Novel action of pituitary urocortin 2 in the regulation of expression and secretion of gonadotropins

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Abstract

Urocortins 2 (Ucn 2), one of the corticotropin releasing factor (CRF) peptide family, is thought to be an endogenous ligand for CRF type 2 receptor (CRF-R2). We previously demonstrated that Ucn 2 is expressed in the corticotrophs of rat pituitary, and the mRNA expression and secretion of Ucn 2 in corticotrophs of rat anterior pituitary are regulated by CRF and glucocorticoids. Since CRF-R2 has been reported to be expressed on gonadotrophs of the rat pituitary, we hypothesized that pituitary Ucn 2 may control the expression and secretion of gonadotropins. Monolayer culture of rat anterior pituitary cells showed that the secretion of gonadotropins was suppressed by Ucn 2. A CRF-R2 selective antagonist, adenoviral-mediated expression of short interfering RNA against CRF-R2, and anti-Ucn 2 rabbit IgG

increased the secretion and mRNA expression of gonadotropins. intraperitoneal injection of anti-Ucn 2 IgG into immature male rats significantly increased the secretion and mRNA expression of gonadotropins compared with those in normal rabbit IgG-injected rats. Daily i.p. injection of anti-Ucn 2 IgG into immature female rats induced a tendency toward earlier occurrence of menarche compared with normal rabbit IgG-injected rats. These findings suggest that pituitary Ucn 2 is involved in the regulatory mechanism of the expression and secretion of gonadotropins through its tonic and inhibitory action on gonadotrophs in a paracrine manner.

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Introduction

Corticotropin-releasing factor (CRF) plays a key role in stress responses in the endocrine system, autonomic nervous system, behavior, and mood (Vale *et al.* 1981, Berkenbosch *et al.* 1987, Sapolsky *et al.* 1987). The various actions of CRF are induced through two subtypes of CRF receptor (CRF-R), the CRF-R1 and the CRF-R2, with a higher affinity to CRF-R1 than to CRF-R2 (Chen *et al.* 1993, Perrin *et al.* 1993, Lovenberg *et al.* 1995a). Urocortins 2 (Ucn 2), one of the CRF family peptides, is thought to be an endogenous ligand for CRF-R2 (Reyes *et al.* 2001). The results of several *in vitro* and *in vivo* experiments have shown that Ucn 2 induces; inhibition of the apoptosis of mesenteric arterial smooth muscle cells (Tao *et al.* 2006), an increase in cardiac output and in mean arterial pressure (Davis *et al.* 2007), a central CRF-R2-mediated inhibition of gastric emptying involving sympathetic α_1 -adrenergic mechanisms (Czimmer *et al.* 2006) and suppression of food intake (Zorrilla *et al.* 2004), and antagonizes the motor activation induced by CRF (Ohata & Shibasaki 2004). Ucn 2-knockout mice exhibit significant increases in basal plasma ACTH and corticosterone levels, a significant decrease in fluid intake, and depressive-like behavior, increased insulin sensitivity, and protection from fat-induced insulin resistance (Chen *et al.* 2006a,b). However,

the details of the physiological role of endogenous Ucn 2 still remain unclarified.

We previously demonstrated that Ucn 2 is expressed in the corticotrophs of rat pituitary but the concentrations of Ucn 2 in culture media of rat anterior pituitary cells are extremely low compared with those of ACTH (Nemoto *et al.* 2007). We also showed that the mRNA expression and secretion of Ucn 2 in corticotrophs of rat anterior pituitary are regulated positively by CRF and negatively by glucocorticoids (Yamauchi *et al.* 2005, Nemoto *et al.* 2007). Two variants of CRF-R2, CRF-R2 α and CRF-R2 β , have been found in rats (Lovenberg *et al.* 1995b). CRF-R2 α is expressed in the hypothalamus, lateral septum, and raphe nuclei, whereas CRF-R2 β is found in peripheral tissues such as heart, intestine, colon, and muscle (Lovenberg *et al.* 1995a). CRF-R2 α has recently been shown to be expressed in more than 50% of gonadotrophs in rat anterior pituitary (Kageyama *et al.* 2003). We therefore hypothesized that pituitary Ucn 2 may be involved in the regulatory mechanism of the expression and secretion of gonadotropins in a paracrine manner.

To clarify the hypothesis, in this study, we examined the effects of Ucn 2 on the expression and secretion of gonadotropins, and the effects of a CRF-R2 antagonist, anti-Ucn 2 rabbit IgG, and adenoviral-mediated expression of short interfering RNA (siRNA) against CRF-R2 on the

expression and secretion of gonadotropins in monolayer-cultured rat anterior pituitary cells. We further examined the effects of single or repeated i.p. injection of anti-*Ucn 2* rabbit IgG on the expression and secretion of gonadotropins in male and female rats and the occurrence of menarche in female rats.

Materials and Methods

Animals

Male and female Wistar rats were maintained at $23 \pm 2^\circ\text{C}$ on a 12 h light: 12 h darkness cycle (light on at 0800 h, off at 2000 h). They were allowed access to chow and distilled water laboratory *ad libitum*. Menarche and the estrous cycle of female rats were determined by vaginal impedance measurements using an MK-10C (Muromachi Kikai Co. Ltd, Tokyo, Japan) and vaginal cytological findings. All experimental procedures were conducted in accordance with the guidelines for use and care of the Laboratory Animals Ethics Committee of Nippon Medical School.

Primary culture of pituitary cells

Male rats aged 6 weeks were killed by decapitation and their pituitary glands were removed under sterile conditions. The anterior and intermediate/posterior pituitaries were separately collected, and mechano-enzymatically dispersed as described previously with several modifications (Nemoto *et al.* 2007). Samples were washed twice in PBS and then incubated at room temperature in the PBS containing 0.047 g/l MgCl_2 , 0.1 g/l CaCl_2 , and 0.01% dispase (Gdo-shusei, Tokyo, Japan) with constant stirring for 30 min. After washing dispersed cells with PBS three times a 1-ml aliquot of cell suspension containing 1.0×10^4 cells in a DMEM/F12 was placed in each well of 24-well plates. The anterior pituitary cells obtained from 30 rats were cultured in 72 wells. The cells were subsequently allowed to attach to the surface of the wells in a humidified 95% air–5% CO_2 incubator for 4 days. On the day of the experiment the culture medium was changed. After 4 h incubation with samples, the culture medium from each well was collected. After centrifugation to remove debris at 3500 r.p.m. for 10 min at 4°C , the culture media were frozen and kept at -80°C until assay. Total RNA was extracted using guanidium HCl from cells attached to the surface of wells.

Recombinant adenovirus construction

siRNAs against rat CRF-R1 (NM_030999), CRF-R2 α (NM_022714), and EGFP (U57608) were synthesized by Takara (Shiga, Japan). Each siRNA was cloned into pBASi-mU6Neo DNA expression vector (Takara), and cut by EcoRV. The DNA fragments, which include siRNA expression unit, were then cloned into pAxcwit2 cosmid vector (Takara; pAx-siCRF-R1 and pAx-siCRF-R2).

Recombinant adenovirus was constructed by the COS-TPC method using pAx-siCRF-R1 and pAx-siCRF-R2 cosmid according to the manufacturer's instruction (Takara).

Recombinant CRF-R1 or CRF-R2 α expressing cells

Rat CRF-R1 and CRF-R2 α cDNA were amplified from λ TriplEx2 rat brain large-insert cDNA library (BD Biosciences Clontech) using CRF-R1: 5'-ggatcatgggacggc-gcccgag-3' and 5'-ctcgagtcacactgctgtggactgcttg-3' and CRF-R2 α : 5'-agatctatggacggcgctgctcctag-3' and 5'-ctcg-agtcacacggcagctgtctgcttg-3' primers. The amplified cDNA was subcloned into pGEM-T vector (Promega). Plasmid vector was digested by *Bam* HI and *Xho* I and cloned into pCMV-tag2B vector (FLAG-CRF-R1 and FLAG-CRF-R2). Each plasmid was transfected into HeLa cells using Lipofectamine 2000. After 72 h, HeLa cells overexpressing CRF-R1 or CRF-R2 were infected by recombinant adenovirus expressing siRNA against CRF-R1 or CRF-R2. The cells were collected 72 h later and total RNA and protein were extracted. Specificities of siRNA against CRF-R1 and CRF-R2 are shown in Fig. 3A and B. The effects of siCRF-R1 and siCRF-R2 on CRF-induced change in POMC mRNA expression and *Ucn 2*-induced gonadotropin secretion were studied. Cultured rat anterior pituitary cells infected by recombinant adenovirus expressing each siRNA were treated with 1 nM CRF for 4 h, and total RNA was extracted and POMC mRNA expression was analyzed by real-time RT-PCR. Cultured rat anterior pituitary cells infected by recombinant adenovirus expressing siRNA against CRF-R2 were also treated with *Ucn 2* at concentrations of 1, 10, and 100 pM for 4 h, and concentrations of LH and FSH in the culture media were measured.

RNA extraction and real-time PCR analyses

Total RNA was extracted from monolayer-cultured pituitary cells and anterior pituitaries using Isogen (Takara). To avoid false positive results caused by DNA contamination, samples were treated with RNase-free DNase (Takara) for 60 min at 37°C . First-strand cDNA was synthesized using 1 μg of denatured total RNA at 42°C for 60 min, 99°C for 5 min, and 5°C for 5 min using an ExScript RT reagent kit (Takara). PCR was performed by denaturation at 94°C for 5 s and annealing extension at 60°C for 30 s for 40 cycles, using SYBR premix Ex Taq (Takara) and specific primers for rat *Ucn 2*, LH β -subunit, FSH β -subunit, and GAPDH (Table 1).

Purification of anti-*Ucn 2* rabbit IgG

We had previously generated antiserum against mouse *Ucn 2* (Yamauchi *et al.* 2005). Synthetic mouse *Ucn 2* was coupled to porcine thyroglobulin through water soluble carbodiimide hydrochloride, and the conjugate was used to immunize female New Zealand white rabbits. The specificity of

Table 1 Primer sequences of the studied genes

Genes	Primers	Length (bp)	Accession number (GenBank)
Ucn 2	GAGCAACTCTAAAGCCAGCCCTTAC TGATTCCTGGCAGCCTTGTC	384 (169–285)	NM_13385
LHβ	TGAGCCCAAGTGTGGTGTG GGCAGCTGGCAGTACTCGAA	167 (48–214)	NM_012858
FSHβ	TGAAGTACCAACATCACCATCTC CAGCCAGGCAATCTTATGGTCTC	189 (66–254)	NM_001007597
GAPDH	GGCAGTCAAGGCTGAGAATG ATGGTGGTGAAGACGCCAGTA	143 (242–384)	NM_017008

anti-Ucn 2 IgG has been described in the previous report (Yamauchi *et al.* 2005). The antiserum obtained after the fifth booster was used to purify IgG fraction with a protein A-sepharose column (Amersham Biosciences). The specificity of anti-Ucn 2 IgG against rat Ucn 2 was studied by dot blotting.

Passive immunization

Monolayer-cultured anterior pituitary cells were treated with 10 µg anti-Ucn 2 IgG or normal rabbit IgG. After 4, 8, and

24 h, culture media and cells were collected to examine FSH secretion and LH and FSH β-subunit mRNA expressions respectively. Twenty-one-day-old male rats were given an i.p. injection of 50 or 150 µg anti-Ucn 2 IgG or normal rabbit IgG. Rats were killed 8 or 24 h after injection and their trunk blood and pituitaries were collected to examine plasma LH and FSH concentrations and LH and FSH β-subunit mRNA expressions respectively. Twenty-one-day-old female rats were injected daily (twice per day) with 100 µg anti-Ucn 2 IgG or normal rabbit IgG until the second metestrus stage. Rats were killed and their trunk blood and tissues were collected at

Figure 1 Ucn 2 suppresses gonadotropins secretion. Anterior pituitary cells were treated with Ucn 2 at concentrations of 1, 3, 10, 30, 100, and 1000 pM for 4 h or 10 pM for 4, 8, and 24 h. After incubation, culture media were collected for assays of LH and FSH (A, B). Total RNA was extracted from cells and assayed for levels of LH and FSH β-subunit mRNA expressions using the Thermal Cycler Dice Real Time PCR System (C). The cells were treated with 10 pM Ucn 2 and/or 10 pM antisauvagine-30 for 4 h. After incubation, culture media were used for assays of LH and FSH (D). Values are the mean ± s.e.m. There were six wells for each treatment. **P* < 0.05, compared with control. Co, control; U2, Ucn 2; AS30, antisauvagine-30.

the second metestrus stage, and plasma gonadotropin concentrations, anterior pituitary LH and FSH β -subunit mRNA expressions, and tissue weight were examined.

Plasma hormone assay

Rats were decapitated and trunk blood was collected in tubes containing EDTA 2Na (1 mg/ml blood) and centrifuged at 3000 r.p.m. for 20 min at 4 °C. Plasma of 1 ml was transferred into 1.5 ml Eppendorf tubes and stored at -80 °C for measurement later. Rat plasma LH and FSH concentrations were measured using a rat LH [¹²⁵I] Biotrak assay system (Amersham Biosciences) and a rat FSH ELISA kit (Biocode, Liege, Belgium) respectively.

Statistical analysis

Statistical significance was performed using two-way ANOVA, unpaired *t*-test, and Mann–Whitney's *U*-test using Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Cell culture in same experimental protocol was repeated two to four times and data were combined and analyzed. For real-time RT-PCR data, all results were combined for the statistical analysis and expressed as percent of controls. Statistical significance was established at the $P < 0.05$ level.

Results

Ucn 2 suppresses basal secretion of gonadotropins in primary culture of anterior pituitary cells

Since 4 h incubation of monolayered anterior pituitary cells with Ucn 2 significantly decreased LH and FSH secretion at concentrations of 3–30 pM (Fig. 1A), 10 pM of Ucn 2 was used in the following experiments. Secretion of LH and FSH was significantly suppressed by 2 h, 4 h, and 8 h treatment with 10 pM Ucn 2 ($42.7 \pm 5.3\%$ for 2 h, $46.0 \pm 13.1\%$ for 4 h, and $51.5 \pm 16.0\%$ for 8 h for LH; and $33.3 \pm 8.3\%$ for 2 h, $26.8 \pm 9.3\%$ for 4 h and $54.0 \pm 7.7\%$ for 8 h for FSH respectively: $P < 0.05$, $n = 6$; Fig. 1B), although the mRNA expression of β -subunit of each gonadotropin was unchanged by 24 h treatment (Fig. 1C). Antisauvagine 30, a selective CRF-R2 antagonist, completely blocked Ucn 2-induced suppression of LH and FSH secretion (Fig. 1D).

Gonadotropin secretion and mRNA expression are increased by blocking Ucn 2 action in vitro and in vivo

To clarify the role of endogenous Ucn 2 in the regulation of expression and secretion of gonadotropins, we examined the *in vitro* effects of antisauvagine-30 and recombinant adenovirus expressing siRNA and *in vitro* and *in vivo* effects of anti-Ucn 2 rabbit IgG on the secretion and mRNA expression of gonadotropins. Antisauvagine-30 significantly increased LH

secretion in 4-h and 8-h incubation (1.3 ± 0.1 -fold and 1.3 ± 0.1 -fold respectively: $P < 0.05$, $n = 6$; Fig. 2A) and its β -subunit mRNA expression in 8 h and 24 h incubation (2.1 ± 0.3 -fold and 1.7 ± 0.1 -fold respectively: $P < 0.05$, $n = 6$; Fig. 2B). Antisauvagine-30 also significantly increased FSH secretion in 4 h and 8 h incubation (1.4 ± 0.1 -fold and 1.4 ± 0.1 -fold respectively: $P < 0.05$, $n = 6$; Fig. 2A) and its β -subunit mRNA expression in 8 h and 24 h incubation (1.8 ± 0.3 -fold and 1.8 ± 0.1 -fold respectively: $P < 0.05$, $n = 6$; Fig. 2B).

Specificities of siRNA against CRF-R1 and CRF-R2 were shown in Fig. 3. Recombinant adenovirus expressing siRNA against CRF-R2 significantly suppressed CRF-R2 mRNA expression by $79.5 \pm 6.0\%$ ($P < 0.05$, $n = 4$) and protein by $78.8 \pm 13.5\%$ ($P < 0.05$, $n = 4$; Fig. 3A and B) with significant suppression of CRF-R1 mRNA expression by $57.7 \pm 3.6\%$ ($P < 0.05$, $n = 4$) and no significant effect on CRF-R1 protein, while recombinant adenovirus expressing siRNA against CRF-R1 significantly decreased CRF-R1 mRNA by $67.9 \pm 8.0\%$ ($P < 0.05$, $n = 4$) and protein by $63.5 \pm 3.8\%$ ($P < 0.05$, $n = 4$) without significant effect on CRF-R2 mRNA or protein as shown in Fig. 3A and 3B. The

Figure 2 CRF-R2 antagonist increases gonadotropins secretion and their β -subunit mRNA expression *in vitro*. Anterior pituitary cells were treated with antisauvagine-30 (AS) at a concentration of 10 pM or vehicle (Co) for 4, 8, and 24 h. After incubation, culture media were collected and used for assays of LH and FSH (A), and cells were analyzed for their β -subunit mRNA expression using the Thermal Cycler Dice Real Time PCR System (B). Values are the mean $\pm$ S.E.M. There were four wells for each treatment. * $P < 0.05$, compared with control.

effects of siCRF-R1 and siCRF-R2 on CRF-induced change in POMC mRNA expression were studied (Fig. 3C). siRNA against CRF-R1 significantly suppressed CRF-induced expression of POMC mRNA, while siRNA against CRF-R2 did not affect the CRF-induced POMC mRNA expression. Recombinant adenovirus expressing siRNA against CRF-R2, but not CRF-R1, significantly increased LH and FSH secretion (1.3 ± 0.1 -fold and 1.4 ± 0.1 -fold respectively: $P < 0.05$, $n = 4$; Fig. 4A) and LH and FSH β -subunit mRNA expressions (2.3 ± 0.1 -fold and 1.4 ± 0.1 -fold respectively: $P < 0.05$, $n = 4$; Fig. 4B). Moreover, the suppression of CRF-R2 mRNA expression by siRNA blocked Ucn 2-induced suppression of LH and FSH secretion (Fig. 4C). These results indicate that recombinant adenovirus expressing siRNA against CRF-R1 or CRF-R2 was specifically active for the suppression of each CRF-R subtype.

The cross-reactivity of the anti-Ucn 2 antiserum used for the present study with rat Ucn 2 is 83.3% at ED50% in RIA (Yamauchi *et al.* 2005). As shown in Fig. 5, anti-Ucn 2 IgG at a concentration of 10 μ g/ml was able to detect 100 pg of rat Ucn 2 and the binding was completely abolished by preincubation of anti-Ucn 2 IgG with 10 μ g rat Ucn 2.

Anti-Ucn 2 IgG significantly increased FSH secretion (1.4 ± 0.1 -fold: $P < 0.05$, $n = 6$) and its β -subunit mRNA expression with 24-h incubation compared with those of normal rabbit IgG treatment (2.3 ± 0.1 -fold: $P < 0.05$, $n = 6$; Fig. 6). Anti-Ucn 2 IgG also significantly increased LH β -subunit mRNA expression in 8-h and 24-h incubation compared with that of normal rabbit IgG treatment (2.9 ± 0.5 -fold for 8 h, and 3.1 ± 0.3 -fold for 24 h respectively: $P < 0.05$, $n = 6$; Fig. 6). The concentrations of LH in culture media could not be measured by rat LH [125 I] Biotrak assay system because of interference by rabbit IgG in the assay system.

A single i.p. injection of anti-Ucn 2 IgG into 3-week-old male rats significantly increased plasma LH (1.2 ± 0.1 -fold for 150 μ g IgG: $P < 0.05$, $n = 9$) and plasma FSH levels (1.3 ± 0.1 -fold for 50 μ g and 1.4 ± 0.0 -fold for 150 μ g respectively: $P < 0.05$, $n = 9$), 24 h after the injection compared with those of normal rabbit IgG-injected rats (Fig. 7A and B). Anti-Ucn 2 IgG at a dose of 150 μ g also significantly increased β -subunit mRNA expression of LH and FSH 24 h after the injection compared with normal rabbit IgG-injected rats (1.8 ± 0.1 -fold for LH and 2.4 ± 0.1 -fold for FSH: $P < 0.05$, $n = 9$; Fig. 7C and D).

Figure 3 Specificity of siRNA against CRF-R1 and CRF-R2. Adenovirus expressing siRNA against CRF-R1 or CRF-R2 were added to culture media of rat anterior pituitary cells at 10^6 MOI. After 72 h incubation, cells were analyzed for CRF-R1 and CRF-R2 mRNA and their proteins (A and B). There were four wells for each treatment. Effects of CRF-R1 or CRF-R2 suppression on CRF-induced POMC mRNA expression were examined (C). Adenovirus expressing siRNA against CRF-R1 or CRF-R2 were added to culture media of anterior pituitary cells at 10^6 MOI. After 72 h incubation, cells were treated with 1 nM CRF for 4 h. There were six wells for each treatment.

Figure 4 Adenovirus expressing siRNA against CRF-R2 increases gonadotropins secretion and their β -subunit mRNA expression *in vitro*. Adenovirus expressing siRNA against CRF-R1 and CRF-R2 was added to culture media of anterior pituitary cells at 10^6 MOI. After 72 h, basal LH and FSH secretion in anterior pituitary cells infected by adenovirus expressing siRNA against CRF-R1 or CRF-R2 for 4 h were measured (A). Total RNA was extracted from cells and analyzed for gonadotropin β -subunit mRNA expression (B). After 4-h treatment with Ucn 2 at concentrations of 1, 10, and 100 pM, culture media were collected and assayed for LH and FSH (C). Values are the mean $\pm$ S.E.M. There were six wells for each treatment. * $P < 0.05$, compared with control siRNA expressing adenovirus (siEGFP).

Anti-Ucn 2 IgG induced a tendency toward earlier occurrence of menarche in female rats

A tendency toward earlier occurrence of menarche was found in anti-Ucn 2 IgG-injected female rats compared with that of normal rabbit IgG-injected rats (41.7 ± 0.5 days after birth versus 43.3 ± 0.8 days after birth: $P = 0.0973$, Mann-Whitney $U = 28.00$, $n = 10$). Seventy percent of anti-Ucn 2 IgG-injected female rats (7 out of 10) exhibited menarche by day 42 while 40% of normal rabbit IgG-injected rats (4 out of 10) did by that day (Fig. 8C), although their body weight and pituitary, ovarian, and uterine weights as ratios of body weight, and time of vaginal opening and estrous cycle did not differ (Fig. 8A–C). Daily i.p. injection of anti-Ucn 2 IgG showed a tendency toward an increase in plasma FSH ($P = 0.0625$), although plasma LH was unchanged. This treatment slightly increased LH and FSH β -subunit mRNA expression, although the change was not statistically significant (Fig. 8D).

Figure 5 Immunoneutralization of anti-Ucn 2 IgG with rat Ucn 2. Rat Ucn 2 blotted at doses of 100 ng, 10 ng, 1 ng, and 100 pg was detected by anti-Ucn 2 IgG at a concentration of 10 μ g/ml. The antibody against mouse Ucn 2 was able to detect 100 pg rat Ucn 2. The signals were abolished by 30-minute preincubation of anti-Ucn 2 IgG with rat Ucn 2 at a dose of 10 μ g.

Figure 6 Anti-Ucn 2 IgG increases gonadotropin secretion and their β -subunit mRNA expression *in vitro*. Anterior pituitary cells were treated with 10 μ g/ml of anti-Ucn 2 IgG (Anti-Ucn 2) or normal rabbit IgG (Cont IgG) for 4, 8, and 24 h. After incubation, culture media were collected and assayed for FSH and cells were analyzed for gonadotropin β -subunit mRNA expression. Values are the mean $\pm$ S.E.M. There were six wells for each treatment. * $P < 0.05$, compared with normal rabbit IgG injection.

Discussion

We previously reported that Ucn 2 is biosynthesized by corticotrophs of rat anterior pituitary, and that the mRNA expression level and secretion of Ucn 2 in corticotrophs are positively and negatively regulated by CRF and glucocorticoids respectively (Nemoto *et al.* 2007). Although CRF-R2, which is bound by Ucn 2, is reported to be expressed mainly on gonadotrophs in rat anterior pituitary (Kageyama *et al.* 2003), it has been unclear whether Ucn 2 modulates the expression and secretion of gonadotropins. The present study has shown that Ucn 2 suppresses the secretion of gonadotropins *in vitro*, and that its action is blocked by antisauvagine-30, a CRF-R2 selective antagonist. These results indicate that Ucn 2 inhibits gonadotropins secretion through CRF-R2.

Our findings also showed that adenovirus expressing siRNA against CRF-R2 and a CRF-R2 antagonist each significantly increased LH and FSH secretion and their β -subunit mRNA expression *in vitro*. Furthermore, *in vitro* and *in vivo* passive immunization experiments using specific anti-Ucn 2 IgG revealed increases in the secretion of gonadotropins and their β -subunit mRNA expression. Our previous study showed that concentrations of Ucn 2 in the culture media of rat anterior pituitary cells are extremely low, at only a few percent of those of ACTH (Nemoto *et al.* 2007). Together with these results, the present findings thus suggest that pituitary Ucn 2 tonically inhibits the expression and secretion of gonadotropins through CRF-R2 in a paracrine fashion.

The dose-response curve found in the present study was U-shaped, and the effective doses for Ucn 2 to inhibit gonadotropins secretion were 3–30 pM. The reported average EC₅₀ value for CRF-R2 activation of Ucn 2 is 0.14 (0.03–0.52) nM (Reyes *et al.* 2001). The difference in the effective doses of Ucn 2 between the studies may be induced by the cells and the marker of cell response used for each study, normal rat anterior pituitary cells and gonadotropins for our study, and Chinese hamster ovary cells and cAMP for the latter study.

Ucn 2 inhibited the secretion of gonadotropins without changing the mRNA expression level of gonadotropins, while the reduction in CRF-R2 by siCRF-R2 and the blockade of endogenous Ucn 2 action by antisauvagine-30 or anti-Ucn 2 IgG increased both the secretion and the mRNA expression of gonadotropins. These findings may suggest that the expression level of gonadotropin mRNA is already suppressed by endogenous Ucn 2 secreted by POMC cells, and that the exogenous Ucn 2 could not induce further inhibition of the gene expression. By contrast, since the basal secretion of gonadotropins gradually increased during the incubation as shown in Fig. 1B, the inhibitory effect of endogenous Ucn 2 seems to be weak on the secretion of gonadotropins. Furthermore, the intracellular signals that participate in the regulatory mechanisms of secretion of gonadotropins and their gene expression may be different,

Figure 7 Anti-Ucn 2 IgG increases secretion by gonadotropins and their β -subunit mRNA expression *in vivo*. Twenty-one-day-old male rats were injected i.p. with anti-Ucn 2 IgG (Anti-Ucn 2; 50 or 150 μ g) or normal rabbit IgG (Cont IgG; 50 or 150 μ g). After 8 and 24 h, rats were killed by decapitation and trunk blood was collected for plasma LH (A) and FSH (B) assays. Total RNA was extracted from anterior pituitary and used for studies of LH (B) and FSH (C) β -subunit mRNA expression levels using the Thermal Cycler Dice Real Time PCR System. Values are the mean $\pm$ S.E.M. There were six rats for each treatment. * $P < 0.05$, compared with normal rabbit IgG injection.

thus causing the different changes in the secretion and gene expression of gonadotropins in response to Ucn 2.

Menarche is one of the main manifestations of puberty. Daily injection of anti-Ucn 2 IgG into immature female rats starting at the age of 21 days induced a tendency toward earlier occurrence of menarche compared with normal rabbit IgG-injected rats. Prior to puberty, circulating levels of gonadotropins are very low, due to the suppression of GnRH release from the hypothalamus via two possible mechanisms: a central inhibitory mechanism that has not yet been specified and a peripheral mechanism that is mediated by the negative feedback effect of gonadal steroids (Wennink *et al.* 1990, Ebbling 2005). As puberty draws near, the pulsatility of release of the two gonadotropins is increased, and with the onset of puberty the pulsatile release of them is amplified. These changes in gonadotropin secretion reflect increased pulsatile secretion of GnRH, and the increases in circulating gonadotropin levels stimulate follicle maturation and estrogen synthesis in the ovaries. It has been hypothesized that before puberty, the hypothalamus is extremely sensitive to estrogen,

the levels of which are very low in blood (Mauras *et al.* 1996, Ebbling 2005). In addition to the high sensitivity of the hypothalamus to estrogen, suppression of the secretion of gonadotropins from gonadotrophs also occurs before puberty (Foster *et al.* 2006). The findings of the present study suggest that pituitary Ucn 2 may play some role in the suppression of gonadotropin secretion before puberty. Since Ucn 2 is secreted by corticotrophs that secrete ACTH in response to stress, Ucn 2 may be involved in stress-induced gonadal dysfunction.

Anti-Ucn 2 IgG increased LH β -subunit mRNA expression after 8 h or 24 h period of incubation in cultured anterior pituitary cells, while it increased FSH β -subunit mRNA expression only after 24 h period of incubation as shown in Fig. 6. Plasma FSH was increased by both 50 and 150 μ g of anti-Ucn 2 IgG injection, while plasma LH and pituitary LH and FSH β -subunit mRNA expressions were increased only by 150 μ g of anti-Ucn 2 IgG injection as shown in Fig. 7. The findings presented indicate that the gene expression and the secretion of LH and those of FSH are

Figure 8 Repeated i.p. injection of anti-Ucn 2 IgG into immature female rats shows earlier occurrence of menarche. Twenty-one-day-old female rats were injected i.p. with anti-Ucn 2 IgG (Anti-Ucn 2; 100 µg/twice a day) or normal rabbit IgG (Cont IgG; 100 µg/twice per day). Body weight (A) and vaginal impedance (C) were measured at 0900 and 1330 h every day respectively (A). Metestrus rats were killed by decapitation, and the pituitary, ovaries, and uterus were removed and weighed (B). Plasma samples were separated for assays of LH and FSH (D). Total RNA was extracted from anterior pituitary and assayed for levels of Ucn2 and LH and FSH β-subunit mRNA expressions using the Thermal Cycler Dice Real Time PCR System (D). Values are the mean ± S.E.M. There were ten rats for each treatment. **P* < 0.05, compared with normal rabbit IgG injection.

differentially regulated by Ucn 2. In addition, as shown in Fig. 8, a tendency toward an increase in plasma FSH (*P* = 0.0625) was found in female rats injected daily with anti-Ucn 2 IgG, while plasma LH was unchanged by the same treatment. These results are presumably due to the different responsiveness of each gonadotropin to GnRH and the complex mechanisms of regulation of mRNA transcription of gonadotropins (Shupnik 1996). It is generally accepted that the mechanisms of regulation of LH and FSH secretion and their β-subunit mRNA expression are complex, and that differences in mechanisms of regulation between the two gonadotropins may have been responsible for the differences in responses to anti-Ucn 2 IgG observed in the present study (MacNeilly *et al.* 2003, Winters & Moore 2007).

A recent study has found that earlier occurrence of menarche is associated with increases in body mass index, elevation of plasma insulin levels, and elevation of blood pressure in humans (Kaplowitz 2008). Although it has been

reported that Ucn 2 reduces glucose utilization and insulin sensitivity (Chen *et al.* 2006b), we found no significant change in serum insulin or plasma glucose levels in the anti-Ucn 2 IgG-injected rats (data not shown). Our findings therefore suggest that neither hyperinsulinemia nor hyperglycemia is related to the tendency toward earlier occurrence of menarche in anti-Ucn 2 IgG-treated rats.

In conclusion, the findings of our both *in vitro* and *in vivo* experiments suggest that Ucn 2 secreted by POMC cells acts on gonadotrophs through CRF-R2 in a paracrine fashion to tonically inhibit the expression and secretion of gonadotropins.

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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