Estrogens downregulate urocortin 2 expression in rat uterus

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Abstract

Urocortin 2 (Ucn2) is a member of the corticotropin-releasing factor peptide family and is expressed by various tissues, including reproductive tissues such as the uterus, ovary, and placenta. However, the regulatory mechanisms of Ucn2 expression and the physiological significance of Ucn2 in these tissues remain unclear. We previously showed that passive immunization of immature female rats by i.p. injection of anti-Ucn2 IgG induces earlier onset of puberty. Therefore, this study was designed to clarify the site and regulatory mechanisms of *Ucn2* expression in the uterus. Expression levels of *Ucn2* mRNA in the uterus were higher in immature (2- and 4-week-old) and aged (17-month-old) rats than in mature (9-week-old) rats in the proestrus phase. In 9-week-old rats, mRNA expression levels and contents in the uterus were lower in the proestrus phase than in the diestrus phase, while plasma Ucn2 concentrations did not differ between the two phases. Ucn2-like immunoreactivitiy was detected in the endometrial gland epithelial cells of the uterus. S.c. injection of estradiol benzoate or an estrogen receptor α (ERα) agonist significantly reduced mRNA expression levels and contents of Ucn2 in the uterus when compared with vehicle-injected ovariectomized rats. By contrast, estradiol benzoate increased *Ucn2* mRNA expression levels in the lung. Thus, estrogens downregulate Ucn2 expression in the uterus in a tissue-specific manner, and Ucn2 may play a role in the regulatory mechanisms of maturation of the uterus through ERα and estrous cycle.

Key Words

- estrogen
- urocortin 2
- uterus
- estrous cycle

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Introduction

Corticotropin-releasing factor (CRF) has a variety of physiological roles in the endocrine system, autonomic nervous system, mood, behavior, and gastrointestinal system under stress (Bale & Vale 2004, Heinrichs & Koob 2004, Smith & Vale 2006). Urocortin 2 (Ucn2) is a member of the CRF peptide family, which acts through binding to CRF type 2 receptor (CRF-R2). Others and we previously demonstrated that Ucn2 is expressed in both the brain and peripheral tissues, including the lung, adrenal, uterus, ovary, and testis (Hsu & Hsueh 2001, Yamauchi *et al.* 2005, Lee *et al.* 2011). It has been reported that expression of *Ucn2* mRNA in various tissues is controlled by stress, inflammation, and hypoxia (Tanaka *et al.* 2003, Tao *et al.* 2006, Chang *et al.* 2007, Buhler *et al.* 2009, Imperatore *et al.* 2010, Nemoto *et al.* 2010) and that factors involved in the regulation of *Ucn2* mRNA expression are CRF, glucocorticoids, and endotoxins (Chen *et al.* 2003, 2004, Tanaka *et al.* 2003, Chang *et al.* 2007, Nemoto *et al.* 2007, Yuan *et al.* 2012). *Ucn2* mRNA is strongly expressed in the uterus during the proliferative phase in healthy women, and the expression is abolished in patients with endometriosis (Novembri *et al.* 2011). However, the regulatory

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mechanisms of Ucn2 expression in reproductive organs are not well understood.

Estrogens regulate many physiological processes in both the reproductive system and the cardiovascular systems, metabolism, behavior, and mood. In the uterus, estrogens trigger the precisely timed physiological and biochemical responses required for establishing the menstrual cycle and maintaining pregnancy. The effects of estrogens are mediated by receptors in large part through the regulation of gene transcription, and estrogen receptors (ERs) are involved in either activating or repressing transcription of target genes. It has not been studied whether and how the expression of Ucn2 in the uterus is influenced by estrogens. In this study, we therefore studied the influences of age, estrous phase, ovariectomy (OVX), and estrogen administration on *Ucn2* mRNA expression levels and contents in the uterus, as well as plasma Ucn2 concentrations, of female rats.

Materials and methods

Animals

Female Wistar rats were maintained at 23±2 °C on a 12 h light:12 h darkness cycle (lights on at 0800 h, off at 2000 h). They were allowed *ad libitum* access to laboratory chow and distilled water. Estrous cycle of rats was determined by vaginal impedance measurements using an MK-10C (Muromachi Kikai Co. Ltd, Tokyo, Japan) and vaginal cytological findings and was confirmed by measuring plasma estrogen concentrations. All experimental procedures were reviewed and approved by the Laboratory Animals Ethics Committee of Nippon Medical School.

Ovariectomized and estrogen administration

Forty-two female rats underwent bilateral OVX through a dorsal approach or sham surgery under sodium pentobarbital anesthesia (5 mg/kg body weight, i.p.). After surgery, all rats were housed in their home cages and allowed a week of recovery. For estrogen replacement, 2 μg estradiol benzoate (Sigma Chem. Co.) dissolved in 0.5 ml sesame oil were injected s.c. into OVX rats at 0930 h. An ERα-specific agonist propylpyrazoletriol (PPT; Tocris Bioscience, Minneapolis, MN, USA) and an ERβ agonist diarylpropionitrile (DPN; Tocris Bioscience) were dissolved in dimethyl sulfoxide (DMSO), and 2.5 mg/kg body weight of PPT or DPN was injected s.c. into OVX rats at 0930 h. The dose of estradiol benzoate elicits maximum plasma estradiol concentrations that appear to be in the proestrus range (Kuriyama & Shibasaki 2004), and doses of PPT and DPN were determined based on previous studies (Asl *et al*. 2013).

Plasma estradiol assay

Rats were decapitated and trunk blood was collected into tubes containing EDTA 2Na (1 mg/ml blood) followed by centrifugation at 3000 ***g*** for 20 min at 4 °C. Blood samples from two 2-week-old rats were pooled for assays because the amount of blood obtained from one 2-week-old animal was small. A 1 ml aliquot of plasma was transferred into 1.5 ml Eppendorf tubes and stored at −80 °C for later measurement. Plasma 17β-estradiol and Ucn2 concentrations were measured using an estradiol EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Tissue and plasma Ucn2 assay

Fifteen of 2-week-old, ten of 4-week-old, 32 of 9-week-old, and five of 17-month-old rats were used. Two to five uteri from each experimental group were pooled and analyzed as one sample because contents of Ucn2 were too small for analysis. Uteri were weighed, homogenized in 15-fold extract buffer solution (PBS contained 0.2% Nonidet P-40), and centrifuged at 10 000 ***g*** for 20 min at 4 °C. One milliliter of each plasma was mixed with 2 μl Nonidet P-40 and centrifuged at 10 000 ***g*** for 20 min at 4 °C. Supernatants of tissue or plasma samples were moved to an HLB 3cc Extraction Cartridge (Waters, Milford, MA, USA), pretreated with 6 ml ethanol, followed by rinsing with 6 ml distilled water. Columns were washed with 6 ml distilled water, and Ucn2 was eluted using 2 ml acetonitrile containing 0.075% TFA solution. Eluates were evaporated to dryness using a centrifugal evaporator. Samples were dissolved in 200 μl buffered solution (provided from Rat Urocortin 2 EIA kit, Yanaihara Institute, Inc., Shizuoka, Japan). Tissue Ucn2 contents and plasma Ucn2 concentrations were assayed using a Rat Ucn2 EIA kit (Yanaihara Institute, Inc.) in accordance with the manufacturers' protocol and data were corrected by uterine weight or plasma volume respectively.

RNA extraction and real-time RT-PCR analysis

Rats were decapitated, their uteri were removed, and total RNA was extracted using Isogen II (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized using 0.5 μg denatured total RNA at 37 °C for 15 min, 84 °C for 5 s, and

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4 °C for 5 min using a PrimeScript RT reagent kit (Takara, Shiga, Japan). 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 Ucn2 (GenBank number: NM_13385, forward primer: GAGCAACTCTAAAGCCAGCCCTTAC and reverse primer: TGATTCCTGGCAGCCTTGTTC) and GAPDH (GenBank number: NM_017008, forward primer: GGCACAGTCAAGGCTGAGAATG and reverse primer: ATGGTGGTGAAGACGCCAGTA). To normalize each sample for RNA content, GAPDH, a housekeeping gene, was used. Diluted uterine cDNA and the second-derivative method (Nolan *et al.* 2006) were used to calculate *Ct* values respectively.

Immunohistochemistry

Female Wistar rats were anesthetized with pentobarbital (50 mg/kg body weight by i.p. injection) and perfused via an intracardiac cannula with PBS followed by 4% paraformaldehyde. The uterus was removed, left overnight in 4% paraformaldehyde, and was then transferred to 20% sucrose/PBS. Tissues were sectioned at 10 μm using a cryostat, collected onto poly L-lysine-coated slides, and air-dried.

Sections were incubated with the specific polyclonal antiserum against Ucn2 (1:1000) (Yamauchi *et al.* 2005) for overnight at 4 °C. Tissues were rinsed in PBS and incubated in biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. This was followed by another 1-h incubation in avidin–biotin complex solution (Vectorstain ABC Elite kit, Vector Laboratories) at room temperature. The antibody–peroxidase complex was visualized using diaminobenzidine (Vector DAB kit; Vector Laboratories). When staining had reached appropriate intensity, tissue was rinsed in PBS and then tissue was counterstained with methyl green. After rinsed with pure water, tissue was dehydrated through a graded alcohol series, cleared in xylene, and coverslipped with VectaMount (Vector Laboratories). To test the specificity of staining, anti-Ucn2 serum preincubated with 10 μg rat Ucn2 was used in the experiments. These treatments did not affect the intensity of staining. Preincubation of anti-Ucn2 serum with 10 μg rat Ucn2 completely abolished the staining.

Western blotting of CRF-R2

The uterine tissues were lysed with TNE buffer (10 mM Tris–HCl, pH 7.8, 1% NP-40, 150 mM NaCl, and 1 mM EDTA) containing the Complete proteinase inhibitor cocktail (Roche Diagnostics). Lysates were subjected to centrifugation to remove debris and the protein

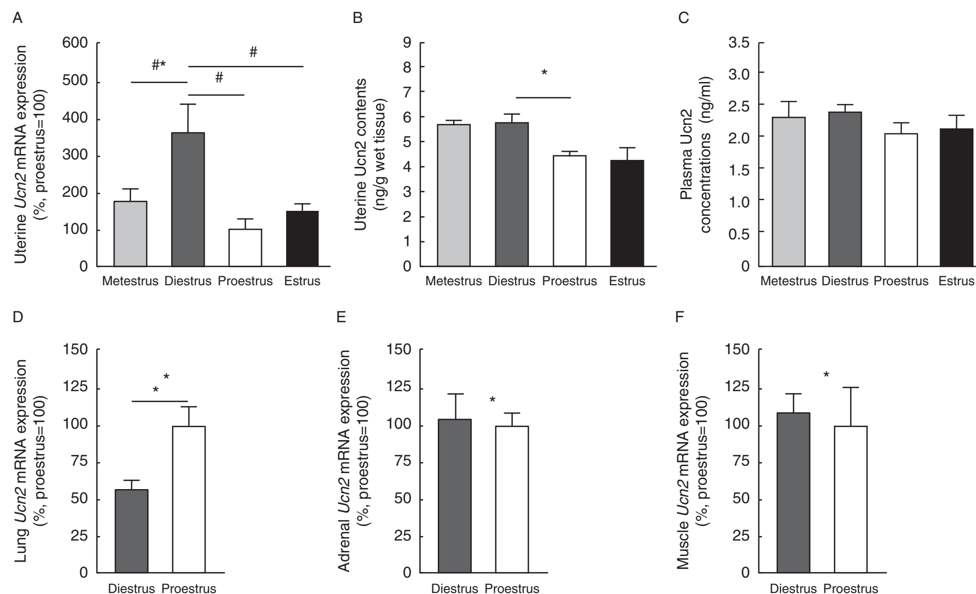

Figure 1

Ucn2 mRNA expression levels and contents during estrous cycle of 9-week-old female rats. Uterine (A), lung (D), adrenal (E) and muscle (F) *Ucn2* mRNA, peptide contents (B), and plasma concentrations (C) were assayed during the estrous cycle of 9-week-old female rats. mRNA expression levels are shown as a percentage of those in rats in the proestrus phase. Values are expressed as the means±S.E.M. *P* values of <0.05 were considered to be significant. $^{\#}P<0.01$ vs diestrus, $^{*}P<0.05$ vs proestrus. $n=8$.

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concentrations in each supernatant were measured. Each sample of protein extract (20 μg) was mixed with 3× SDS sample buffer (200 mM Tris–HCl, pH 6.8, 30% glycerol, 6% SDS, 0.03% bromophenol blue, and 3% β-mercapto-ethanol); each mixture was then boiled for 5 min. Proteins in each sample were then separated by SDS–PAGE and transferred onto PVDF membranes. The membranes were blocked with 10% nonfat dried milk and incubated with goat anti-CRF-R2 antibody (N-20, sc-1826, Santa Cruz Biotechnologies) (1:200). The membranes were washed and then incubated with HRP-conjugated anti-goat IgG (Jackson Immuno Research Laboratory, West Grove, PA, USA). ChemiDoc XRS (Bio-Rad Laboratories, Inc.) and SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA) were used to detect and measure CRF-R2 immunoreactive signal. After detecting CRF-R2 signal, each membrane was incubated in restore PLUS western blot stripping buffer (Thermo Scientific) to remove the antibodies. The membrane was reprimed with mouse anti-β-actin, MAB (Progen Biotechnik, Heidelberg, Germany), and then incubated with HRP-conjugated anti-mouse-IgG (Jackson Immuno Research Laboratory). The β-actin immunoreactive signals were detected and measured to normalize for equal loading and blotting efficiency.

Statistical analysis

Statistical analysis was performed using two-way ANOVA and an unpaired *t*-test using Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). For real-time RT-PCR data, all results were expressed as a percentage of control levels. Statistical significance was established at the $P<0.05$ level.

Results

Comparison of mRNA expression levels and contents of Ucn2 in each estrous phase

Plasma estrogen concentrations in the metestrus, diestrus, proestrus, and estrus phases were 13.4±1.1, 19.3±2.6, 29.9±2.8, and 13.8±1.1 pg/ml (mean±S.E.M., $n=6$) respectively and those in the proestrus phase were significantly higher than those in the other three phases ($P<0.0001$ vs metestrus, $P<0.01$ vs diestrus, and $P<0.0001$ vs estrus). Expression level of *Ucn2* mRNA in the uterus of proestrus phase was the lowest among other phases. The expression levels of *Ucn2* mRNA were significantly higher in the diestrus than in the proestrus phase (3.6±0.8-fold of proestrus, $n=8$, $P<0.01$) in the uterus (Fig. 1A). Ucn2 contents in the uterus in the proestrus phases were significantly lower than those in the diestrus phase (4.4±0.2 ng/g wet tissue for proestrus and 5.7±0.4 ng/g wet tissue for diestrus, $n=8$, $P<0.05$) (Fig. 1B), while there were no significant differences in

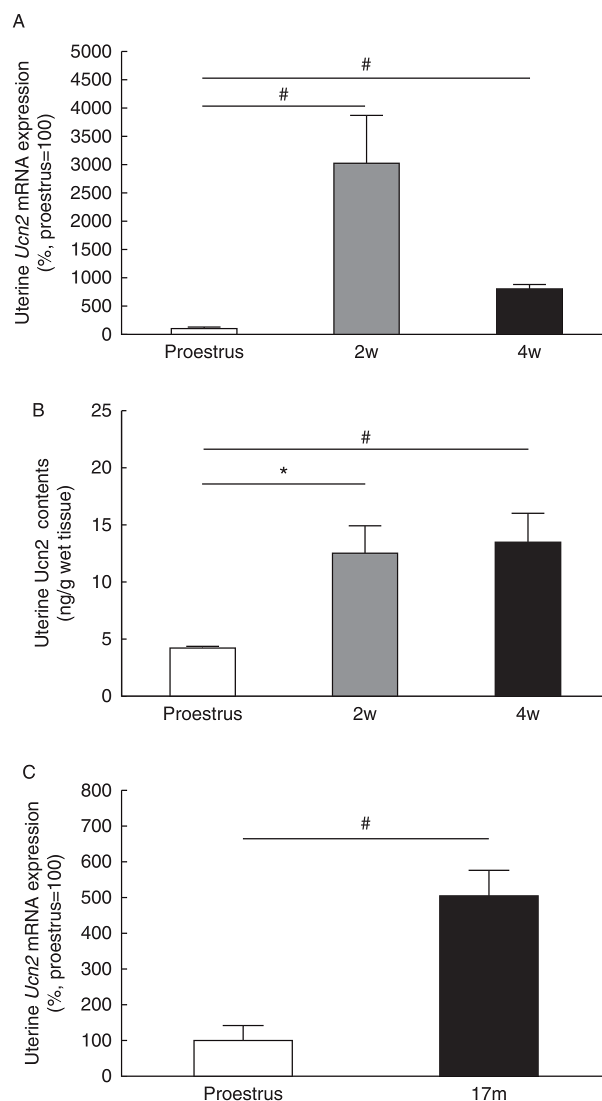

Figure 2
Ucn2 mRNA expression levels and contents in uterus of 2- and 4-week-old immature female rats, 9-week-old rats in the proestrus phase, and 17-month-old female rats. Uterine *Ucn2* mRNA expression (A and C) and contents (B) were assayed in 2- and 4-week-old immature female rats, 9-week-old female rats in the proestrus phase, and 17-month-old postmenopausal female rats. Uterus samples were pooled and extracted for Ucn2 EIA assay. mRNA expression levels are shown as a percentage of those in 9-week-old rats in the proestrus phase ($n=8$). Values are expressed as the means±S.E.M. *P* values of <0.05 were considered to be significant. [#]$P<0.001$ vs proestrus, *$P<0.01$ vs proestrus.

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plasma Ucn2 concentrations among each estrous phase (Fig. 1C). The expression levels of *Ucn2* mRNA in the lung were significantly higher in the proestrus phase than in the diestrus phase (1.8±0.3-fold of diestrus, $n=8$, $P<0.05$) (Fig. 1D).

Ucn2 mRNA and contents in uterus in 2-, 4- and 9-week and 17-month-old female rats

Plasma estrogen concentrations of 2- and 4-week-old immature female rats, 9-week-old mature female rats in the proestrus phase, and 17-month-old rats were 10.4±0.7, 9.8±1.1, 29.9±2.8, and 7.3±1.0 pg/ml respectively. The expression levels of *Ucn2* mRNA in the uterus were significantly higher in 2- and 4-week-old rats than in 9-week-old rats in the proestrus phase (30.2±8.5- and 8.0±8.3-fold of proestrus of 9-week-old rats respectively, $n=8$, $P<0.001$) (Fig. 2A). The contents of Ucn2 in the uterus were significantly higher in 2- and 4-week-old rats when compared with 9-week-old rats in the proestrus phase (12.5±2.4 ng/g wet tissue for 2-week-old rats ($P<0.01$), 13.5±2.5 ng/g wet tissue for 4-week-old rats ($P<0.001$), and 4.2±0.2 ng/g wet tissue for 9-week-old rats at proestrus phase) (Fig. 2B). Similarly, expression level of *Ucn2* mRNA in the uterus of 17-month-old rats was significantly higher than that in 9-week-old rats in the proestrus phase (5.0±0.7-fold 9-week-old rats at proestrus phase, $P<0.001$) (Fig. 2C).

Localization of Ucn2 in uterus

Ucn2-like immunoreactivity (LI) was detected in the endometrial gland epithelial cells of the uterus (Fig. 3A and B), although the signal densities in the diestrus phase did not differ from those in the proestrus phase. Ucn2-LI in the diestrus or proestrus phase was abolished by preincubation of anti-Ucn2 antibody with 10 μg Ucn2 (Fig. 3C and D).

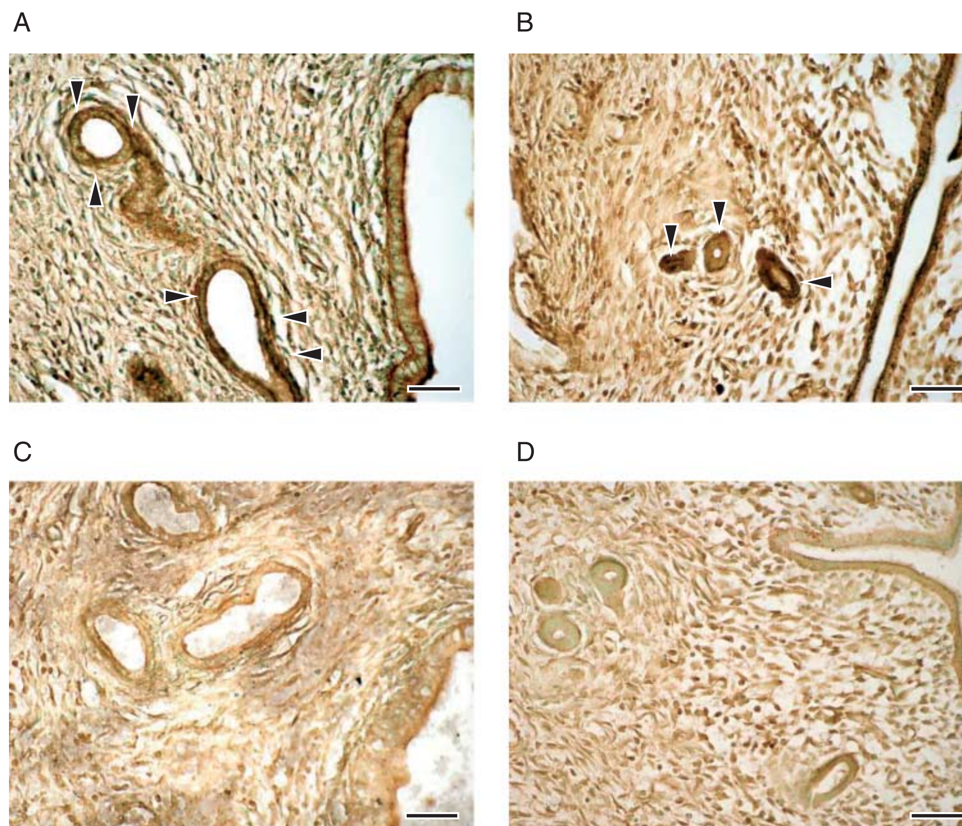

Figure 3
Localization of Ucn2 in the uterus of 9-week-old female rats at diestrus and proestrus phases. Ucn2-like immunoreactivity was observed in the endometrial gland endothelial cells in the proestrus (A) and diestrus (B) phases. Signals were abolished by preincubation of anti-Ucn2 antisera with 10 μg of rat Ucn2 in the diestrus (C) and proestrus (D) phases. Arrowheads indicate endometrium gland epithelial cells. Scale bar=50 μm.

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Administration of estradiol benzoate decreased mRNA and content of Ucn2 in uterus of OVX rats

Plasma estrogen concentrations were undetectable in both estradiol benzoate- and vehicle-administered OVX rats. Twenty-four and 48 h after s.c. administration of estradiol benzoate, expression levels of *Ucn2* mRNA (4.2±1.0% for 24 h and 4.6±1.2% for 48 h vs vehicle-administered control respectively, $n=6$, $P<0.01$, Fig. 4A) and Ucn2 contents (5.6±0.4 vs vehicle, 8.6±1.3 ng/g wet tissue for 24 h ($P<0.01$), 6.3±1.0 vs vehicle, 11.0±0.7 ng/g wet tissue for 48 h ($P<0.001$), $n=6$, Fig. 4B) were significantly lowered when compared with vehicle-administered controls in the uterus of OVX rats, while there were no significant differences in plasma Ucn2 concentrations between the groups at 24 and 48 h after sample administration (Fig. 4C). Twenty-four hours after s.c. administration of estradiol benzoate, the expression levels of *Ucn2* mRNA in the lung were significantly higher than those of vehicle-administered OVX rats (2.9±0.3-fold of vehicle-administered control, $n=6$, $P<0.001$, Fig. 4D) with no changes in *Ucn2* mRNA expression in the adrenal gland (Fig. 4E) or in the skeletal muscle (Fig. 4F).

ERα agonist decreased mRNA expression levels and peptide contents of Ucn2 in uterus of OVX rats

Twenty-four hours after s.c. administration of each ER agonist, plasma estrogen concentrations were undetectable in each ER agonist- and vehicle-administered OVX rats. An ERα agonist, PPT, significantly decreased *Ucn2* mRNA (69.7±7.9% of vehicle administration, $n=8$, $P<0.01$, Fig. 5A) and Ucn2 contents (9.4±0.6 ng/g wet tissue for PPT administration vs 17.9±1.1 ng/g wet tissue

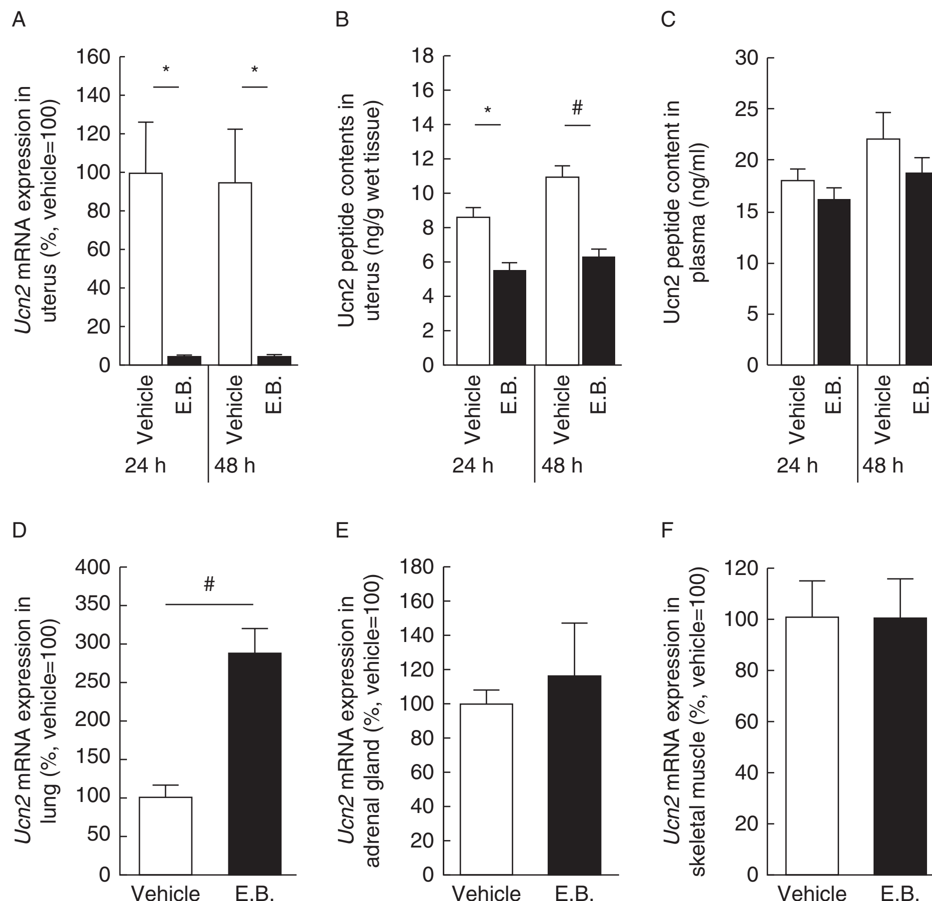

Figure 4
Effects of estrogen administration on uterine *Ucn2* mRNA expression levels and contents and plasma Ucn2 concentrations in OVX rats. Uterine *Ucn2* mRNA expression levels (A), contents (B), and plasma Ucn2 concentrations (C) were assayed at 24- and 48 h after s.c. administration of 2 μg estradiol benzoate or vehicle. *Ucn2* mRNA expression levels in the lung (D), adrenal gland (E) and muscle (F) were assayed at 24-h after s.c. administration of estradiol benzoate or vehicle. mRNA expression levels are shown as a percentage of those in vehicle-injected rats. EB, estradiol benzoate. Values are expressed as the means±S.E.M. *P* values of <0.05 were considered to be significant. *$P<0.01$ and #$P<0.001$ vs vehicle control. $n=6$.

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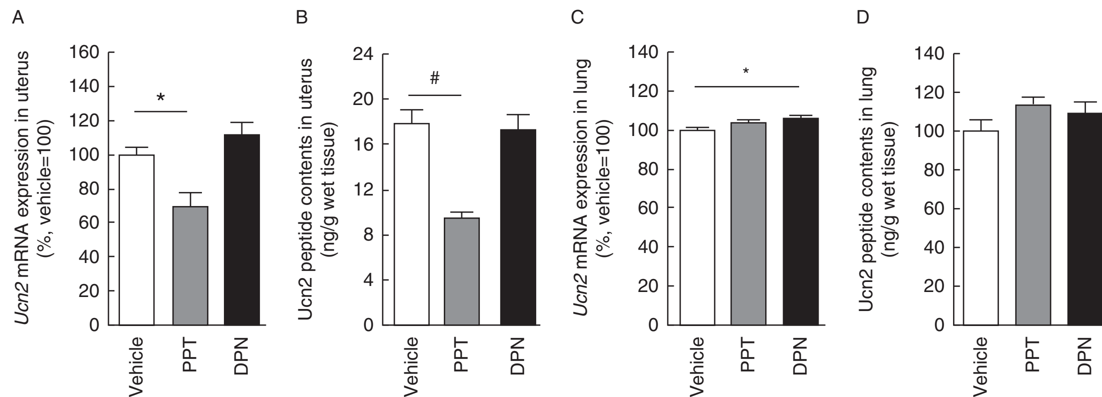

Figure 5
Effect of ERα or β agonist on *Ucn2* mRNA expression and tissue content of uterus and lung of OVX rats. mRNA expression levels (A for uterus and C for lung) and peptide contents (B for uterus and D for lung) of Ucn2 were assayed at 24 h after s.c. administration of 2.5 mg PPT, an ERα agonist, DPN, an ERβ agonist, or vehicle. mRNA expression levels are shown as a percentage of those in vehicle-injected rats. Values are expressed as the means $\pm$ S.E.M. *P* values of <0.05 were considered to be significant. *$P<0.01$ and #$P<0.001$ vs vehicle control. $n=6$.

for vehicle administration, $n=8$, $P<0.001$, Fig. 5B), while an ERβ agonist, DPN, did not show significant changes in the expression levels and peptide contents of Ucn2 in uterus of OVX rats (Fig. 5A and B). By contrast, DPN slightly but significantly increased *Ucn2* mRNA expression levels (1.06$\pm$0.01-fold of vehicle administration, $n=8$, $P<0.01$, Fig. 5C) without affecting in its peptide contents (Fig. 5D) in the lung, while PPT showed no significant changes in mRNA expression levels (Fig. 5C) or peptide contents of *Ucn2* (Fig. 5D) in the lung of OVX rats.

Expression levels of CRF-R2 mRNA and protein in uterus of proestrus rats, diestrus rats, and estradiol benzoate-administered OVX rats

Expressions of CRF-R2 mRNA and protein in the uterus of diestrus rats were significantly higher than those of proestrus rats (1.1$\pm$0.02-fold of proestrus for mRNA expression and 1.3$\pm$0.1-fold of proestrus for protein expression, $n=8$, $P<0.05$, Fig. 6A, B, and C). Estradiol benzoate administration in OVX rats did not change the expression of CRF-R2 mRNA or protein in the uterus (Fig. 6D, E, and F).

Discussion

We have previously shown that *Ucn2* mRNA is strongly expressed in reproductive tissues including the uterus (Yamauchi *et al*. 2005). In this study, Ucn2-LI was detected in endometrial gland epithelial cells of the uterus in rats. We also showed that *Ucn2* mRNA expression levels and contents in the uterus were higher in immature and aged female rats than in mature female rats in the proestrus phase; plasma estradiol concentrations in the two former groups were significantly lower than those in the latter group. *Ucn2* mRNA expression levels and contents in the uterus of mature rats were significantly higher in the diestrus phase than those in the proestrus phase, while plasma estrogen concentrations were significantly lower in the diestrus phase than in the proestrus phase. These results are consistent with a previous human study showing that endometrial *Ucn2* mRNA levels show peak in the early proliferative phase (Novembri *et al*. 2011). Furthermore, we demonstrated that estradiol benzoate or ERα agonist, but not ERβ agonist, significantly decreases *Ucn2* mRNA expression levels and contents in the uterus of OVX rats. Taken together, this suggests that Ucn2 is negatively regulated by estrogens through ERα.

In this study, we also found that estradiol benzoate administration increased *Ucn2* mRNA expression in the lung without changing in the skeletal muscle and adrenal gland, in contrast to the suppressive effects on Ucn2 expression in the uterus. Previous studies demonstrated that glucocorticoids alter *Ucn2* mRNA expression in various tissues in a tissue- or cell-specific manner (Chen *et al*. 2003, 2004). Thus, estrogen-induced modification of *Ucn2* mRNA expression also occurs in a tissue- or cell-specific manner. We were unable to perform Ucn2 promoter assay because the rat Ucn2 promoter has not been identified yet and we were unable to identify it.

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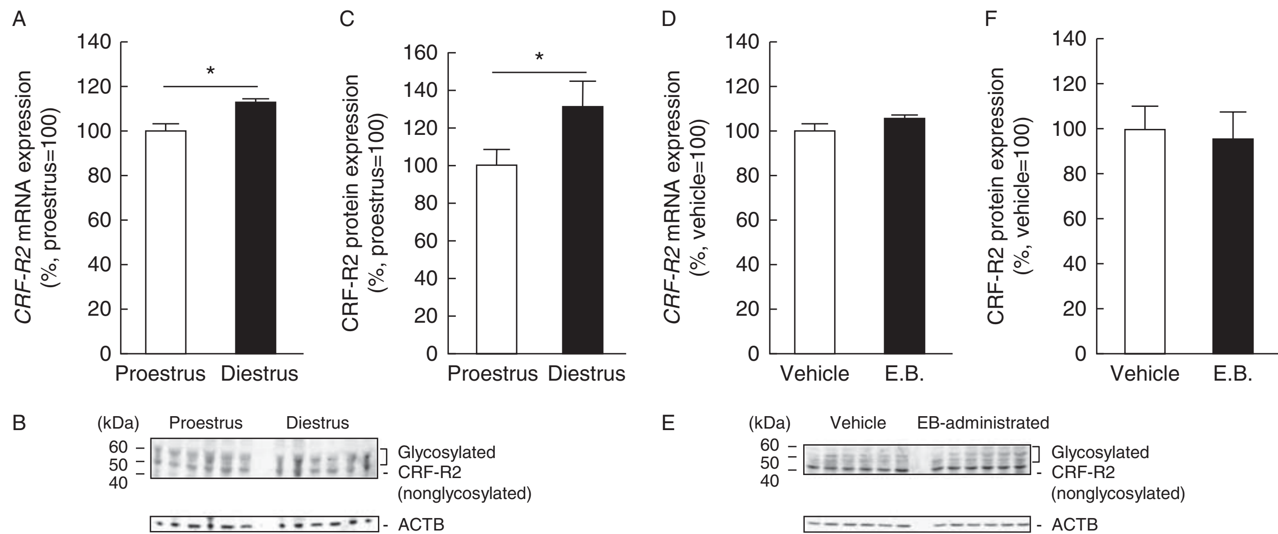

Figure 6
CRF-R2 mRNA and protein expression levels during estrous cycle of intact rats and after estrogen administration in OVX rats. mRNA (A and D) and protein (B, C, E, and F) expression of CRF-R2 in the uterus were assayed during the estrous cycle of 9-week-old female rats (A, B, and C), or 2 μg estradiol benzoate- or vehicle-administered OVX rats (D, E, and F). The mRNA and protein expressions of CRF-R2 are shown as a percentage of those in rats in the proestrus phase or in vehicle-administered rats. Values are expressed as the means ± S.E.M. *P* values of <0.05 were considered to be significant. **P*<0.05 vs proestrus or vehicle. $n=8$.

Database analysis suggests that there are several half palindrome estrogen-responsive elements in the 5′-site of the *Ucn2* gene. As there may be multiple mechanisms involved in estrogen-induced gene repression, further studies are needed to clarify the mechanisms underlying estrogen-induced repression of *Ucn2* mRNA expression. On the other hand, ERβ agonist-induced increase in *Ucn2* mRNA expression was very small in the lung of OVX rats, and no change in *Ucn2* mRNA expression was induced in the lung of OVX rats by ERα agonist. Therefore, the major mediator through which estrogens increase *Ucn2* mRNA expression in the lung is not ERβ.

Although we have previously shown that secretion of Ucn2 from the anterior pituitary is regulated by CRF and dexamethasone (Nemoto *et al*. 2007), the details of the regulatory mechanisms of Ucn2 secretion from peripheral tissues are unknown. The mRNA expression levels and contents of Ucn2 in uterus were decreased in estradiol benzoate-administered OVX rats, as shown in this study. However, this study also showed that Ucn2 contents in the uterus did not change in parallel with mRNA expression during estrous phase or during aging. It therefore seems likely that the regulatory mechanisms of Ucn2 secretion from the uterus are not necessarily estrogen dependent. Taken together, these results suggest that factors other than estrogens are involved in the mechanisms underlying Ucn2 secretion from the uterus in rats.

The role of Ucn2 secreted from the uterus remains unclear. It has been reported that Ucn2 and other members of CRF family peptides delay gastric emptying, and their expression is induced by lipopolysaccharide injection in rats (Czimmer *et al*. 2006, Yuan *et al*. 2012); Ucn2 inhibits the hypoxia-induced apoptosis in mesenteric arterial smooth muscle cells from spontaneously hypertensive rats (Tao *et al*. 2006), and i.v. injection of Ucn2 increases plasma angiotensin II and noradrenaline concentrations, cardiac output, heart rate and left ventricular ejection fraction, and renin activity in healthy humans (Davis *et al*. 2007). However, plasma Ucn2 concentrations are not significantly affected by age differences, estrous phase, or estrogen administration in female rats as shown in this study. We have previously demonstrated that the amount of Ucn2 secreted from rat anterior pituitary cells is ∼1/40 of that of ACTH (Nemoto *et al*. 2007) and that Ucn2 has a paracrine tonic and inhibitory effect on gonadotropin (Nemoto *et al*. 2009). We found that expressions of CRF-R2 mRNA and protein in the uterus of diestrus rats were higher than those of proestrus rats. However, estradiol benzoate treatment did not significantly change the expression of CRF-R2 mRNA and protein compared with that of vehicle treatment in OVX rats. These results therefore suggest that the expressions of CRF-R2 mRNA and protein in the uterus may be independent of estrogens. Further studies are

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necessary in order to clarify the regulatory mechanisms underlying CRF-R2 expression in the uterus. It has been reported that Ucn2-LI is present in human placenta, amnion, and chorion and that the concentrations of Ucn2 in placenta are 1/50 of those of CRF (Pepels *et al.* 2009). Moreover, a previous study using knockout mice showed that Ucn2 is a local negative regulator of glucose uptake in skeletal muscle (Chen *et al.* 2006). These results suggest that uterine Ucn2 plays a paracrine role. Furthermore, our previous study showed that daily passive immunization of immature female rats with an i.p. injection of anti-Ucn2 IgG induces earlier onset of puberty. It has been reported that estrogen release and P450 aromatase mRNA expression are enhanced in the presence of Ucn2 in cultured human trophoblast cells (Imperatore *et al.* 2009). Taken together with these results, this study suggests that the uterine Ucn2/CRF-R2 system may cross talk with the estrogen system and may have regulatory roles in the estrous cycle and/or reproductive events.

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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