Effects of ovarian hormones on GPR120 mRNA expression in mouse pituitary gonadotrophs

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Abstract. GPR120 is a G-protein-coupled receptor that is activated by long-chain fatty acids. In our previous study, GPR120 expression was detected in gonadotrophs of the mouse anterior pituitary gland. It is well known that the function of anterior pituitary cells is largely under the influence of circulating sex steroids. Thus, in the present study, we investigated the modulatory roles of the ovarian hormones, estrogen (E2) and progesterone (P), on the expression levels of GPR120 mRNA in mouse pituitary glands. GPR120 mRNA expression levels in the pituitary gland were increased after ovariectomy or P treatment, and were decreased after the administration of E2. Simultaneous injection of E2 and P interfered with the action of E2 on GPR120 mRNA expression. The GnRH antagonist, Cetrotide, did not inhibit the increase in GPR120 expression in ovariectomized (OVX) animals. In addition, immunohistochemistry revealed that more than 95.4% of GPR120 immunoreactive cells colocalized with the luteinizing hormone β (LHβ) in the anterior pituitary gland of intact, ovariectomized (OVX), estradiol-primed OVX (OVX+E2), or progesterone-primed OVX (OVX+P) animals. Furthermore, GPR120 mRNA expression levels were not significantly different in the pituitary gland of females throughout the ovarian cycle. It is suggested that low levels of P may mask the inhibitory effect of estradiol on the synthesis of GPR120 in the estrous stage in intact animals. These results demonstrate that ovarian hormones may directly regulate GPR120 expression in the reproductive cycle at the pituitary level.

Key words: GPR120, Pituitary, Gonadotroph, Progesterone, Estrogen

FATTY ACIDS are not only nutritional components but also act as signaling molecules. G-protein-coupled receptor 120 (GPR120) is a receptor for long-chain fatty acids. It has been reported that GPR120 mediates adipogenesis and glucagon-like peptide-1 secretion during lipid metabolism [1], and is also involved in the anti-inflammatory effects of macrophages [2]. Furthermore, GPR120 is considered a fatty acid sensor that controls energy balance, since the dysfunction of GPR120 leads to obesity in both humans and rodents [3]. In our previous study, GPR120 expression was detected in gonadotrophs of the mouse anterior pituitary gland [4]. This result suggested that GPR120 could play a role in regulating gonadal function by affecting the synthesis and/or secretion of gonadotropic hormones, such as the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH). However, the long-chain fatty acid receptor agonist GW9508 did not affect LH secretion [5] or the transcription of the gonadotropin subunit genes [6] in an LβT2 gonadotropic cell line. Nevertheless, fasting induced an increase in GPR120 mRNA expression in mouse gonadotrophs [4] and free fatty acids (FFAs) directly suppressed Fshb mRNA expression in the LβT2 gonadotropic cell line [7], suggesting that GPR120 could serve as a sensor for the detection of serum fatty acid levels in pituitary gonadotrophs.

It is well known that the function of anterior pituitary cells is largely under the influence of circulating sex steroids in both males and females [8].
the present study, we hypothesized that the ovarian hormones, estrogen and progesterone (P), regulate GPR120 expression levels in pituitary gonadotrophs. To test this hypothesis, we studied the effect on GPR120 expression levels when ovarian hormones were depleted through ovariectomy and replaced by estradiol-17β (E2) or P in ovariectomized (OVX) mice.

Materials and Methods

Animals

Adult female ICR mice that were obtained from Japan SLC, Inc. (Hamamatsu, Japan) through a local distributor (Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) were individually housed in a controlled environment (12 h light and 12 h dark; lights on at 0700 h; temperature, 24 ± 2 °C). Animals had free access to food (Labo-MR stock, Nihon Nosan Kogyo Co., Yokohama) and water until the day of sampling. Female mice (10-12 weeks of age; n = 6 per group) that had at least two consecutive regular 4-day estrous cycles were used in this study. The estrous cycle stage was monitored by daily vaginal smears that were taken around 1,000 h. Some mice were sacrificed by rapid decapitation at the four different stages of the estrus cycle, and their pituitary glands were collected form 1,000 h to 1,200 h for RNA extraction. The study was approved by the Committee on Animal Experiments of Kindai University for the care and use of experimental animals.

Ovariectomy and hormonal replacement

Six days before sampling, female mice were bilaterally OVX. E2 (250 ng dissolved in 0.1 mL of peanut oil; Sigma-Aldrich Co., St Louis, MO) and/or P (2 mg dissolved in 0.1 mL of peanut oil; Sigma-Aldrich Co.) were administered subcutaneously to some of the OVX mice, at 1,000 h for five consecutive days [9,10]. Three days before sampling, the GnRH antagonist cetrorelix (10 µg in 0.2 mL saline/daily, S.C.; Cetrotide, Merck KGaA, Darmstadt, Germany) was administered subcutaneously to some of the OVX mice, at 1,000 h for three consecutive days [11]. Six days after ovariectomy, the mice were decapitated between 1,000 and 1,200 h, and their pituitary glands and uteri were collected for RNA extraction and wet weight measurements, respectively. In addition, diestrous female mice that underwent a sham ovariectomy operation were used as controls.

RNA extraction and cDNA synthesis

Total RNA was prepared from pituitary glands using TRI Reagent (Sigma-Aldrich Co.), and treated with RNase-free DNase I to remove genomic DNA contamination. cDNA was synthesized using a Superscript II kit with an oligo(dT)12-18 primer (all reagents were purchased from Life Technologies Co., Carlsbad, CA).

Real-time PCR

GPR120 mRNA expression levels were determined by real-time PCR, using the SYBR Premix Ex Taq II master mix (Takara Bio Inc., Tokyo, Japan) containing SYBR Green I, and run on a 7,500 Real-time PCR System (Applied Biosystems, Darmstadt, Germany). The following conditions were used: denaturation at 95 °C for 30 s and amplification by cycling 40 times at 95 °C for 5 s, 60 °C for 34 s. Data were analyzed using the standard curve method [12], and normalized to the L19 ribosomal protein gene, which was used as a reference gene. The forward and reverse primers (Nippon EGT, Toyama, Japan) used for mouse GPR120 (NM_181748) were 5ʹ-TCGCTGTTCAGGAACGAATG-3ʹ and 5ʹ-CACCGAGGCTAGTTAGCTG-3ʹ, respectively, and those used for ribosomal protein L19 were 5ʹ-CCAAAGAAGATTGACCGCCATA-3ʹ and 5ʹ-CAGCTTGCTGCTCCAT-3ʹ, respectively.

Tissue preparation for immunohistochemistry

Tissues were fixed and processed as previously described [4,13]. First, mice were anesthetized using a ketamine/xylazine mixture (8.75 mg ketamine and 1.25 mg xylazine/100 g BW), and then perfused with 0.05 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.05 M phosphate buffer (PB). Pituitary glands were collected immediately, incubated in 4% paraformaldehyde/PB overnight at 4°C, and then cryoprotected in PB containing 30% sucrose. A cryostat was used to prepare frozen 10-µm horizontal sections that were mounted on MAS-coated glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan).

Dual-immunostaining for GPR120 and luteinizing hormone beta (LHβ)

For dual-immunostaining, an anti-LHβ rabbit antibody (1:2,000; kindly provided by Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA) was pre-conjugated with anti-rabbit Alexa Fluor 488 Fab fragments for 5 min at room
temperature using a Zenon Alexa Fluor 488 Rabbit IgG Labeling Kit (Life Technologies Co.), according to the manufacturer’s protocol. Tissue sections were incubated with 10% normal goat serum (NGS) for 1 h at room temperature to block non-specific binding, then incubated with the rabbit anti-GPR120 antibody (1:166; LS-A2004; LifeSpan BioSciences Inc., Seattle, WA) in blocking solution at 4 °C overnight. After four washes in PBS, the sections were incubated at room temperature for 90 min with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:200), followed by four washes in PBS. Sections were then incubated with the pre-conjugated anti-LHβ rabbit antibody overnight at 4 °C, and washed four times in PBS at room temperature. Finally, cover slips were placed over the sections using VECTASHIELD Mounting Medium (Vector Laboratories Inc., Burlingame, CA) and examined with a BX51 fluorescence microscope (Olympus Co., Tokyo, Japan). To test the specificity of the anti-GPR120 antibody, an antigen preadsorption sample was prepared, in which 50 μL of a 6 μg/mL antibody solution was incubated at 4 °C overnight with 15 μg of a synthetic GPR120 peptide antigen (LS-P2004; LifeSpan BioSciences), prior to incubation with the tissue.

Quantification of GPR120 immunoreactivity

Colocalization of GPR120 and LHβ was quantified unilaterally in five non-consecutive horizontal sections obtained from each of the four diestrus, OVX and OVX+E2 animals. The percentages of GPR120 immunoreactivity in LHβ immunoreactive cells and LHβ immunoreactivity in GPR120 immunoreactive cells were calculated. To avoid bias while counting, slides were coded and evaluated in a blinded experimental setup.

Statistical analysis

Data were analyzed using the one-way ANOVA and the Bonferroni’s post-hoc analysis. Data are expressed as mean ± SEM and were analyzed using JMP 9. A p value < 0.05 was considered significant.

Results

GPR120 mRNA levels in the pituitary gland after E2, P, or Cetrotide injection

GPR120 mRNA expression levels were significantly higher in the pituitary glands of OVX, OVX+P, OVX+E2+P and OVX+Cetrotide animals than in sham surgery animals (Fig. 1). This increase in GPR120 mRNA was reduced following treatment with E2.

Colocalization of GPR120 and LHβ in the pituitary gland

GPR120 protein expression in gonadotrophs of the pituitary glands of Sham, OVX, OVX+E2, OVX+P, OVX+E2+P, and OVX+Cetrotide animals was examined using dual immunohistochemistry. GPR120 immunoreactivity was observed in almost all LHβ-immunoreactive cells in intact (96.8 ± 1.7%; Fig. 2 and Table 1), OVX (98.0 ± 1.1%; Fig. 2 and Table 1), OVX+E2 (97.2 ± 2.0%; Fig. 2 and Table 1), OVX+P (98.4 ± 0.0%; Fig. 2 and Table 1), and OVX+E2+P (95.4 ± 0.0%; Fig. 2 and Table 1) animals. Furthermore, almost all GPR120 immunoreactive cells were also LHβ immunopositive in Sham (98.3 ± 1.0%; Fig. 2 and Table 1), OVX (97.0 ± 1.8%; Fig. 2 and Table 1), OVX+E2 (97.4 ± 1.9%; Fig. 2 and Table 1), OVX+P (97.8 ± 0.0%; Fig. 2 and Table 1), and OVX+E2+P (97.6 ± 0.0%; Fig. 2 and Table 1) animals. The number of LHβ immunoreactive cells could not be counted in OVX+Cetrotide animals, because the immunoreactivity of LHβ was weak in these animals (Fig. 2 and Table 1). In addition, the preincubation of the anti-GPR120 antibody with a synthetic GPR120 peptide abolished the immunostaining of GPR120 in frozen pituitary tissue sections of ad-lib fed animals (Fig. 2, Adsorption).
Fig. 2 Dual immunohistochemistry for GPR120 (red) and luteinizing hormone β (LHβ, green) in the pituitary tissues of sham operated, ovariectomized (OVX), estradiol-primed OVX (OVX+ E2), progesterone-primed OVX (OVX+P), both E2 and P-primed OVX (OVX+ E2+P) and GnRH antagonist, Cetrotide-injected OVX animals. The GPR120 protein colocalized with LHβ in the pituitary glands of diestrus, OVX, OVX+E2, OVX+P, OVX+E2+P and OVX+Cetrotide animals. GPR120 immunoreactivity was eliminated by preincubation of the anti-GPR120 antibody with a synthetic antigen peptide (Adsorption) in the pituitary tissues of day 1 diestrus animals. Scale bar = 50 µm.

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<th>Diestrus</th>
<th>OVX</th>
<th>E2</th>
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<th>E2+P</th>
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<tr>
<td>GPR120 / LH</td>
<td>96.8 ± 1.7</td>
<td>98.0 ± 1.1</td>
<td>97.2 ± 2.0</td>
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<tr>
<td>LH / GPR120</td>
<td>98.3 ± 1.0</td>
<td>97.0 ± 1.8</td>
<td>97.4 ± 1.9</td>
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Table 1 Immunohistochemical colocalization of GPR120 and luteinizing hormone β (LHβ) in the pituitary gland of diestrus, ovariectomized (OVX), estradiol-primed OVX (OVX+ E2), progesterone-primed OVX (OVX+P), both E2 and P-primed OVX (OVX+ E2+P), and GnRH antagonist, Cetrotide-injected OVX animals. The top row indicates the percentages of GPR120 immunoreactivity in LHβ immunoreactive cells. The bottom row indicates the percentages of LHβ immunoreactivity in GPR120 immunoreactive cells.
**GPR120 mRNA levels in the pituitary gland of normal cycling females**

GPR120 mRNA expression levels were not significantly different in the pituitary gland of females throughout the phases of the ovarian cycle (Fig. 3).

**Uterine wet weight**

Uterine wet weights were significantly lower in OVX (99.2 ± 10.5 mg), OVX+P (120.7 ± 13.1 mg), and OVX+Cetrotide (82.5 ± 30.8 mg) animals than in Sham (187.7 ± 19.1 mg), OVX+E2 (250.1 ± 38.8 mg), and OVX+E2+P animals (223.4 ± 38.3 mg) (Fig. 4). Furthermore, uterine wet weights of OVX+E2 and OVX+E2+P animals were almost the same as those of sham operated diestrus female animals.

**Discussion**

The present study clearly demonstrates that the expression of GPR120 increased following OVX in female mice. This indicates that GPR120 expression in the pituitary gland is under the effect of a humoral signal, which is altered following OVX. Indeed, the OVX-induced increase in GPR120 mRNA expression levels was markedly reduced by E2, and tended to be elevated by P, suggesting these hormones directly or indirectly regulate GPR120 mRNA expression in the pituitary gland.

Further, this change in GPR120 expression would occur in gonadotrophs, because more than 95.4% of GPR120 immunoreactivities were observed in gonadotrophs.

E2 reduced the OVX-induced increase in GPR120 mRNA expression in the pituitary gland, indicating that estradiol negatively regulates GPR120 mRNA expression in gonadotrophs of the anterior pituitary gland. Interestingly, GPR120 mRNA expression levels in the estrous stage varied, but only slightly. The results suggest that GPR120 mRNA expression is regulated by the presence or absence of estrogen rather than in an estrogen dose-dependent manner. If that should be the case, GPR120 mRNA expression levels may be high in the prepubertal and postmenopausal periods when levels of blood estrogen are very low, and this GPR120 activation may support the inactivation of gonadotropic hormones in these periods. In addition, it has been reported that some growth factors, such as the insulin-like growth factor I and the transforming growth factor β1, are upregulated by E2 in the pituitary gland [14,15]. Thus, these growth factors may be involved in the regulation of GPR120 expression.

The OVX-induced increase in GPR120 mRNA expression levels tended to be elevated by P. Furthermore, simultaneous injection of E2 and P interfered with the action of E2 on GPR120 mRNA expression. Thus, P may counteract the inhibitory effect.

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**Fig. 3** GPR120 mRNA expression, analyzed using real-time PCR, in the pituitary glands of normal cycling female animals. D1, day 1 diestrus; D2, day 2 diestrus; P, proestrus; E, estrus. All data are represented as mean ± SEM (n = 6).

**Fig. 4** Mean uterine wet weights (mg) of diestrus, ovariectomized (OVX), estradiol-primed OVX (OVX+E2), progesterone-primed OVX (OVX+P), both E2 and P-primed OVX (OVX+ E2+P), and GnRH antagonist, Cetrotide-injected OVX animals. Uterine wet weights were lower in the OVX, OVX+P, and OVX+Cetrotide groups compared to those in diestrus animals. * indicates p < 0.05.
of estradiol on the synthesis of GPR120 in the pituitary gland. The results suggest that the activation of GPR120 expression in response to P, which is increased in the luteal phase and during pregnancy, may contribute to the inactivation of gonadotropic hormones during these phases. Indeed, FFAs directly suppress Fshb mRNA levels in the LβT2 gonadotropic cell line [7], and the plasma levels of FFA in female rodents are commonly elevated during pregnancy [16,17].

The hypothalamus, which is also regulated by E2 and P, may regulate GPR120 mRNA expression in the pituitary gland indirectly through GnRH secretion in response to E2 and P injection, because cells in the hypothalamus also express E2 and P receptors [18]. Therefore, we decided to investigate whether E2 directly regulates GPR120 mRNA expression in the pituitary gland by using the LβT2 gonadotropic cell line in vitro. However, the data were inconsistent and the results of the study were inconclusive (data not shown). Thus, we conducted another experiment that involved GnRH antagonist, Cetrotide, treatment to examine the direct effects of E2 and P on GPR120 mRNA expression in the pituitary gland. Cetrotide did not reduce the increase in GPR120 mRNA expression levels in OVX animals. This suggests that E2 and P act not on the hypothalamus but directly on the pituitary gland to regulate GPR120 mRNA expression.

Uterine weight is classically used as a bioassay for estrogen levels [19, 20]. In the present study, the uterine wet weights of OVX+E2 and OVX+E2+P animals were almost equal to those of sham operated diestrus animals, indicating that the blood estrogen levels of OVX+E2 and OVX+E2+P animals were almost the same as those for diestrus animals in this study.

In summary, the current study clearly demonstrates that the expression of GPR120 in the pituitary gland, and particularly in gonadotrophs, is augmented by ovariectomy or P, and is down regulated by E2. Furthermore, P can interfere with the activity of E2 in GPR120 expression. These results suggest that ovarian hormones may directly regulate GPR120 expression at the pituitary level. Additional studies are needed to further elucidate the physiological function of GPR120 in the pituitary gland.

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References

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