

Regulation of PRL expression and secretion by PS in immature rats

Following the *in vitro* results showing the elevation of PRL by PS in GH3 cells, we next examined the effects of PS *in vivo* using immature female rats. As it is known that PRL is regulated by sex steroid hormones such as E₂ and P4, we performed the experiment in sexually immature rats. The rats were treated with PS or corn oil as a vehicle control from PNDs 17 to 19, and then killed on PND 20. For comparison, P4, an endocrine metabolite of P5, was also administered. Expression of PRL in the pituitary of immature female rats was tested first because the pituitary is the main organ that produces PRL. Consistent with the results of GH3 cells, transcription of PRL increased by approximately two-fold in response to PS (Fig. 4A). To measure the amount of PRL secreted from the pituitary, PRL levels in serum were analyzed by Western blotting. PRL protein levels were upregulated by PS in serum, similar to that in the pituitary (Fig. 4B). However, E₂ and P4 showed

Figure 5
Regulation of PRL target genes in the pituitary of immature rats. After treatment of immature rats with PS, transcription levels of GNRH1 (A), gonadotropin-α (B), FSHB (C) and LHB (D) in the pituitary were analyzed by Q-PCR. Data are expressed as mean $\pm$ s.d. The mRNA expression level was normalized to that of an internal control gene, β-actin. * $P < 0.05$ compared with the control group.

no significant change in serum. Then, the expression of PRL target genes such as GNRH1, gonadotropin- α , FSHB and LHB in the pituitary was then evaluated. Among the target genes, mRNA expression of GNRH1, FSHB and LHB was reduced significantly, whereas gonadotropin- α expression was unchanged (Fig. 5).

Discussion

Secretion of pituitary PRL is regulated by DA, and gonadal and pituitary hormones (Fitzgerald & Dinan 2008). E_2 regulates PRL gene expression within the anterior pituitary gland by binding to its nuclear receptor and then conferring DNA binding and transcriptional activation of the gene. The ability of physiological E_2 to elicit cellular Ca^{2+} influx via a membrane version of ER α was demonstrated previously (Wozniak *et al.* 2005). P4 is a neurosteroid that decreases PRL synthesis and secretion, leading to inhibition of E_2 -induced PRL gene expression. P5, a precursor of steroid hormones, downregulates PRL via conversion to P4 by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (Assairi *et al.* 1974).

In this study, we examined the effects of PS on pituitary PRL regulation *in vivo* and *in vitro*. GH3 rat pituitary-derived cells that synthesize and secrete PRL were treated with E_2 , P4, P5 and PS. PS significantly increased PRL mRNA and protein levels, whereas P4 and P5 reduced PRL levels. PRL levels were elevated in growth medium by PS in a time-dependent manner compared with the negative control. These results indicate that PS upregulates both PRL synthesis and secretion. Although it has been known that E_2 upregulates PRL production in GH3 cells, it did not significantly change in our experimental condition.

Because PRL secretion and synthesis increased in response to PS, we examined the mechanism of action of PS. To accomplish this, GH3 cells were pretreated with signaling inhibitors of PS action in the brain. PS is an excitatory neurosteroid in the brain that acts as a potent negative allosteric modulator of GABA $_A$ R and a weak positive allosteric modulator of NMDAR (Majewska *et al.* 1988, Wu *et al.* 1991). GABA $_A$ R is an ionotropic receptor and a ligand-gated ion channel that increases the membrane conductance for Cl $^-$ ions upon activation, leading to hyperpolarization and reduced excitability of neurons (Majewska *et al.* 1988). NMDAR is an ion channel protein that enables the flow of positively charged ions through the cell membrane when activated (Irwin *et al.* 1992). Transcription of PRL induced by PS was unchanged following pretreatment with GABA $_A$ R and

NMDAR inhibitors, indicating that PS may have different signaling pathways for the PRL regulation than its known signaling pathways. Activation of protein kinases, including PKA, PKC or MAPK, leads to phosphorylation of specific PRL regulating factors such as PIT-1 and ETS-1. Therefore, we tested PRL expression in the absence and presence of PKA, PKC and MAPK inhibitors. The results showed that PS regulated PRL gene expression via the activation of protein kinases. In addition, total ERK1/2 levels were elevated in a time-dependent manner after PS treatment, whereas the levels of phosphorylated ERK1/2 did not change. Although ERK1/2 proteins are mainly regulated by phosphorylation, evidence shows that posttranslational modifications of ERK1/2 other than phosphorylation could be involved in cellular response (Luanpitpong *et al.* 2012). Taken together, these findings indicate that activities of these factors may be linked via PKA, PKC and MAPK.

Although E_2 and P4 regulated the mRNA expression of PRL in the pituitary, they did not affect PRL levels in the serum. The distinct results between mRNA and serum PRL levels may be due to the presence of other regulatory systems rather than the pituitary. Regarding the effects of PS, the results of *in vivo* study were similar to those of *in vitro* study. Transcription levels of PRL in the pituitary were elevated by approximately two-fold in response to PS. The amount of PRL was also analyzed by Western blotting assay in rat serum, which showed that PRL was upregulated by PS. However, these results demonstrate that both secretion and synthesis of PRL were increased in response to PS in immature rats. Elevated PRL may affect reproduction through its action on the GnRH neurons of the hypothalamus and/or on the pituitary gland, and secretion of the gonadotropins, LH and FSH (Grattan *et al.* 2007). Increased PRL expression tends to suppress expression and secretion of GnRH through PRL receptors from the hypothalamus, which reduces FSH and LH secretion from the anterior pituitary. Therefore, abnormal PRL concentration such as hyperprolactinemia and hypoprolactinemia can cause infertility in woman through interference of ovarian function (Kauppila *et al.* 1988). LH and FSH are heterodimers composed of α - and β -subunits, which are associated noncovalently (Kooy *et al.* 1990). In this study, the elevated PRL after PS treatment reduced the expression of GNRH1, FSHB and LHB. However, gonadotropin- α levels were not significantly altered. Many studies have shown that gonadotropin- α is differentially regulated from β -subunits, and that regulation of β -subunits is sufficient to modulate FSH and LH production (Kaiser *et al.* 1997).

Taken together, these findings indicate that PS triggered activation of PKA and PKC, followed by MAPK signaling, enhanced PRL synthesis and secretion, and enhanced the proliferation of GH3 cells. The effects of PS were further evidenced *in vivo* based on the increased expression of PRL and the downregulation of PRL target genes. These results suggest that PS is a potential steroid hormone that can be applied to patients with PRL-related disorders such as hypoprolactinemia and milk supply.

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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