—Original Article—

GPR30 mediates estrone, estriol, and estradiol to suppress gonadotropin-releasing hormone-induced luteinizing hormone secretion in the anterior pituitary of heifers

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Abstract. Recent studies demonstrated that G-protein-coupled receptor 30 (GPR30) on the plasma membrane of gonadotroph cells mediates picomolar, but not nanomolar, levels of estradiol (E2) to rapidly suppress gonadotropin-releasing hormone (GnRH)-induced luteinizing hormone (LH) secretion in the anterior pituitary (AP). While estrone (E1) and estriol (E3) are considered "weak" estrogens that exert suppressive effects through estrogen receptors α and β, it is conceivable that they also strongly suppress GnRH-induced LH secretion via GPR30. Both E1 and E3 are likely present within the blood at picomolar or nanomolar concentrations, indicating that such concentrations are sufficient to suppress GnRH-induced LH secretion. To evaluate this possibility, bovine AP cells were cultured under steroid-free conditions and then incubated with various concentrations (0.01 pM to 10 nM) of E2, E1, or E3, prior to stimulation with GnRH. Notably, GnRH-induced LH secretion from AP cells was inhibited by 1–100 pM E2, 1–10 pM E1, and 1–100 pM E3. GnRH-induced LH secretion from AP cells was not inhibited by lower (0.01–0.1 pM) or higher (1–10 nM) concentrations of E2, E1, and E3. These suppressive effects were inhibited by pre-treatment of AP cells with the GPR30 antagonist G36, but not with the estrogen receptor alpha antagonist. Treatment with E1 or E3 also yielded decreased cytoplasmic cAMP levels in cultured AP cells pre-treated with dopamine and phosphodiesterase inhibitors. Therefore, these results suggest that GPR30 mediates the suppressive effects of E1, E3, and E2 on GnRH-induced LH secretion from bovine AP.
Key words: Anterior pituitary, Gonadotrope, G protein-coupled estrogen receptor-1, GPR30, Ruminant

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Estradiol (E2) modulates the transcriptional expression of the genes that produce gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) within the hypothalamus and the anterior pituitary (AP), respectively, via binding to nuclear-localized estrogen receptors α or β (ERα or ERβ). Moreover, E2 was found to suppress LH, but not follicle stimulating hormone (FSH), production in a rapid manner within the AP via interaction with an as yet uncharacterized membrane receptor [1, 2]. Candidate membrane receptors for this rapid regulatory pathway include membrane ERα, membrane ERβ, STX receptor, and G protein-coupled receptor 30 (GPR30; or G protein-coupled estrogen receptor 1); however, multiple studies indicate that GPR30 is the primary receptor governing this process within the AP [1, 3–5].

We previously reported that the nonsteroidal mycoestrogen zearalenone (ZEN), as well as the five known ZEN metabolites, rapidly suppressed GnRH-induced LH secretion [5, 6]. Moreover, some of these compounds, even those that exhibit weak suppression via ERα and ERβ signaling, can yield rapid and strong suppression of GnRH-induced LH secretion via GPR30.

The endogenous estrogens, estrone (E1) and estriol (E3), are weaker agonists for ERα and ERβ than E2, and have therefore been designated "weak" estrogens [7]. However, Watson *et al.* [7] reported that both E1 and E3 are strong, rapid regulators in rat lactotroph-like tumor cells. Moreover, Selles *et al.* [8] reported that physiological concentrations of E1 (0.1–10 nM) non-genomically increase aortic nitric oxide synthase and cyclooxygenase activities in female rats. As such, it is possible that E1 and E3 also modulate GnRH-induced LH secretion from the bovine AP via GPR30. Indeed, intramuscular injection of E3 was found to induce an LH surge earlier than an E2 injection in cows and ewes [9, 10].

Notably, previous studies demonstrated that GPR30 mediates rapidly (within a few minutes) picomolar, but not nanomolar, levels of E2, as detected in the blood of luteal phase cows [11, 12], to suppress GnRH-induced LH secretion from the bovine AP [3–5]. During pregnancy, cows were found to exhibit increases in the blood concentration of E1, from picomolar levels during the first trimester to a maximum of 7.4 nM [13]. To the best of our knowledge, however, the levels of E3 in the blood of cows are yet to be evaluated. In animals, E3 is synthesized from E1 and E2 [14], and the blood concentration of E3 in non-pregnant women is always significantly higher (3–19-fold) than that of E1 and E2 combined [15]. Thus, the physiological levels of E3 within the blood of cows are also likely within the picomolar or nanomolar range.

Cyclic AMP (cAMP) plays a key role in the cytoplasmic pathway that increases LH secretion by ovine gonadotrophs by modulating Ca^{2+}-activated K^+ channels [16, 17]. In the previous study, we de-

scribed the suppressive effects of E2 pre-treatment on cAMP levels in cultured AP cells; the pre-treatment caused rapid suppression of LH secretion [5].

In this study, we evaluated whether GPR30 mediates picomolar and nanomolar levels of E1 and E3 to suppress GnRH-induced LH secretion from bovine AP cells. We also evaluated the levels of cAMP in cultured AP cells in the presence and absence of pre-treatment with E2, E1, and E3.

Materials and Methods

All experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and approved by the Committee on Animal Experiments of the School of Veterinary Medicine, Yamaguchi University.

Effects of E2, E1, and E3 on GnRH-induced LH secretion from bovine AP cells

G*Power 3 for windows [18] was used to estimate the required number of APs with an α-error probability of 0.05 and a statistical power of 0.95. APs were obtained from post-pubertal Japanese Black heifers (n = 11, 26 months of age). The experiment was repeated eleven times with each of the eleven different pituitary glands, using four wells per treatment. Each experiment began with enzymatic dispersal of AP cells via a previously described method [19] and confirmation of cell viability of greater than 90% by Trypan blue exclusion. Total cell yield was $20.1 \times 10^6 \pm 0.9 \times 10^6$ cells per pituitary gland. The dispersed cells were then suspended in phenol red-free Dulbecco's modified Eagle's medium (DMEM; 21063-029, Gibco, Grand Island, NY, USA) containing 1% nonessential amino acids (100 ×; Gibco), 100 IU/ml penicillin, 50 µg/ml streptomycin, 10% horse serum (Gibco), and 2.5% fetal bovine serum (FBS; Gibco). The horse serum and the FBS had previously been treated with dextran-coated charcoal for removal of steroid hormones. After the cells (2.5×10^5 cells/ml, total 0.5 ml) had been plated in 24-well culture plates (MS-80240, Sumitomo Bakelite, Tokyo, Japan), the cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 82 h. The wells were washed twice with PBS and then incubated with 490 µl of DMEM containing 0.1% BSA for 2 h. Cells were pre-treated with 5 µl of DMEM alone or with 5 µl of DMEM containing either 0.001 nM to 1,000 nM E2, E1, or E3 (all were from Wako Pure Chemicals, Osaka, Japan). The cells were incubated while gently shaking for 5 min. Cells were then treated for 2 h with 5 µl of 100 nM GnRH (Peptide Institute, Osaka, Japan) dissolved in DMEM to stimulate LH secretion. Pre-treatment with 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, or 1,000 nM E2, E1, or E3 resulted in final concentrations of 0.00001 nM, 0.0001 nM, 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, or 10 nM after GnRH treatment, respectively. The final concentration of GnRH was 1 nM in all treatments [20], except the "control". Control wells were treated with 5 µl of DMEM, but were not incubated with GnRH. "GnRH" wells were pre-treated with 5 µl of DMEM for 5 min and were then incubated with GnRH for 2 h. After incubation for 2 h, the medium from each well was collected for radioimmunoassay (RIA) analysis of LH levels.

Analysis of the effects of the GPR30 antagonist on E2-, E1-, or E3-induced suppression of GnRH-induced LH secretion from bovine AP cells

APs were obtained from post-pubertal Japanese Black heifers (n = 11, 26 months of age). The experiment was repeated 11 times with each of the 11 different pituitary glands, using four wells per treatment. After the enzymatic dispersal of AP cells, the cells were cultured as described previously at 37°C in 5% CO_2 for 82 h. After washing with PBS, the cells were incubated with 485 µl of DMEM containing 0.1% BSA for 2 h. Cells were pre-treated with 5 µL of DMEM alone or with 5 µl of DMEM containing 1 nM of the GPR30 antagonist G36 (Azano Biotech, Albuquerque, NM, USA). After 5 min of gentle shaking, either 5 µl of DMEM alone or 5 µl of DMEM containing 1 nM E2, E1, or E3 was added to each well (final concentration, 0.01 nM). The cells were then incubated with gentle shaking for 5 min, after which they were incubated for 2 h with 5 µl of 100 nM GnRH dissolved in DMEM to stimulate LH secretion. After 2 h of incubation, the medium was collected from each well for RIA analysis of LH levels.

Analysis of the effects of ERα antagonist on E2-, E1-, or E3-induced suppression of GnRH-induced LH secretion from bovine AP cells

We repeated the same design described in the previous section to evaluate the effect of ERα-specific antagonist, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole (MPP) (Sigma-Aldrich, Saint Louis, MI, USA), on E2-, E1-, and E3-induced suppression of GnRH-induced LH secretion from cultured AP cells obtained from post-pubertal Japanese Black heifers (n = 11; 26 months of age). The final concentration of MPP, E2, E1, and E3 were 0.01 nM.

RIA to measure LH concentrations in culture media

LH concentrations in culture media were assayed in duplicate using double antibody RIA with bovine LH and anti-oLH-antiserum (AFP11743B and AFP192279, National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, CA, USA). This assay has been previously described in detail [5] and has been used to measure changes in blood LH concentrations in Holstein heifers before and after GnRH treatment [21]. The limit of detection was 0.40 ng/ml. At 2.04 ng/ml, the intra- and inter-assay coefficients of variation (CV) were 3.5% and 6.1%, respectively.

Effects of E2, E1, and E3 on the levels of cAMP in cultured AP cells

APs were obtained from post-pubertal Japanese Black heifers (n = 6, 26 months of age). The experiment was repeated six times with each of the six different pituitary glands, using two wells per treatment. After enzymatic dispersal, AP cells were cultured as described previously at 37°C in 5% CO_2 for 82 h. After washing with PBS, the cells were incubated with 490 µl of DMEM containing 0.1% BSA for 2 h. Cells were pre-treated with 5 µl of DMEM alone or with 5 µl of DMEM containing 1 nM of E2, E1, or E3 (final concentration, 0.01 nM). After gently shaking for 5 min, cells were treated for 2 h with 5 µl of 100 nM GnRH (final concentration, 1 nM, except

the "control") dissolved in DMEM containing dopamine (0.5 μM, Nacalai Tesque, Kyoto, Japan) and phosphodiesterase inhibitor [0.5 mM 3-isobutyl-1-methyl-xanthine (MIX; Sigma-Aldrich)]. Dopamine and MIX are required to measure cAMP in cultured gonadotroph cells from heterogeneous AP cells because the amount of cAMP in lactotrophs fluctuates; moreover, phosphodiesterase decreases the amount of cAMP to below the detection limits [16]. The concentrations of dopamine and MIX used in the present study were identical to those used in a previous investigation of cAMP in cultured ovine AP cells [16]. There were five treatment conditions: (1) Control, cells treated with 5 μl of DMEM, but not GnRH; (2) GnRH, cells treated with 5 μl of DMEM and GnRH; (3) E2, cells pre-treated with E2 and then treated with GnRH; (4) E1, cells pre-treated with E1 and then treated with GnRH; and (5) E3, cells pre-treated with E3 and then treated with GnRH.

After the 2 h of treatment, the wells were washed twice with PBS and were then used for cAMP extraction with a cAMP Select Enzyme Immunoassay (EIA) Kit (Cayman Chemical, Ann Arbor, MI, USA), as described in detail previously [5]. The limit of detection was 0.09 pmol/ml. At 22.2 pmol/ml, the intra- and inter-assay CV was 5.9 and 7.4%, respectively. The specificities of the cAMP EIA kit evaluated by the company were 100% for cAMP and < 0.01% for cGMP, AMP, ATP, adenosine, and dibutyryl cAMP.

Statistical analysis

The concentrations of LH and cAMP within the control samples from each pituitary gland were averaged, and the mean values were set at 100%. Meanwhile, the concentrations of LH and cAMP in the treated samples from each pituitary gland were averaged, and the mean values were expressed as percentages of the control value. Differences in LH or cAMP concentration were analyzed by one-factor analysis of variance (ANOVA), with concentrations of LH or cAMP as the dependent variable and treatment as the independent variable, followed by post-hoc comparisons using Fisher's protected least significant difference test (StatView version 5.0 for Windows, SAS Institute, Cary, NC, USA). The level of significance was set at $P < 0.05$. Data were expressed as mean ± standard error of the mean (SEM).

Results

Effects of E2, E1, or E3 on GnRH-induced LH secretion

Fig. 1 depicts the effects of various concentrations of E2, E1, or E3 on GnRH-induced LH secretion from cultured AP cells. The media from cells treated with 1 nM GnRH exhibited significantly greater concentrations of LH than that of the control cells. Meanwhile, pre-treatment with 0.001–0.1 nM E2, 0.001–0.01 nM E1, or 0.001–0.1 nM E3, but not with 1–10 nM E2, 0.1–10 nM E1, or 1–10 nM E3,-resulted in marked suppression of GnRH-induced LH secretion from AP cells. In contrast, pre-treatment with low (0.00001–0.0001 nM) or high (1–10 nM) concentrations of E1, E2, and E3 did not decrease the LH concentration.

Effects of G36 on E2, E1, and E3-mediated suppression of GnRH-induced LH secretion

As summarized in Fig. 2, treatment with 1 nM GnRH resulted in a significant increase in LH secretion from cultured AP cells, compared to the untreated control cells, and treatment with the GPR30 antagonist G36 alone had no effect on this process. Notably, however, the observed decreases in LH secretion, mediated by pre-treatment with 0.01 nM E2, E1, and E3, were abrogated by pre-treatment of cells with G36.

Effects of MPP on E2-, E1-, and E3-mediated suppression of GnRH-induced LH secretion

Treatment with the ERα antagonist, MPP, alone had no effect on the GnRH-induced LH secretion (Fig. 3). The observed decrease in LH secretion caused by pre-treatment with 0.01 nM E2, E1, and E3 was not abrogated by pre-treatment with MPP.

Effects of E2, E1, or E3 on the levels of cAMP in AP cells

While AP cells treated with 1 nM GnRH exhibited significantly higher levels of cAMP production than the control cells, this effect was abrogated by pre-treatment with 0.01 nM E2, E1, or E3 (Fig. 4).

Discussion

In the present study, we demonstrated that both E1 and E3, in addition to E2, rapidly suppressed the GnRH-induced LH secretion from cultured bovine AP cells, and that this effect was inhibited by pre-treatment of cells with a GPR30 antagonist. The results of the present study suggested that femtomolar levels of E2, E1, and E3 could not suppress the GnRH-induced LH secretion from the cultured bovine AP cells. ERα is another candidate membrane receptor for the rapid regulatory pathway. However, the present study did not show any significant effect of ERα antagonists on the E2-, E1-, and E3-mediated inhibition of GnRH-induced LH secretion. In addition, multiple studies have shown that GPR30 is the primary receptor governing this process within the AP [1, 3–5]. Together, these findings imply that GPR30 mediates the E1-, E2-, and E3-dependent suppression of GnRH-induced LH secretion from AP cells. However, the physiological significance of these results must be carefully considered; in particular, (1) whether the suppressive effects of E1 and E3 are important negative feedback from the ovary or other organs to the AP, and (2) the reason behind the absence of any significant effect of 1–10 nM E1, E2, and E3 on the GnRH-induced LH secretion.

To date, very few reports have been published on the effect of E1 and E3 on LH secretion *in vivo*. Forrest *et al.* [9] had reported that intramuscular injection of E3 or E2 could induce an LH surge; interestingly, E3 exerts this effect earlier than E2 in cows and ewes [9]. In addition, Schoenemann *et al.* [10] reported that an E3 injection could induce an earlier LH surge in ovariectomized cows. Plasma concentration of E1 was found to be positively correlated ($r = 0.71$) with the number of ovulations in superovulated heifers [22]. Therefore, both E1 and E3 could affect the GnRH-induced LH secretion *in vivo*. Unlike E2, ZEN, and environmental estrogens [23], the affinity of E1 and E3 for GPR30 has yet to be clarified. However, previous studies have reported that E1, E2, and E3 could non-genetically activate the extracellular signal-regulated kinase (ERK) pathway in rat pituitary cell lines [7, 24]. The ERK pathway is an important pathway downstream of GPR30 that suppresses GnRH-induced LH
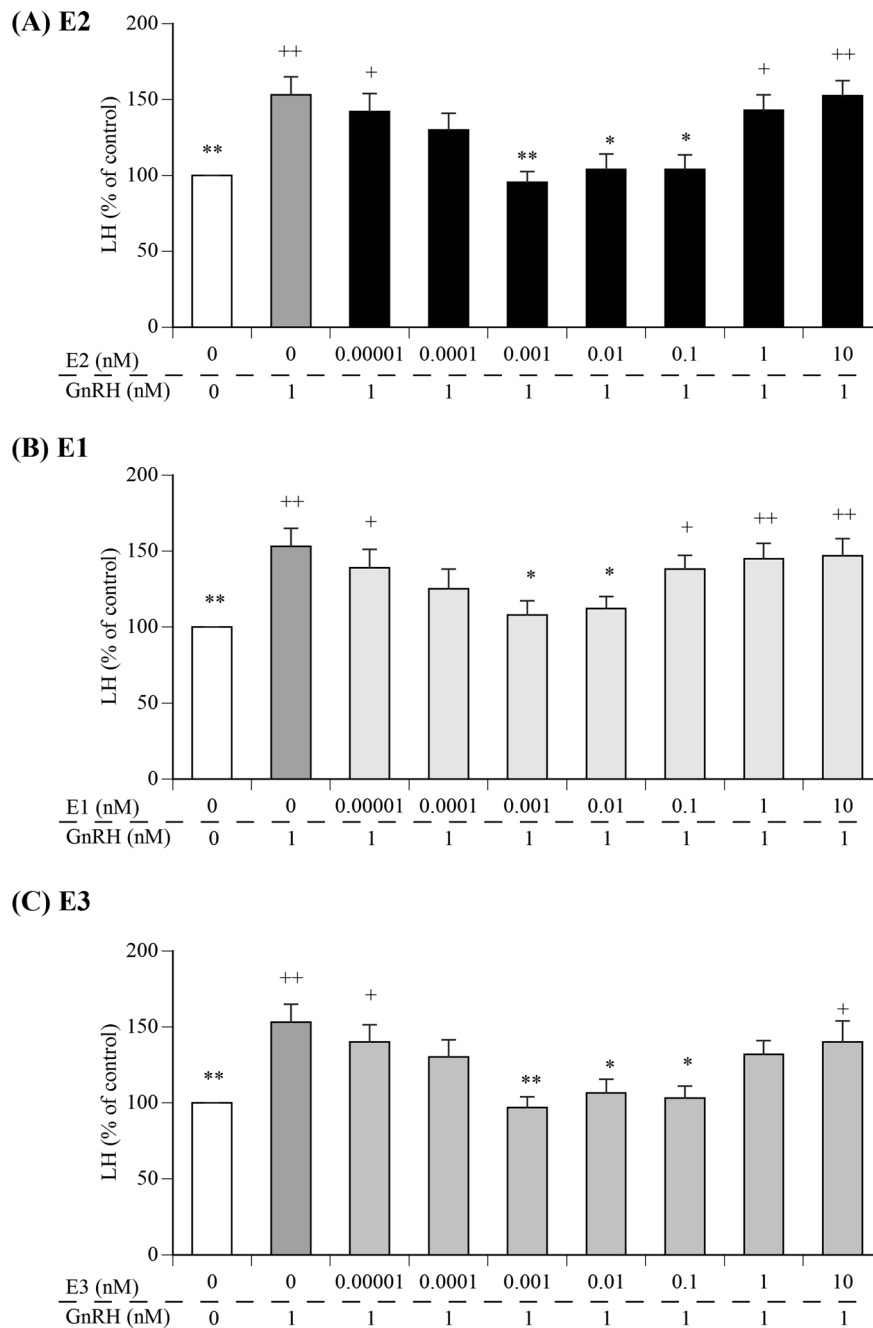
Fig. 1. Comparison of the effects of various concentrations of estradiol (E2), estrone (E1), or estriol (E3) in media containing 1 nM gonadotropin-releasing hormone (GnRH) on luteinizing hormone (LH) secretion from cultured bovine anterior pituitary (AP) cells. The concentrations of LH in the control cells (cultured in medium alone; shown as white bars) were averaged and set at 100%, and the mean LH concentration for each treatment group is expressed as a percentage of the control value. + P < 0.05 and ++ P < 0.01 compared to the control; * P < 0.05 and ** P < 0.01 compared to GnRH alone.

secretion in bovine AP cells [25]. Therefore, GPR30 might bind with E1 and E3 to activate the ERK pathway to suppress GnRH-induced LH secretion in bovine AP cells.

While little is known regarding the physiological blood concentrations of E1 in ruminants, a previous study indicated that blood E1

concentrations in cows ranged from picomolar to nanomolar levels [13]. Meanwhile, a separate study demonstrated that E3 is a much weaker hormone than E2 and E1 in women [15]. Notably, however, the serum levels of E3 in both pregnant and non-pregnant animals, including cows, have yet to be compared. Although the current opinion
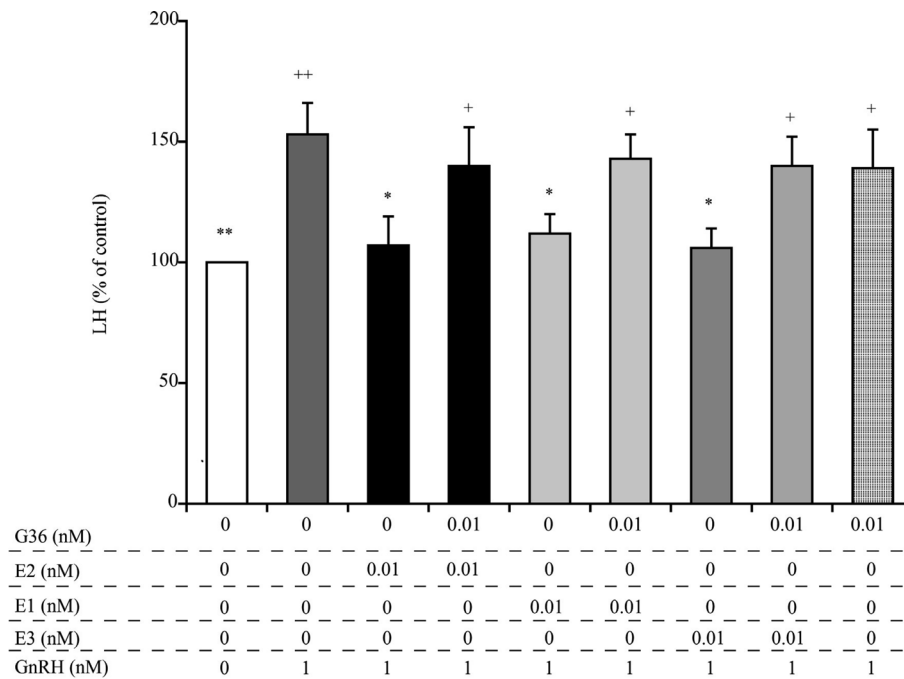
Fig. 2. Comparison of the effects of the GPR30 antagonist G36 on E2, E1, and E3-mediated suppression of GnRH-induced secretion of LH from cultured bovine AP cells. The final concentrations of G36, E2, E1, E3, and GnRH were 0.01 nM, 0.01 nM, 0.01 nM, 0.01 nM, and 1 nM, respectively. The concentrations of LH in the control cells (cultured in medium alone; shown as white bars) were averaged and set at 100%, and the mean LH concentration for each treatment group is expressed as a percentage of the control value. + $P < 0.05$ and ++ $P < 0.01$ compared to the control; * $P < 0.05$ and ** $P < 0.01$ compared to GnRH alone.

held by the medical community is that E3 plays no significant role in non-pregnant animals relative to the other estrogens, Wright *et al.* [15] found that the blood concentration of E3 (0.6–8.4 nM) in non-pregnant premenopausal women was consistently higher (3–19-fold) than the sum of the concentrations of E1 and E2. Moreover, they found that the blood concentration of E3 fluctuated less in this group of women than that of E1 or E2 [15]. Given that pregnant women exhibit blood E3 concentrations within the picomolar or nanomolar range [26], this might also be the physiological range for E3 in cows. Therefore, the suppressive effect of E1 and E3 on GnRH-induced LH secretion may have important roles at different life stages, for e.g., reproductive cycle, pregnancy, and menopause.

The present study showed that 1–10 nM of E1, E2, and E3 could not suppress GnRH-induced LH secretion. Iqbal *et al.* [27] had suggested that E2 might have a biphasic effect on LH secretion by gonadotropes, with a rapid initial suppression of LH release (negative feedback) followed by a positive feedback event many hours later. Iqbal *et al.* [27] had also reported that E2 might activate the MAPK pathway for exerting its time-delayed positive feedback effect. Therefore, the superpharmacological concentration of E1, E2, and E3 may have activated the MAPK pathway earlier. However, the term "positive feedback" must be used with caution, because the GnRH-induced LH secretion from the AP cells pre-treated with 1–10 nM of E1, E2, and E3 was not higher than that from AP cells treated with GnRH only. A more appropriate phrase would be "no suppressing effect," for 1–10 nM E1, E2, and E3.

The weak suppressive effect of 0.01–0.1 nM E2, compared to 0.001 nM E2, is reasonable, as gonadotropes are surrounded by a high concentration of estradiol and must respond to the increased GnRH in the hypophyseal portal blood to release LH in a state of positive estradiol feedback. E2 rapidly stimulates GnRH secretion from cultured GnRH neurons derived from the embryonic olfactory placode, and the rapid estradiol action is mediated by GPR30 [28]. Therefore, further studies are required to understand the importance of gonadotrope GPR30 in controlling sensitivity to GnRH.

cAMP promotes LH secretion from ovine gonadotroph cells by acting as an intracellular second messenger that rapidly modulates Ca^{2+}-activated K^+ channels [16, 17]. Meanwhile, GPR30 forms a plasma membrane complex with membrane-associated guanylate kinase, discs, large homolog 4 (DLG4; or postsynaptic density protein 95), and protein kinase A-anchoring protein 5 (AKAP5), and this complex was shown to inhibit cAMP production in HEK293 cells and CHO cells ectopically expressing GPR30 [29]. In the present study, pre-treatment of AP cells with small amounts of E1, E2, or E3 resulted in decreased cAMP production, suggesting that the GPR30-mediated decreases in cAMP production play an important role in the inhibition of GnRH-induced LH secretion.

However, we must ensure that the cultured bovine AP cells contain not only gonadotrophs, but also lactotrophs. Both E1 and E3 are strong, rapid regulators found in rat lactotroph-like tumor cells [7]. cAMP promotes prolactin synthesis and secretion in lactotrophs [30]. GnRH may indirectly stimulate prolactin secretion in cultured
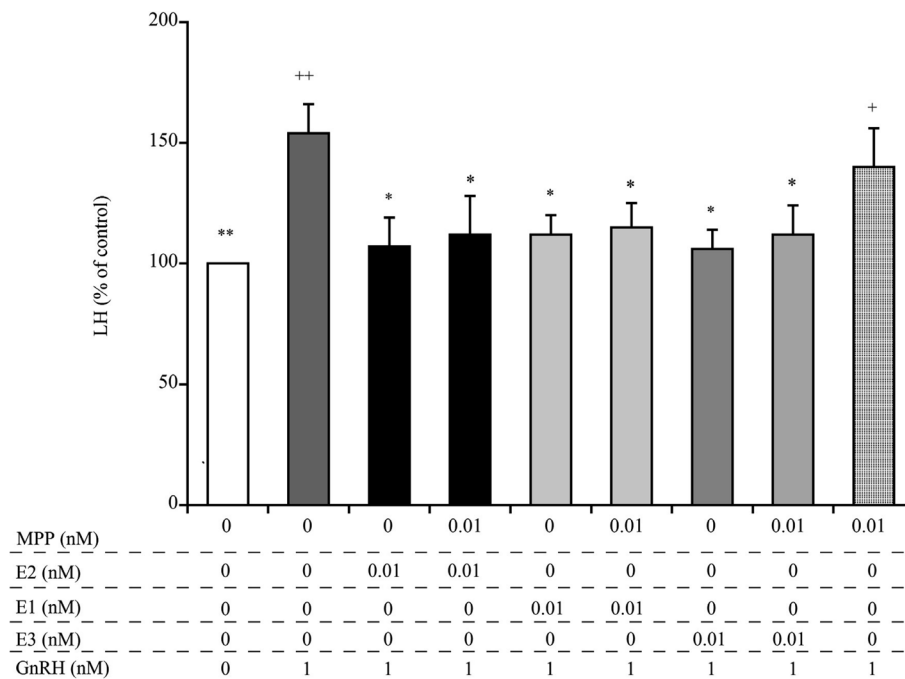
Fig. 3. Comparison of the effects of the estrogen receptor α antagonist, MPP, on E2-, E1-, and E3-mediated suppression of GnRH-induced secretion of LH from cultured bovine AP cells. The final concentrations of MPP, E2, E1, E3, and GnRH were 0.01 nM, 0.01 nM, 0.01 nM, 0.01 nM, and 1 nM, respectively. The concentration of LH in the control cells (cultured in medium alone; shown as white bars) was averaged and set at 100%, and the mean LH concentration for each treatment group was expressed as a percentage of the control value. + $P < 0.05$ and ++ $P < 0.01$ compared to the control; * $P < 0.05$ and ** $P < 0.01$ compared to GnRH alone.
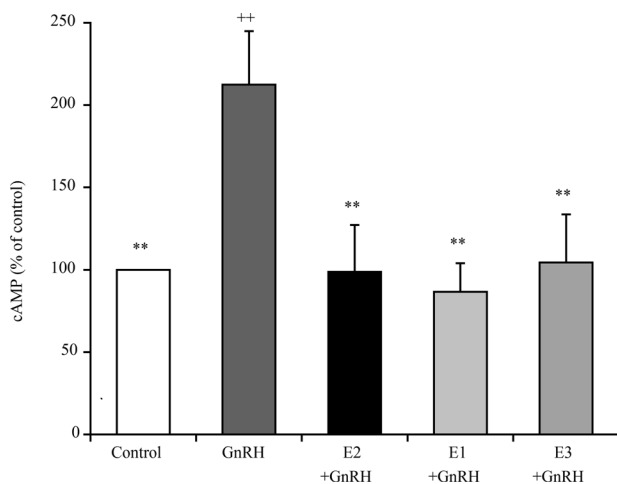

Fig. 4. Comparison of the effects of 0.01 nM of E2, E1, and E3 on the levels of cyclic AMP (cAMP) in cultured bovine AP cells treated with 1 nM GnRH. The concentrations of cAMP in the control cells (cultured in medium alone; shown as white bars) were averaged and set at 100%, and the mean cAMP concentration for each treatment group is expressed as a percentage of the control value. ++ $P < 0.01$ compared to the control; ** $P < 0.01$ compared to GnRH alone.

AP cells of ewes in the breeding season [31]. Therefore, some of the observed cAMP maybe derived from lactotrophs. However, this study used DMEM containing dopamine at the same concentration as used in previous studies [5, 16]. Dopamine inhibits GnRH-induced prolactin secretion from cultured AP cells of ewes in breeding season [31]. Dopamine also decreases intracellular cAMP concentrations and inhibits prolactin synthesis and secretion in lactotrophs [32, 33]. Therefore, changes in measured cAMP likely reflect the intracellular cAMP concentration in cultured bovine gonadotrophs.

In conclusion, the results presented in this study suggest that GPR30 mediates the suppressive effects of E1 and E3 as well as E2 on GnRH-induced LH secretion from bovine AP cells.

Acknowledgements

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