Evidence that estrogen mediates the positive feedback effect on GnRH

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ABSTRACT

Kisspeptin (Metastin), a protein encoding by the KiSS-1 gene, is a ligand for the G protein-coupled receptor GPR54. To investigate the molecular consequences of estrogen secretion on brain, estradiol benzoate was intravenously injected to sheep, and the expression of kisspeptin and Fos-like protein were scanned in the neurons of arcuate nucleus (ARC) by immunohistochemistry. In the case of Fos, the results showed that the number of immunopositive-Fos cells in ARC was significantly greater in the estradiol benzoate-treated OVX ewes 1 h after the injection. Similarly, estrogen injection activated the expression of kisspeptin within 1 h of injection. The results suggest an acute effect of estrogen -through kisspeptin, initiating the events leading to the positive feedback effect on pulsatile secretion of gonadotrophin releasing hormone (GnRH).

Key words: Arcuate nucleus, Estrogen, Immediate early gene, Metastin, Immunohistochemistry

Introduction

The pulsatile secretion of gonadotrophin releasing hormone (GnRH) activates the pituitary gonadotropes and leads to the release of the gonadotrophins [2]. The Gonadotropins act on the gonads to evoke the release of steroids hormones, which then feedback on GnRH neurons [1,17]. Surges in GnRH and LH secretion, are crucial to the process of ovulation are known to involve a switch from negative to positive feedback regulation of GnRH, but the precise molecular mechanism for this change in regulation is not completely known. With respect to the impact of GnRH and estrogen in critical phenomena like fertility and infertility, there is a great attention in understanding and dissecting their molecular actions. Kisspeptin is a newly discovered protein encoded by the KiSS-1 gene and is a ligand for the G protein-coupled receptor GPR54 [13]. Loss of function of GPR54 leads to infertility in human [4] and impairs the reproductive axis in mice [8], suggesting a key role for kisspeptin in reproduction. Furthermore, kisspeptin stimulated the secretion of FSH and LH, but with a GnRH antagonist no effect of kisspeptin was observed [10,12]. It has been suggested that kisspeptin may influence on the reproductive axis through GnRH pathways. Kisspeptin neurons in the periventricular nucleus were activated by estrogens during the proestrus in the rat [15]. This region is known to be a centre for initiating the GnRH/LH surge [14]. In the sheep, the arcuate nucleus (ARC) is the site at which estrogen exerts its positive feedback effects on GnRH/LH secretion [11]. In this species, KiSS-1 mRNA is up-regulated in the ARC just prior to the LH surge [6]. Whether, this increase in KiSS-1 is coupled with increased activation of kisspeptin neurons is unknown. The major goal of this study was to investigate the effect of increased level of estrogen on kisspeptin in ARC. EB causes the production of immediate early gene (Fos protein) in estrogen-responsive cells [3]. Immunohistochemical localization of c-Fos immunoreactivity has been used successfully to determine neural activity of estrogen.

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in the brain [16]. In fact, Fos protein can be assumed as a reliable marker of estrogen increase [16]. Kisspeptin was recently shown to increase co-expression of c-Fos and GnRH in rat [15]. Virtually, all kisspeptin cells in the ARC express estrogen receptor-a and may be involved in the positive feedback effects of estrogen on GnRH secretion [6]. One of the best–known actions of steroids is the rapid activation of the Ras/Raf/Erk pathway. In the nervous system, estradiol evokes Erk1/2 fast, resulting in c-Fos gene expression [5]. 17β-estradiol increased c-Fos immunoreactive cells in the ovine ARC. Our hypothesis was that kisspeptin in the ARC may show co-expression with the Fos protein (indicative of EB) in c-Fos immunoreactivity. This experiment was aimed to test whether kisspeptin-producing cells in the ARC of the EB-treated sheep show c-Fos immunoreactivity, indicative of estrogen effect.

Materials and methods

Animals and treatments

Six ewes (2-3 yr old with weight of 50-60 kg) that had been ovariectomised for at least one month, were used during the time of the year when intact ewes would normally mate. The ewes were randomly allowed into two groups of 3 ewes each. Within each group, three ewes received intravenous (i.v.) injections of estradiol benzoate (50 mg per ewe in 1 ml sunflower oil) and three ewes served as the control, receiving 1 ml of 10% ethanolic saline by i.v. route. Estrasdiol benzoate was purchased from sigma (St Louis, MO, USA). Computed traits were the expression of kisspeptin, and Fos protein (indicative of estrogen), and co-expression of kisspeptin and Fos in a cell. The total numbers of kisspeptin-immunopositive cells, Fos-immunopositive cells, and kisspeptin-and Fos-immunopositive cells were counted in six sections evenly situated throughout the rostral, medial and caudal regions of the ARC. Data were analysed by pairwise sample T test (SPSS software, version 11.5). P<0.05 was considered as a level of significance.

Tissue collection

The ewes were euthanized 1 hour after EB injection with 20 ml of lethobarb (i.v.), decapitated and their brains were perfused through the carotid arteries with 2 l of heparinized saline followed by 2 l Zamboni's fixative and 1 l of the same fixative containing 20% sucrose. The hypothalamus was removed and postfixed in 30% sucrose and fixative for 24 h and then in 30% sucrose in 0.1 M neutral phosphate-buffered saline (PBS) until it sank. The tissue blocks were then rinsed in buffer, frozen on the dry ice, wrapped in parafilm and stored in a container at -20°C until sectioning. Coronal sections (40 mm) were cut on a cryostat and collected into individual tissue culture wells containing cryoprotectant, and stored at -20°C prior to use.

Immunohistochemistry

All incubations were carried out on an orbital mixer at room temperature except for incubations with primary antibody which were performed at 4°C. The sections were washed (three times for 10 min) in PBS between solution changes. Sections were first incubated for 20 min in 0.1% sodium borohydride and then for 30 min in dH₂O, containing 3% H₂O₂ to block endogenous peroxidase activity and were then given a 30-40 min preincubation in 0.1 M phosphate buffer (PB) containing Triton X-100 and 2% normal goat serum. These sections were then incubated for 2-3 days in primary antibody of onconege rabbit c-Fos at the dilution of 1:3000 plus normal goat serum, Triton X-100 and 0.1 M PB. The second antibody was biotinylated goat antirabic serum (Vector, Burlingame, CA, USA) (1:250); visualisation was with nickel-enhanced diaminobenzidine (Ni-DAB) for Fos, and diaminobenzidine (DAB) for double-labelling of kisspeptin. The sections were first incubated with the Fos antibody, and the nickel DAB reaction was performed before incubation with kisspeptin antibody (1:1500). The peroxidase reaction was developed over approximately 3 min using 0.05% diaminobenzidine and 0.01% H₂O₂ in PB with 1% ammonium nickel sulphate and 1% cobalt chloride. The sections were mounted on gelatinised slides, dehydrated in ascending series of ethanol, cleared and cover slipped. All sections that enclosed kisspeptin-immunoreactive neurons were observed under bright-field illumination. All kisspeptin neurons were counted bilaterally at 20X magnification. Single-labelled kisspeptin neurons were counted if brown bipolar neurons were observed with clear nuclei lacking dark staining. Double-labeled neurons (kisspeptin/Fos positive) were counted if brown bipolar neurons were observed with dark nuclei.

Results

Estradiol benzoate greatly increased kisspeptin expression, Fos protein expression, and kisspeptin+Fos protein expression (Fig.1, Table 1). Expression of kisspeptin in ARC increased from a means of 4.67 cells to 42 cells confirming the positive effect of estrogen on kisspeptin expression (Fig.1). Similarly, a significant expression increase for Fos and kisspeptin+Fos, from a mean of 3.33 to 32.67 and 1.33 to 26.67 respectively, was observed (Fig.1). Co-immunooassay image clearly showed the existence of Fos protein in kisspeptin positive cells (Fig.2).

Table 1: Effect of estradiol benzoate injection in the expression of Kp, Fos and Kp + Fos (mean ± SD)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Kp</th>
<th>Fos</th>
<th>Kp + Fos</th>
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<tbody>
<tr>
<td>EB</td>
<td>42 ± 11.79</td>
<td>32.67 ± 6.81</td>
<td>26.67 ± 5.77</td>
</tr>
<tr>
<td>Control</td>
<td>4.67 ± 1.15</td>
<td>3.33 ± 1.15</td>
<td>1.33 ± 1.15</td>
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†: Mean number of cells showing immunoreactivity

Discussion

Successful reproduction is dependent on the highly controlled interactions between the brain, hypophysis and gonads. The release of the gonadotropins from hypophysis is important for ovulation and spermatogenesis, and is regulated, in turn, by the feedback effects of gonadal steroid (including estrogen) on the brain. The final common factor in the control of hypophysis-gonad is the GnRH neurons which are located in POA of the brain. The GnRH neurons do not express estrogen-alfa receptor (ER-α). Instead, they express the receptor beta (ER-β) with a minor role in relaying the feedback effects of estrogen to GnRH neurons. Therefore, other steroid-sensitive neurons must mediate the feedback effects of estrogen (via ER-α) on GnRH secretion. On the basis of our observation and others [9,15], it seems that kisspeptin cells are good candidates for relaying the estrogen information to GnRH neurons.

The present study demonstrated that estrogen has a rapid effect on kisspeptin expression. The question as to whether the estrogen activation of the arcuate nucleus (Fos) is in the kisspeptin cells is an important one, since it has been shown that the positive feedback effect is generated here. If kisspeptin mediates the positive feedback effect, one would expect Fos to be localised to kisspeptin cells within 1 h of estrogen injection. We showed that injection of estrogen causes expression of immunoreactive Fos in the kisspeptin cells of ARC in OVX ewes within 1 h. Our data are in agreement with the results of Smith et al. [15] who reported that in the rat, kisspeptin cell bodies in the anterioventralperiventricular (AVPV) area, which is
a surge site, contain Fos concomitantly with the LH surge. Our data demonstrated that estrogen stimulated a rapid response in kisspeptin cells of the ARC where implantation of estrogen known to cause a positive feedback effect on GnRH secretion. It has been shown that neurokinin B (NKB) neurons are involved in the preovulatory surge of LH and that most of NKB neurons in ARC co-localise with the estrogen receptor. This issue is consistent with our results, since we recently showed that most of NKB neurons of the ARC are co-expressed with kisspeptin cells [9]. We found that over 90% kisspeptin cells co-localised with Fos, a marker of estrogen (Fig.2). This is in agreement with previous results showing that most of kisspeptin neurons contain ER [7]. Clarke et al. [3], examined cellular responses to estrogen in the ARC using Fos immunohistochemistry and determined the cell type that shows an acute response to estrogen. They found that only less than 30% of all the cells were Fos-positive and that majority of cells that produce Fos following estrogen injection are of unknown phenotype. Now, we know that kisspeptin-producing cells are abundant in the ARC of sheep and have ER-α [7]. Number of kisspeptin positive-cells in the ARC were higher in estrogen-treated ewes (Fig.1). The lack of ER-α expression in GnRH neurons indicates that estrogen-receptive interneurons are essential to convey the estrogen signal to GnRH neurons. In summary, we showed that kisspeptin cells in the ARC initiated the events leading to the positive feedback effect of estrogen on GnRH secretion.

References

