Role of urocortin 2 secreted by the pituitary in the stress-induced suppression of luteinizing hormone secretion in rats

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Nemoto T, Iwasaki-Sekino A, Yamauchi N, Shibasaki T. Role of urocortin 2 secreted by the pituitary in the stress-induced suppression of luteinizing hormone secretion in rats. Am J Physiol Endocrinol Metab 299: E567–E575, 2010. First published July 27, 2010; doi:10.1152/ajpendo.00163.2010.—We have previously demonstrated that Ucn 2 is expressed in proopiomelanocortin (POMC) cells of the anterior pituitary and that its secretion and expression are increased by CRF in both the anterior and intermediate lobes and suppressed by glucocorticoids in the anterior lobe. We have also shown that Ucn 2 secreted by POMC cells acts on gonadotrophs expressing CRF type 2 receptors and inhibits the expression and secretion of gonadotropins. In the present study, we examined whether pituitary Ucn 2 is involved in stress-induced inhibition of gonadotropin secretion. A 90-min period of immobilization stress increased POMC mRNA expression without influencing Ucn 2 mRNA expression and suppressed luteinizing hormone (LH) β-subunit mRNA expression in the anterior lobe and plasma LH levels, while it increased both POMC and Ucn 2 mRNA expression in the intermediate lobe of the pituitary. Pretreatment with anti-CRF IgG blocked immobilization-induced increases in plasma ACTH and corticosterone and in POMC mRNA expression in both pituitary lobes and Ucn 2 mRNA expression in the intermediate pituitary. It also blocked immobilization-induced suppression of plasma LH and LH β-subunit mRNA expression. Pretreatment with anti-Ucn 2 IgG blocked immobilization-induced suppression of plasma LH and LH β-subunit expression without affecting immobilization-induced ACTH and corticosterone release and POMC or Ucn 2 mRNA expression. These results suggest that CRF suppresses the secretion and expression of LH probably through pituitary Ucn 2 in stress.

luteinizing hormone; corticotropin-releasing factor; pituitary

STRESS INHIBITS REPRODUCTIVE function (12, 13, 23). The hormones composing the hypothalamic-pituitary-adrenal (HPA) axis such as corticotropin-releasing factor (CRF), adrenocorticotropin (ACTH), β-endorphin, and corticosteroids reportedly play important roles in the suppressive influence of stress on reproductive function (41—43). CRF is a key stress mediator in the endocrine system, autonomic nervous system, emotion, and behavior (4, 19, 46). The various actions of CRF are mediated through two subtypes of CRF receptors (CRF-R), CRF-R1 and CRF-R2. CRF binds with a higher affinity to CRF-R1 than to CRF-R2 (4, 18). Urocortin 2 (Ucn 2) is a CRF peptide family and shows higher affinities to both CRF-R1 and CRF-R2 compared with CRF, in particular to CRF-R2 (40). Hypothalamic CRF inhibits gonadotropin-releasing hormone (GnRH) neuron activity either directly or indirectly through β-endorphin in the arcuate nucleus (42, 43). Intracerebroventricular infusion of CRF in the third ventricle inhibits the estrous cycle and ovulation and reduces immunoreactive GnRH stores in the median eminence (39). ACTH has been found to decrease the response of plasma luteinizing hormone (LH) to GnRH and inhibited the LH surge (38). Numerous GnRH neurons receive β-endorphinergic input, and β-endorphin suppresses the activity of GnRH neurons via the μ-opioid receptor (25, 47).

Furthermore, several studies show that the secretion of gonadotropins is suppressed by peripheral administration of glucocorticoids in various species (16, 48). Glucocorticoids are thought to act at the hypothalamus and/or anterior pituitary to inhibit the secretion of gonadotropins (16).

We have previously demonstrated that Ucn 2 is expressed in proopiomelanocortin (POMC) cells of the anterior and intermediate lobes of rat pituitary (50) and that the expression of Ucn 2 mRNA is increased by CRF in the anterior and intermediate lobes and decreased by glucocorticoids only in the anterior lobe (31). CRF-R2 is expressed on gonadotrophs (22). We have recently reported that Ucn 2 suppresses the secretion and expression of gonadotropins and that a selective CRF-R2 antagonist and small-interfering RNA (siRNA) against CRF-R2 significantly increase the secretion and expression of gonadotropins in vitro (32). We also have shown that anti-Ucn 2 IgG significantly increases the secretion and mRNA expression of gonadotropins both in vitro and in vivo and that intraperitoneal injection of anti-Ucn 2 IgG in immature female rats induces a tendency toward earlier occurrence of menarche (32). These findings suggest that pituitary Ucn 2 is involved in the regulatory mechanism of the expression and secretion of gonadotropins. Because the amount of Ucn 2 secreted in culture media is much lower than that of ACTH, it appears that Ucn 2 secreted by POMC cells acts on gonadotrophs in a paracrine manner.

It is unclear whether Ucn 2 plays some role in stress-induced suppression of gonadotropin secretion, although the secretion of Ucn 2 by POMC cells is stimulated by CRF. We therefore attempted to clarify, in this study, whether Ucn 2 is involved in the mechanism by which stress suppresses the expression and secretion of gonadotropins. For this purpose, we examined the secretion of ACTH, corticosterone, and LH and follicle-stimulating hormone (FSH) and the expression of POMC, Ucn 2, LH, and FSH β-subunit mRNA in the pituitary in immobilization-exposed rats and tested the effect of intraperitoneal administration of anti-CRF IgG or anti-Ucn 2 IgG on the secretion and expression of these hormones in these rats.

MATERIALS AND METHODS

Animals. All procedures involving rats in this study were reviewed and approved by the Laboratory Animals Ethics Review Committee of Nippon Medical School. Male Wistar rats (7 wk old) were maintained at 23 ± 2°C on a 12:12-h light-dark cycle (lights on at 0800,
off at 2000). They were allowed ad libitum access to laboratory chow and distilled water.

**Stress exposure.** For immobilization stress, rats were wrapped in a flexible wire mesh and immobilized for 30, 60, 90, or 120 min between 1300 and 1500 in an isolated room (3). Rats were killed in the adjacent room immediately after each period of immobilization; their trunk blood was collected to examine plasma ACTH, corticosterone, LH, and FSH levels; and their pituitary was divided into the anterior and posterior intermediate lobes for measurement of POMC, Ucn 2, LH, and FSH β-subunit mRNA expression. As controls, nonstressed rats were used, and they were killed 5–8 min before the decapitation of the rats exposed to 30 min of immobilization stress.

**Primary culture of pituitary cells.** Thirty male rats aged 6 wk were killed by decapitation, and their pituitary glands were removed under sterile conditions. The anterior pituitary lobes were collected and kept at 2000. They were allowed ad libitum access to laboratory chow and distilled water.

**Trunk blood was collected into tubes containing EDTA 2Na (1 mg/ml blood) and centrifuged at 3,000 rpm for 10 min at 4°C to remove debris, the culture media were frozen and kept at 80°C until used.** After 2, 4, and 8 h of incubation with CRF at concentrations ranging from 1 to 103 pM, the culture medium from each well was collected. After centrifugation at 3,500 rpm for 10 min at 4°C to remove debris, the culture media were frozen and kept at −80°C until used. After 2, 4, and 8 h of incubation with CRF at a concentration of 105 pM, cells were harvested, and total RNA was extracted using a CellAmp Direct RNA Prep Kit (Takara Bio, Shiga, Japan).

**Passive immunization.** We had previously generated antisera against rat CRF and mouse Ucn 2. An IgG fraction was purified from each antisera obtained after the fifth booster using a protein A sepharose column. The specificity of the anti-Ucn 2 antisera has been described in our previous reports (32, 50). The cross-reactivity of the anti-CRF antisera has been described in previous reports (10, 45), and the immunostaining results obtained using the anti-CRF antisera were not influenced by 1 or 10 μg of rat Ucn 1, Ucn 2, or Ucn 3 (data not shown). Rats were injected intraperitoneally with anti-Ucn 2 IgG, anti-CRF IgG, or normal rabbit serum (NRS) IgG (1 mg/kg body wt dissolved in 1 ml of normal saline). Later (2 h), they were exposed to immobilization stress for 90 min. Rats were then killed immediately, and their trunk blood and pituitaries were collected for the hormone assays described in the following sections.

**Plasma hormone assay.** Trunk blood was collected into tubes containing EDTA 2Na (1 mg/ml blood) and centrifuged at 3,000 rpm for 20 min at 4°C. A 1-ml aliquot of plasma was transferred to 1.5-ml Eppendorf tubes and stored at −80°C for later measurement. Plasma ACTH, corticosterone, LH, and FSH concentrations were measured using an ACTH ELISA (M046006, BD Bioscience, St. Paul, MN), a corticosterone EIA kit (500651; Cayman Chemical, Ann Arbor, MI), a rodent LH ELISA test (EKRR7010; Endocrine Technologies, Newark, CA), and a rat FSH ELISA (AER004; Biocode-Hycel, Liege, Belgium), respectively. According to the manufacturers’ literature supplied with the kits, the assay sensitivities were 5 pg/ml for ACTH ELISA, 16.4 pg/ml for corticosterone EIA, 0.5 ng/ml for LH ELISA, and 0.78 ng/ml for FSH ELISA.

**RIA for plasma Ucn 2.** For the extraction of Ucn 2, 0.5 ml of trunk plasma of nonstressed or stress-exposed rats diluted with 3 ml of 5% acetic acid was applied to an Oasis HLB 60-mg column (Waters, Milford, MA) that had been washed with 2 ml of methanol and 2 ml of distilled water. The column was washed with 2 ml of methanol, and the bound fraction was eluted with 2 ml of 0.1% trifluoroacetic acid containing 60% acetonitrile. The samples were then applied to an evaporator and a lyophilizer and then dissolved in RIA buffer [0.1 M PBS containing 1 mM EDTA, 0.01% NP-40 (Nacalai Tesque, Kyoto, Japan), and 0.02% NaN3, pH 7.4] for assay. Synthetic rat Ucn 2 (Yanaihara Institute, Shizuoka, Japan) was iodinated using the chloramine-T method and purified on a Sephadex G-50 column as previously described (50). Standard synthetic rat Ucn 2 or extracted plasma sample was incubated with anti-Ucn 2 antisera in 5-ml plastic tubes for 24 h at 4°C. 125I-labeled Ucn 2 was then added to each tube, and the reactions were incubated for another 24 h. Goat anti-rabbit γ-globulin was used to separate tracer bound to antiserum from free tracer. The anti-Ucn 2 serum was used for RIA at a final dilution of 1:350,000 to yield a maximum binding of −30%. The intra-assay and interassay coefficients of variation were 4.0 and 9.4%, respectively. The sensitivity of Ucn 2 RIA was 0.1 ng/ml.

**RNA extraction and real-time RT-PCR analysis.** Total RNA was extracted from rat pituitaries using RNAiso Plus (Takara Bio). First-strand cDNA was synthesized using 0.5 μg of denatured total RNA at 37°C for 15 min, 84°C for 5 s, and 4°C for 5 min using a PrimeScript RT reagent kit (Takara Bio). 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 Bio) and specific primers for rat Ucn 2, POMC, LH and FSH β-subunit, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). To normalize each sample for RNA content, GAPDH, a housekeeping gene, was used. Diluted normal rat pituitary cDNA and the second derivative method (33) were used as the standard and for calculating C values.

**Statistical analysis.** Statistical analysis was performed using two-way ANOVA and an unpaired t-test using Prism 5.0 software (GraphPad Software, La Jolla, CA). Pituitary cell culture using the same experimental protocol was performed two times. For real-time RT-PCR data, all results are expressed as percent of controls. Statistical significance was established at the <0.05 level.

### Table 1. Primer sequences of the studied genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Length, bp</th>
<th>Accession No. (GenBank)</th>
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<tr>
<td>POMC</td>
<td>CTCCTCATAGGCTGGTGAGGCTG</td>
<td>151</td>
<td>NM_139326</td>
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<tr>
<td>Ucn 2</td>
<td>AAGGGCTGTTCTCACTCCGTG</td>
<td>(180–330)</td>
<td>NM_13385.2</td>
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<tr>
<td>LH β</td>
<td>TGATTTGCTGAGAGCTTGTCG</td>
<td>112</td>
<td></td>
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<tr>
<td>FSH β</td>
<td>GGCACTGGAGCAAATCTGAG</td>
<td>(195–306)</td>
<td>NM_012858</td>
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<tr>
<td>GAPDH</td>
<td>GGCGAGTGGGACCTCCTGGA</td>
<td>167</td>
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POMC, proopiomelanocortin; Ucn 2, urocortin 2; LH, luteinizing hormone; FSH, follicle-stimulating hormone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
RESULTS

Effects of immobilization stress on pituitary hormone expression and plasma ACTH and corticosterone levels. Immobilization stress significantly increased plasma ACTH (4.41 ± 0.85-fold at 30 min and 2.42 ± 0.37-fold at 60 min, P < 0.05) and corticosterone (4.88 ± 1.07-fold at 30 min, 4.04 ± 0.66-fold at 60 min, and 2.26 ± 0.43-fold at 90 min, P < 0.05) levels compared with nonstressed controls (Fig. 1, A and B). It also significantly increased POMC mRNA expression (1.48 ± 0.03-fold at 60 min, 1.84 ± 0.19-fold at 90 min, and 1.37 ± 0.02-fold at 120 min, P < 0.05) in the anterior lobe of the pituitary, whereas it induced no significant change in Ucn 2 mRNA expression in the same lobe compared with nonstressed controls (Fig. 1, C and D). In contrast, immobilization stress significantly increased both POMC mRNA expression (1.70 ± 0.20-fold at 30 min, 1.88 ± 0.06-fold at 60 min, 2.20 ± 0.35-fold at 90 min, and 2.37 ± 0.10-fold at 120 min, P < 0.05) and Ucn 2 mRNA expression (2.71 ± 0.04-fold at 90 min and 1.84 ± 0.18-fold at 120 min, P < 0.05) in the intermediate lobe of the pituitary compared with that of nonstressed controls (Fig. 1, E and F). Immobilization stress significantly decreased plasma LH (46.7 ± 0.06% at 60 min, 58.3 ± 6.3% at 90 min, and 56.7 ± 3.1% at 120 min, P < 0.05) and pituitary LH β-subunit mRNA expression (25.0 ± 8.3% at 90 min and 75.0 ± 12.5% at 120 min, P < 0.05)

Fig. 1. Effects of 30-, 60-, 90- and 120-min immobilization stress on plasma adrenocorticotropin (ACTH), corticosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels and on proopiomelanocortin (POMC), urocortin 2 (Ucn 2), LH β-subunit, and FSH β-subunit mRNA expression in rats. Male rats (7 wk old) were exposed to immobilization stress for 30, 60, 90, or 120 min. Their trunk blood was assayed for ACTH (A), corticosterone (B), LH (G), and FSH (I). Pituitaries were divided into anterior (AP) and intermediate (IP) pituitary, and pituitary mRNA was extracted to assay mRNA expression of POMC (C for anterior pituitary and E for intermediate pituitary), Ucn 2 (D for anterior pituitary and F for intermediate pituitary), and gonadotropin β-subunit (H for LH and J for FSH). Each mRNA expression level is shown as %non-stressed controls. The nos. of rats in each experimental group were 10 for A and B and 5 for C–H. *P < 0.05 compared with the nonstressed controls (non).
CRF does not affect LH secretion and LH β-subunit mRNA expression in cultured anterior pituitary cells. Although incubation of monolayered anterior pituitary cells with CRF for 4 h significantly increased ACTH (1.52 ± 0.23-fold at 30 pM, 1.72 ± 0.25-fold at 10^3 pM, and 2.16 ± 0.32-fold at 10^3 pM, \( P < 0.05, n = 8 \)) (Fig. 3C) and POMC mRNA at a concentration of 10^3 pM (1.39 ± 0.29-fold for 4 h and 1.54 ± 0.17-fold for 8 h, \( P < 0.05, n = 8 \)) (Fig. 3D), it did not change LH secretion at concentrations ranging from 1 to 10^3 pM for 4 h and LH β-subunit mRNA expression at a concentration of 10^2 pM for 2, 4, and 8 h (Fig. 3, A and B).

Pretreatment with anti-CRF IgG or anti-Ucn 2 IgG blocks stress-induced suppression of LH secretion and expression. Although plasma ACTH levels at 30 and 90 min and plasma corticosterone levels at 90 min were significantly increased in the anti-CRF IgG-pretreated rats compared with those in the non-stressed anti-CRF IgG-pretreated rats, the plasma ACTH levels at 30 and 90 min in the anti-CRF IgG-pretreated rats were significantly lower than those at 30 and 90 min in NRS IgG-pretreated rats. Moreover, the plasma corticosterone level at 30 min in the anti-CRF IgG-pretreated rats was also significantly lower than that at 30 min in NRS IgG-treated rats (Fig. 4, A and B). Pretreatment with anti-CRF IgG also blocked immobilization stress-induced POMC mRNA expression in the anterior and intermediate lobes and Ucn 2 mRNA expression in the intermediate lobe (Fig. 4, C, E, and F). Furthermore, pretreatment with anti-CRF IgG blocked immobilization stress-induced suppression of plasma LH levels and LH β-subunit mRNA expression at 90 min (Fig. 4, G and H). Pretreatment with anti-Ucn 2 IgG blocked stress-induced suppression of plasma LH levels and LH β-subunit mRNA expression in the anterior lobe at 90 min without any significant changes in plasma ACTH and corticosterone levels, or in POMC and Ucn 2 mRNA expression, in either the anterior or intermediate lobe (Fig. 5). The lack of effect of pretreatment with anti-Ucn 2 IgG on the plasma ACTH and corticosterone levels or on POMC mRNA expression suggests that the IgG does not cross-react with CRF in vivo.

**DISCUSSION**

We previously reported that Ucn 2 is biosynthesized by POMC cells of the anterior and intermediate lobes of rat pituitary and that the mRNA expression and secretion of Ucn 2 by POMC cells are increased by CRF in both the anterior and intermediate lobes (31, 50). We have also reported that the mRNA expression and secretion of Ucn 2 are suppressed by glucocorticoids in the anterior lobe, but not in the intermediate lobe (31). In the present study, we have demonstrated that immobilization stress increases the expression of the mRNA of both Ucn 2 and POMC in the intermediate lobe of the pituitary and POMC in the anterior lobe and plasma ACTH levels and that these immobilization stress-induced changes are significantly attenuated by anti-CRF IgG. These results suggest that immobilization stress increases the secretion and mRNA expression of POMC in the pituitary and the mRNA expression of Ucn 2 in the intermediate lobe through CRF released from the hypothalamus.

Because anti-CRF IgG or anti-Ucn 2 IgG did not affect basal ACTH and corticosterone levels in nonstressed rats compared with these levels in NRS IgG-pretreated nonstressed rats (Figs. 4 and 5), consistent with previous reports that intravenous injection of anti-CRF antibody does not alter basal ACTH levels but blocks the increase in ACTH resulting from stress (35, 44), we propose that anti-CRF IgG or anti-Ucn 2 IgG induces no feedback effect on the HPA axis.

The plasma ACTH and corticosterone response to immobilization stress in nontreated rats in Fig. 1 was lower than the responses to the same stress shown in NRS IgG-injected rats in Figs. 4 and 5, although the responses of POMC mRNA expression in the anterior pituitary were almost the same among those experiments. Several repetitions of these experiments using the same protocol yielded similar results. This has led us to the conclusion that the intraperitoneal IgG injection itself has acted as a stressor to affect the immobilization stress-induced ACTH secretion, since it has been reported that a prior stress facilitates the subsequent stress-induced ACTH response (1a, 11a, 47a). In other words, the intraperitoneal IgG injection affected the HPA axis as a prior stressor, although the precise mechanism involved is unknown.

Our previous in vitro study showed that Ucn 2 inhibits the secretion of LH and FSH and the mRNA expression of their β-subunits (32). Our study using anti-Ucn 2 IgG and siRNA against CRF-R2 has also demonstrated that Ucn 2 secreted by POMC cells in the anterior lobe tonically inhibits the expression and secretion of gonadotropins (32). The present study has shown that immobilization stress suppresses plasma LH levels and pituitary LH β-subunit mRNA expression, although plasma FSH and pituitary FSH β-subunit mRNA expression
are not affected. These differences between the LH and FSH responses to stress are consistent with other reports showing the absence of an FSH response to various stressors (11, 16, 48), although the precise mechanism involved is unclear. Because passive immunization with anti-Ucn 2 IgG blocked the immobilization stress-induced suppression of plasma LH levels and pituitary LH mRNA expression in the present study, we believe that the immobilization-induced suppression of secretion and expression of LH is mediated by Ucn 2.

In the present study, pretreatment with anti-Ucn 2 IgG 2 h before exposure to immobilization stress affected LH β-subunit expression, while in our previous study the effect of anti-Ucn 2 IgG on basal LH β-subunit mRNA expression was detected 24 h but not 8 h after anti-Ucn 2 IgG administration (32). This interval between the administration and effect of anti-Ucn 2 IgG on LH β-subunit mRNA expression appears to depend on the amount of secreted Ucn 2. In our previous study, anti-Ucn 2 IgG was administered to nonstressed rats whose secretion of Ucn 2 from the pituitary was probably much lower, thus causing a weaker inhibitory effect on LH β-subunit mRNA expression compared with that of immobilization stressed rats used in the present study. In contrast, in the present study, 90-min immobilization stress, which would have elevated Ucn 2 secretion, significantly suppressed LH β-subunit mRNA expression in NRS IgG-treated rats, as shown in Figs. 4 and 5. In this case, the blocking effect of anti-Ucn 2 IgG on Ucn 2-induced inhibition of LH β-subunit gene expression appears to manifest in a shorter period with passive immunization. Thus it would have taken longer for the effect of anti-Ucn 2 IgG on the basal levels of LH β-subunit mRNA expression to become significantly different from that in the NRS IgG-treated nonstressed rats of our previous study.

We were unable to determine the origin of the Ucn 2 that inhibits the secretion and mRNA expression of LH during immobilization stress. Under normal conditions, Ucn 2 is expressed in various peripheral tissues, including the adrenal glands, lungs, skeletal muscles, and skin (8, 9, 20, 50). In the present study, we found that basal plasma Ucn 2 concentrations are a few nanograms per milliliter. These concentrations of Ucn 2 reflect the secretion of Ucn 2 by various tissues into the peripheral circulation, since basal plasma concentrations of Ucn 2 secreted from the pituitary are speculated to be \( \leq 100 \) pg/ml, based on the ACTH and β-endorphin levels in the peripheral circulation. The secretion of Ucn 2 from POMC cells of both the anterior and intermediate lobes is stimulated by CRF (31), and the release of CRF from the hypothalamus would increase during immobilization stress since plasma ACTH increased during immobilization stress, as shown in the present study. Thus, the secretion of Ucn 2 from POMC cells most likely increases during immobilization stress. However, the plasma Ucn 2 levels did not change during immobilization stress. This could be explained by the difference in the amount of secreted Ucn 2 between the pituitary and other peripheral tissues, the former being much lower than the latter. In other words, the secretion pattern of Ucn 2 from the pituitary might be obscured by Ucn 2 secreted by various other peripheral tissues (8, 50). It is unlikely that CRF released from the hypothalamus into the pituitary portal vessels stimulates the secretion of Ucn 2.
into the various peripheral tissues after entering the peripheral circulation because of the low concentrations of CRF in the pituitary portal vessels (31). Therefore, the POMC cells of the pituitary are the only likely targets of CRF released from the hypothalamus during stress, and they are the probable origin of the Ucn 2 that inhibits LH secretion during stress.

During stress, CRF and other ACTH secretagogues, such as vasopressin and norepinephrine, are released into the pituitary portal vessels, and the secretion of ACTH is subsequently stimulated by these secretagogues (1, 2, 7, 14, 17). The secretion of glucocorticoids is then increased by ACTH. Although CRF stimulates both the secretion and mRNA expression of Ucn 2 in both the anterior and intermediate lobes, the secretion of Ucn 2 is blocked by glucocorticoids in the anterior lobe but not in the intermediate lobe, as shown in our previous in vitro study (31). Therefore, the elevated secretion of Ucn 2 by POMC cells of the anterior lobe in response to CRF during immobilization stress would return to the basal level when the secretion of corticosterone is increased by ACTH and the corticosterone then counteracts the stimulatory effect of CRF on Ucn 2. The elevated secretion of Ucn 2 appears to last longer in the intermediate lobe than in the anterior lobe. Because local circulation from the intermediate lobe of the pituitary to the anterior lobe has been reported (30), Ucn 2 secreted by POMC cells of the intermediate lobe could act on gonadotrophs through the local circulation. On the other hand, because the amount of secreted Ucn 2 is extremely low compared with ACTH (1/40) in the culture media of anterior

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**Fig. 4. Effects of anti-CRF IgG pretreatment on plasma ACTH, corticosterone, and LH levels and on POMC, Ucn 2, and LH β-subunit mRNA expression in the anterior and intermediate pituitary of immobilization stress-exposed rats.** Male rats (7 wk old) pretreated with anti-CRF IgG (1 mg/kg body wt ip) were exposed to immobilization stress for 30 or 90 min (30 or 90). Their trunk blood was assayed for ACTH (A), corticosterone (B), and LH (G), and their anterior and intermediate pituitary RNA was extracted and assayed for mRNA expression of POMC (C for anterior pituitary and E for intermediate pituitary), Ucn 2 (D for anterior pituitary and F for intermediate pituitary), and LH β-subunit (H). Passive immunization using the same experimental protocol was performed two times, and data were combined and analyzed. Each mRNA expression level is shown as %normal rabbit serum (NRS) IgG-injected non-stressed controls. The no. of rats in each experimental group were 10 for A and B and 5 for C–H. *P < 0.05 compared with the nonstressed controls. NRS IgG, normal rabbit serum IgG-injected rats; anti-CRF, anti-CRF IgG-injected rats; IMO, immobilization.
lobe cells of rat pituitary (31), the Ucn 2 secreted by POMC cells of anterior pituitary lobe may act on gonadotrophs in a paracrine manner.

Intracerebroventricular infusion of CRF reduces immunoreactive GnRH stores in the median eminence (39). Intracerebroventricular injection of CRF attenuates LH secretion by inhibiting immunoreactive GnRH release in the hypophysial-portal circulation in ovariectomized and estradiol-administered rats (37). Intravenous injection of CRF antagonist reverses the inhibitory effect of immobilization stress on LH secretion in rats (27). These findings suggest that hypothalamic CRF modifies reproductive function at the hypothalamic level during stress. In the present study, passive immunization with anti-CRF IgG also blocked immobilization stress-induced suppression of secretion and expression of LH mRNA. In conjunction with the results of our study using anti-Ucn 2 IgG, these findings indicate that immobilization stress-induced suppression of secretion and expression of LH is mediated probably by the hypothalamic CRF-pituitary Ucn 2 axis as well as the hypothalamic CRF-GnRH axis.

A recent study has shown that 13% of gonadotrophs express CRF-R1 (49). However, in the present in vitro study, no significant change in LH secretion or mRNA expression was induced by CRF, which mainly binds to CRF-R1. Therefore, it seems unlikely that CRF released from the hypothalamus directly suppresses the secretion and expression of LH in the pituitary. A study has shown that gonadotropin inhibitory hormone (GnIH) is expressed in the dorsomedial hypothalamus and that GnIH neurons express CRF-R1 (24), and thus CRF

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Fig. 5. Effects of anti-Ucn 2 IgG pretreatment on plasma ACTH, corticosterone, and LH levels and on POMC, Ucn 2, and LH β-subunit mRNA expression in the anterior and intermediate pituitary of immobilization stress-exposed rats. Male rats (7 wk old) pretreated with anti-Ucn 2 IgG (1 mg/kg body wt ip) were exposed to immobilization stress for 90 min (90). Their trunk blood was assayed for ACTH (A), corticosterone (B), and LH (C), and their anterior and intermediate pituitary RNA were extracted and assayed for LH β-subunit mRNA (D), POMC mRNA (E for anterior pituitary and G for intermediate pituitary), and Ucn 2 mRNA (F for anterior pituitary and H for intermediate pituitary) expression. Passive immunization using the same experimental protocol was performed two times, and data were combined and analyzed. Each mRNA expression level is shown as %NRS IgG-injected nonstressed controls. The no. of rats in each experimental group were 10 for A and B and 5 for C-H. *P < 0.05 compared with the nonstressed controls. anti-Ucn 2, anti-Ucn 2 IgG-injected rats.
may also modify the reproductive function by acting on GnIH neurons during stress.

In addition to CRF, ACTH suppresses the LH surge. It has been reported that LH secretion in response to GnRH is inhibited by ACTH in adrenalectomized rams, suggesting that ACTH suppresses LH secretion without involving corticotro-roid pathways, although the precise mechanism of ACTH action is not clear (15). We found no significant effect of Ucn 2 on ACTH secretion in the cultured rat anterior pituitary cells (32). Similarly, neither stresscopin-related peptide, a human homolog of Ucn 2, nor Ucn 2 affect ACTH secretion of cultured rat anterior pituitary cells in vitro (20) and in vivo (36). Therefore, ACTH does not seem to be involved in the mechanism by which Ucn 2 suppresses the secretion and expression of LH although ACTH released by CRF may inhibit LH secretion during stress. Glucocorticoids are reported to inhibit the LH response to GnRH in vivo and in vitro (5, 6, 26, 34). However, because anti-Ucn 2 IgG blocked the stress-induced suppression of plasma LH and LH β-subunit mRNA expression without affecting POMC mRNA expression in the anterior and intermediate lobes, it would appear that the increase in corticosterone secretion induced by ACTH during immobilization stress does not play a major role in stress-induced suppression of LH secretion.

Intracerebroventricular injection of Ucn 2 reportedly suppresses LH pulsatile secretion, and intracerebroventricular injection of CRF-R2 antagonist blocks stress-induced suppression of LH secretion in ovariectomized and estrogen-replaced rats (28). There have been no reports demonstrating the expression of CRF-R2 on GnRH neurons, although CRF-R1, which has low affinity for Ucn 2 (inhibitory constant >100 nM) (40), is reportedly expressed on GnRH neurons (21, 29). Therefore, the sites of action in the central nervous system of Ucn 2 and CRF-R2 antagonist injected intracerebroventricularly are unclear. The peptide and antagonist injected intracerebroventricularly might act on the pituitary by leaking into the peripheral circulation.

In summary, the present study suggest that Ucn 2 secreted by POMC cells of the pituitary in response to CRF acts on gonadotrophs and suppresses the expression and secretion of LH in stress. This is the first report outlining a novel pathway, namely a hypothalamic CRF-pituitary Ucn 2-pituitary LH axis, through which CRF suppresses the reproductive system in addition to the hypothalamic CRF-GnRH pathway in stress.

GRANTS

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DISCLOSURES

Nothing to disclose.

REFERENCES

PITUITARY Ucn 2 INHIBITS LH SECRETION DURING STRESS


