Effects of 17β-estradiol on leptin signaling in anterior pituitary of ovariectomized rats

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Abstract: Leptin is secreted predominantly by adipocytes and exerts its role mainly by interaction with the long form of leptin receptor (LEPR_V2). It has been identified that LEPR_V2 is widely distributed in various tissues, including the anterior pituitary. Cross-talk between leptin and estrogens has been indentified. Estrogen is known to modulate the tissue-specific expression of LEPR_V2 and leptin in ovariectomized (OVX) rats, a model of postmenopausal condition. Our previous data showed that 17β-estradiol (E2) up-regulated the expression of LEPR_V2 protein and mRNA in rat dorsal root ganglion (DRG) in an estrogen receptor alpha (ERα)-dependent manner. But it is still unclear whether estrogen can regulate leptin signalling in the pituitary of OVX rats. In the present study, we found that ovariectomy decreased the expressions of LEPR_V2. Administration of E2 increased the expressions of LEPR_V2 in a dose-dependent manner. In addition, E2 improved LEPR_V2, STAT3, and SOCS3 protein levels in OVX rats. The effects of exogenous E2 were attenuated by ICI 182,780, a specific estrogen receptors antagonist. However, E2 did not change the Lepr_v1, a type of short form of leptin receptor (LEPR), or leptin mRNA levels. Thus, E2 plays a crucial role in regulating pituitary sensitivity to leptin in OVX rats. Our findings implied that exogenous E2 had potential roles in modification of the function of pituitary in postmenopausal women.

Key words: 17β-estradiol, anterior pituitary, leptin, leptin receptor, ovariectomized rat

Introduction

Menopause, as the culmination of reproductive aging, is characterized by an altered hormonal status including estrogens. The progressive loss of estrogen is associated with an increased risk of developing obesity, diabetes, and cardiovascular disease [3, 17, 20]. Furthermore, manopause also causes pituitary dysfunction [21]. Estrogen is an important class of hormones which has vital effects on pituitary. Estradiol (E2) is able to exert a rapid apoptotic action in anterior pituitary cells, especially in lactotropes and somatotrophs, through binding the estrogen receptors [38]. Administration of E2 stimulates the increase of mammatrophs in a dose-dependent manner [37]. In gonadectomized rats, estradiol increases adrenocorticotropic hormone (ACTH) in the plasma.
and ovariectomy decreases the ACTH response to corticotropin-releasing factor [36]. However, to the best of our knowledge, the effects of estrogen on leptin signaling in pituitary of OVX rats have not been fully elucidated.

Leptin is a 16-kDa polypeptide hormone, which is initially reported to be secreted by adipose tissue [39]. Leptin plays an important role in regulating food intake, energy expenditure, body weight homeostasis [9], cardiovascular system [11], reproduction function [6] and neuroendocrine response [2]. Furthermore, leptin is capable of modification the function of pituitary [13, 30].

The leptin receptor (LEPR) belongs to the family of cytokine-like receptors with a single transmembrane domain [33]. The cytoplasmic sequence of the receptor contains a signal transducer and activator of transcription (STAT) activating motif. Stimulation of JAK-STAT pathway that mediates at least some action of leptin [8]. Six isoforms of the LEPR have been characterized that differ in the truncation of the cytoplasmic sequence. The long isoform of LEPR (LEPR_V2) is the active long intracellular form of the receptor and can activate both JAK and STAT proteins [31]. The short form of the LEPR (LEPR_V1) is a transporter molecule for leptin that promotes leptin stability [32]. LEPR_V2 is detected mainly in the peripheral tissues including the testes, pituitary, hypothalamus and ovary. The specific site of expression of LEPR within these tissues has important implications with regard to the function of leptin [14]. Our previous research has demonstrated that LEPR_V2 is expressed in the rat dorsal root ganglion (DRG) [7], and chronic constriction sciatic nerve injury-induced neuropathic pain is alleviated by intrathecal leptin through inhibition the expression of P2X2/3 receptors [19].

Ovariectomized rats were considered a very useful model of postmenopausal condition. This model was useful to investigate the effects of hypoestrogenism and hormonal replacement on pituitary. The present study was designed to elucidate the effects of estrogen therapy on leptin signalling in pituitary of OVX rats. In addition, the mechanisms of E2 in regulation of leptin signalling were also investigated. It is hoped that our novel finding will help to understand the roles of hormone replacement therapy in the regulation of pituitary function in postmenopausal women.

### Materials and Methods

#### Animals

Nine to ten weeks old Sprague-Dawley rats (235.9 ± 5.0 g) were provided by the Center of Laboratory Animal Science of Nanchang University. The rats were fed a commercial diet (Murine bred diet; Slacom, China). The rats were maintained on a 12 L:12 D cycle (0800–2000 h) at an ambient temperature of 22 ± 2°C and a humidity of 45–55% under the conventional condition in the center. All animal procedures were approved by the Animal Care Committee of the Medical College of Nanchang University and were performed in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. During all the experiments, efforts were made to minimize both animal suffering and the number of animals used. Female rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and ovariectomized via two small dorsal incisions. Two weeks after surgery the rats were used.

#### Experimental protocols

Two experiments were conducted. In experiment 1, the female rats were divided into five groups with 6 rats in each group: 1) sham-operated control+vehicle (Sham group); 2) OVX+vehicle (OVX group); 3) OVX+E2 (17β-estradiol, 2 µg/kg per day; OVX+E2 group); 4) OVX+E2 (10 µg/kg per day; OVX+E2 10 group); and 5) OVX+E2 (25 µg/kg per day; OVX+E2 25 group). E2 was dissolved in the ethanol and then diluted with sesame oil. The rats were given all injections subcutaneously and all injections were given daily for 14 days.

In experimental 2, rats were divided into four groups with 8 rats in each group: 1) OVX+vehicle (OVX group); 2) OVX+E2 (25 µg/kg per day; E2 group); 3) OVX+ICI182,780 (500 µg/kg per day; ICI group) and 4) OVX+17β-estradiol+ICI182,780 (E2, 25 µg/kg per day; ICI182,780, 500 µg/kg per day; E2+ICI group). E2 and ICI182,780 were dissolved in the ethanol and then diluted with sesame oil. The rats were given all injections subcutaneously and all injections were given daily for 14 days. ICI182,780 administration 1 h prior to E2 in each injection of E2+ICI Group.
Tissue collection
Rats were sacrificed 24 h after the last drug injection. They were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The anterior pituitaries were carefully detached. Then these tissues were immediately frozen in liquid nitrogen and stored at −80°C. Each anterior pituitary wrapped with aluminum foil was frozen in the liquid nitrogen for 10 s, the pituitary was then hammered into powder quickly. On the dry ice, the powder was separated for total RNA (one quarter powder) and protein (three quarters powder) isolation. Blood was collected by cardiac puncture before the animals were sacrificed. Blood samples were centrifuged and the serum was stored at −80°C prior to being analyzed.

Enzyme-linked immunosorbent assays (ELISA)
A competitive ELISA kit (Cayman Chemical, USA) was used to determine the serum concentration of E2 and leptin according to the procedure. The intra-assay coefficients of variance were 6% and <10%, respectively.

Real-time PCR quantification
Total RNA from the anterior pituitary of rats was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. 1,000 ng total RNA was used as a template for reverse transcription using the Applied Biosystems Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR for leptin, Lepr_v1, and Lepr_v2 was performed using ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression levels of leptin, Lepr_v1, and Lepr_v2 were normalized to GAPDH. Probes were purchased from Applied Biosystems. All assays were performed in triplicate.

Western blot
The anterior pituitaries from ovariectomized rats were lysed with RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), supplementing with protease inhibitor cocktail. Protein concentration was determined using Bradford protein assay reagent with bovine serum albumin (BSA) as a standard. Samples of 30 µg of total protein were separated by 8% (w/v) sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% (w/v) BSA in phosphate buffer saline (pH 7.4) for 3 h at room temperature. After incubation with primary antibody of LEPR polyclonal antibody (Santa Cruz Biotechnology, CA, USA; dilution 1:350), phospho-STAT3 (1:1,000; Abcam, USA), STAT3 (1:1,000; Abcam, USA), or SOCS3 (1:1,000; Abcam, USA) overnight at 4°C, the membrane was then incubated with peroxidase conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Immunodetection was completed using Pierce-enhanced chemiluminescence substrate (Thermo Scientific, USA) and then exposed to X-ray film. GAPDH (1:10,000; ABclonal, USA) was used as a loading control.

Statistical analysis
Data were showed as the mean ± S.D. Comparisons of means between two groups were carried out using a t-test. Statistical comparisons were performed by analysis of variance (ANOVA) with Dunnett's test for multiple comparisons. A value of *P* < 0.05 was considered to be significant.

Results

Body weight, serum E2 and leptin levels
At the endpoint of the experiment 1, the body weight, serum E2 and leptin levels were evaluated. As expected, ovariectomy lead to a significant increase body weight and a decrease in serum E2 levels when compared with the sham group. E2 suppress the O VX-induced body weight increase (Fig. 1A). Serum E2 levels were higher (P < 0.01) in the OVX+E2 10 group (1.68-fold) and OVX+E2 25 group (2.25-fold) when compared to the OVX group (Fig. 1B). The serum leptin level also increased in the OVX rats, and administration of E2 (10 µg/kg and 25 µg/kg) for 14 days decreased the serum leptin level when compared to the OVX group (Fig. 1C).

E2 increased the Lepr_v2 mRNA expression in the anterior pituitary of OVX rats
Real-time PCR was performed to detected the expression of leptin, Lepr_v1, and Lepr_v2 mRNA in the anterior pituitary of all the animal groups. Ovariectomy led to a significant decrease in Lepr_v2 mRNA levels as compared with the sham group. Administration of E2 (10 µg/kg and 25 µg/kg) for 14 days suppressed the ovariectomy-induced decrease in expression of Lepr_v2 mRNA (Fig. 2). Thus, E2 increased the Lepr_v2 mRNA in a dose-dependent manner. Furthermore, the effects
were efficiently attenuated by ICI 182,780 (Fig. 3).

**E2 increased the LEPR_V2, p-STAT3 and SOCS3 protein expression in the anterior pituitary of OVX rats**

To investigate the potential mechanism of E2 in reversing of ovariectomy-induced decrease of leptin signaling at protein levels, the LEPR_V2, p-STAT3 and SOCS3 proteins were measured by Western blot. The results indicated that ovariectomy led to a significant decrease in LEPR_V2 protein when compared with the sham group. E2 suppressed the ovariectomy-induced decrease in expression of LEPR_V2 protein in a dose-dependent manner (Fig. 4). In addition, ICI 182,780 attenuated the E2-induced increase of LEPR_V2, p-STAT3, and SOCS3 at protein levels (Fig. 5). The results suggested that E2 activated the leptin signalling in an estrogen receptor-dependent manner.

**Discussion**

The progressive loss of estrogens induced by menopause leads to typical menopausal symptoms which may seriously compromise the quality of life. Hormone replacement therapy (HRT) with estrogens used to be standard therapy for treatment of menopausal symptoms such as hot flushes and depression [29]. Estrogens is also
involved in the regulation of the neuroendocrine response. Estradiol short-term therapy modulates the neuroendocrine control of the hypothalamus-pituitary unit and induces the recovery of both gonadotropins synthesis and secretion in hypogonadotropic patients with functional hypothalamic amenorrhea [10]. Pituitary dysfunction is associated with estrogen-deficiency [21].

Leptin is a powerful stimulator of PRL release and its actions occur in part through stimulation of extracellular signal-regulated kinase [34]. Leptin exerts a stimulatory effect on growth hormone releasing hormone (GHRH)-induced GH secretion in GH-secreting pituitary adenomas [12]. In addition, leptin stimulates the expression of GH in primary pig pituitary cells and bovine pituitary tissues [1]. It has been postulated that leptin might increase thyroid-stimulating hormone (TSH) secretion, since there is a synchronicity between leptin and TSH secretion in normal humans. And the pulsatile and circadian rhythm was disorganized in leptin-deficient patients [23]. By contrast, leptin is identified to inhibited rat pituitary TSH release [27]. Leptin induces pituitary cells to produce and secrete both LH and FSH [26]. It is also showed that the influences of leptin on FSH secretion are biphasic [18]. The actions of leptin are mediated by LEPR which is widely distributed in many tissues including hypothalamus and pituitary [15, 16]. The factors of influence leptin or LEPR expression in pituitary may lead to the physiological function abnormal.

Administration of E₂ for short term improves the serum leptin level in both normal and O VX rats [5, 28]. In addition, E₂ stimulate, in vivo and in vitro, the expression of leptin in adipocytes [5, 22]. O VX induced up-regulation of body weight and fat mass, which is associated with the increase of serum leptin level. Treatment of E₂ for long term causes the body weight and fat mass down-regulation and lead to the serum leptin decrease in the
OVX rats [25]. In the present study, OVX caused the serum leptin level up-regulation with the body weight increase. And administration of E2 for 14 days reduced the serum leptin level with the body weight decrease as compared to the OVX rats.

Estrogen can also modulate leptin actions by changing LEPR expression [25, 28]. In the previous report, we identify that estrogen can improve the expression of LEPR in rat DRG [7]. In addition, estrogen regulates the expression of LEPR in growth plate chondrocytes via estrogen receptor and activation of ERK1/2 signaling pathways [35]. However, the effect of estrogen on leptin signaling in pituitary of OVX rat remains unclear.

In the present study, we investigated the effects of estrogen on expressions of leptin, LEPR_V1 and LEPR_V2 in anterior pituitary of OVX rats. The results indicated that E2 significantly increase Lepr_v2 mRNA and protein in a dose-dependent manner in the anterior pituitary. Similar to our findings, ovariectomy decrease the LEPR_V2 expression and administration of E2 reversed the ovariectomy-induced down-regulation of LEPR_V2 in the hypothalamus, DRG and skeletal muscle [4, 7, 25]. By contrast, ovariectomy increase the LEPR_V2 expression in the adipose tissue, and E2 administration reverse the ovariectomy-induced up-regulation of LEPR_V2 [25]. Thus, estrogen regulated LEPR_V2 expression in a tissue-specific way. Our results indicated that the actions of estrogen in promotion LEPR_V2 expression was prevented by ICI182,780, a specific estrogen receptor antagonist. However, E2 could not modulate the expressions of leptin and LEPR_V1. To further investigate the role of E2 on leptin signalling in the pituitary, we examined the downstream effectors of LEPR_V2 activation, STAT3, and SOCS3 in the pituitary of OVX rats, and thereby might play a role in regulating pituitary sensitivity to leptin. However, the exact mechanism of estrogen in modulation leptin signaling in OVX pituitary is still not well indentified and need to be further investigated.

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