Increased expression of miR-325-3p by urocortin 2 and its involvement in stress-induced suppression of LH secretion in rat pituitary

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Nemoto T, Mano A, Shibasaki T. Increased expression of miR-325-3p by urocortin 2 and its involvement in stress-induced suppression of LH secretion in rat pituitary. Am J Physiol Endocrinol Metab 302: E781–E787, 2012. First published January 17, 2012; doi:10.1152/ajpendo.00616.2011.—Urocortin 2 (Ucn2) is a member of the corticotropin releasing factor (CRF) peptide family, which binds to CRF type 2 receptor. We previously reported on expression of Ucn2 in proopiomelanocortin cells of rat pituitary and its inhibitory action on LH secretion. We also demonstrated that Ucn2 is involved in the mechanism underlying immobilization-induced suppression of LH secretion; the details remain unclear. Here, we found that Ucn2 increased the expression of miR-325-3p, one of three microRNAs with predicted sequence for binding to LH β-subunit 3’-untranslated region (3’-UTR) in monolayer cultured rat anterior pituitary cells, and that miR-325-3p was expressed in LH cells of the anterior pituitary. Immobilization also increased miR-325-3p expression in the anterior pituitary, and its increase was blocked by pretreatment with anti-Ucn2 IgG. Overexpression of miR-325-3p in cultured pituitary cells significantly suppressed intracellular contents and secretion of LH, while miR-325-3p knockdown blocked Ucn2-induced suppression of intracellular contents and secretion of LH. Coexpression of miR-325-3p with LH β-subunit 3’-UTR-fused luciferase vector significantly suppressed luciferase activity compared with that of mock transfectants. These results suggest that miR-325-3p is involved in immobilization-induced suppression of LH translation and secretion and that Ucn2 plays a role in the increase in miR-325-3p expression.

luteinizing hormone; microRNA; stress

REPRODUCTIVE FUNCTION IS SUPPRESSED by corticotropin-releasing factor (CRF), adrenocorticotropin, β-endorphin, and glucocorticoids, composing the hypothalamic-pituitary-adrenal axis under stress exposure (28, 29). We previously demonstrated that urocortin 2 (Ucn2), a member of the CRF peptide family (27), is expressed in proopiomelanocortin (POMC) cells of the anterior and intermediate lobes of the pituitary (32) and that in both sites CRF increases its mRNA expression (20). We also reported that Ucn2 suppresses luteinizing hormone (LH) secretion without influencing LH β-subunit mRNA expression, whereas a selective CRF type 2 receptor (CRF-R2) antagonist and a specific siRNA against CRF-R2 significantly increase LH secretion and LH β-subunit mRNA expression in monolayer cultured anterior pituitary cells of rats (23). A possible explanation for the inconsistency of effects between Ucn2 and its blockade on LH β-subunit mRNA expression is proposed; the expression level of LH β-subunit mRNA is already suppressed by endogenous Ucn2 secreted by POMC cells into the culture medium, and exogenous Ucn2 could not induce further suppression of LH β-subunit mRNA expression (6). Furthermore, we have shown that anti-Ucn2 IgG partially blocks immobilization-induced suppression of LH secretion and LH β-subunit mRNA expression (21). Considered together, these findings suggest that pituitary Ucn2 inhibits LH secretion and LH β-subunit mRNA expression and is involved in immobilization-induced suppression of reproductive function, although the details of the mechanism remain unclear.

Glycoprotein hormones such as LH, follicle-stimulating hormone (FSH), thyroid-stimulating hormone, and human chorionic gonadotropin are members of a family of cysteine-rich proteins, each consisting of α- and β-subunits; the α-subunit is common to all, and the β-subunit confers specificity (26). Expression of gonadotropins is regulated by positive and negative feedback mechanisms (25). Sex steroid hormones control the synthesis of each subunit separately but in a coordinated fashion (10). Regulation of transcription and translation differs for LH and FSH: inhibin-induced inhibition of SMAD signaling prevents activation of the FSH but not the LH β-subunit promoter (8). Regulatory mechanisms involved in the posttranscriptional and translational processes during LH β-subunit synthesis, especially their suppressive mechanisms, are unclear at present.

MicroRNAs (miRNAs) are short RNA molecules, ~22 nucleotides long on average, that are found in most types of eukaryotic cells (3). They are posttranscriptional regulators that usually induce translational repression and gene silencing in animals after binding to complementary sequences on target mRNAs. To the best of our knowledge, there have been no previous reports of the involvement of miRNAs in the regulation of pituitary hormone secretion. We hypothesized that Ucn2 might induce expression of miRNAs, which may then be involved in stress-induced suppression of LH secretion. To test this hypothesis, through database searches, we identified miRNAs predicted to bind to the LH β-subunit 3’-UTR, determined their expression levels in the Ucn2-treated monolayer cultured anterior pituitary cells of rats and pituitaries of immobilization-exposed rats, and then assessed the effects of miRNA overexpression and knockdown on LH β-subunit mRNA expression, intracellular LH content, and LH secretion.

MATERIALS AND METHODS

Animals. Six- and seven-week-old male Wistar rats were maintained at 23 ± 2°C in 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. All experimental procedures were reviewed and approved by the Laboratory Animals Ethics Review Committee of Nippon Medical School.

Primary culture of pituitary cells. Thirty male rats (6 wk old) were killed by decapitation, and their pituitary glands were removed under sterile conditions. The anterior pituitary lobes were collected, pooled at 2000). They were allowed ad libitum access to laboratory chow and distilled water. All experimental procedures were reviewed and approved by the Laboratory Animals Ethics Review Committee of Nippon Medical School.

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described (20), but with several modifications. Briefly, lobes were washed twice in PBS and then incubated at room temperature in the PBS containing 0.01% dispase (Godoshuei, Tokyo, Japan) with constant stirring for 30 min. After washing with PBS three times, cells were plated in 24-well plates or 60-mm dishes and cultured with 10% FBS containing DMEM-F10 HAM culture medium (Sigma-Aldrich, St. Louis, MO) supplemented with an antibiotics/antimyotic solution (GIBCO, Auckland, NZ). The cells were subsequently allowed to attach to the plating surfaces at 37°C in a humidified 5% CO2-95% air incubator for 4 days. On the day of experiment, the culture media were changed. Cells were treated with Ucn2 at concentrations of 1, 10, 30, and 100 μM for 1 h or 100 μM Ucn2 for 0.5, 1, and 2 h. After incubations, the cells were assayed for miRNA expression.

Passive immunization. We previously generated antisera against mouse Ucn2 (32). The IgG fraction was purified from serum obtained after the fifth booster by using a protein A-Sepharose column. The specificity of this antisera to Ucn2 was described in our previous reports (32). Cross-reactivities of the antisera with rat Ucn2, rat CRF, Ucn1, and Ucn3 were 83.3, 0.0, 0.014, and 0.023%, respectively. Twenty male rats were administered antisera IgG to Ucn2 or normal rabbit serum (NRS) IgG (1 mg/kg body wt ip) dissolved in 1 ml normal saline. Two hours later, they were exposed to 90 min of immobilization stress (21). Rats were then killed immediately, and their trunk blood and pituitaries were collected. Trunk blood was placed into tubes containing EDTA-2Na (1 mg/ml blood) and centrifuged at 3,000 rpm for 20 min at 4°C. One-milliliter aliquots were transferred into 1.5-ml Eppendorf tubes and stored at -80°C until use.

Stress exposure. Twenty male rats (7 wk old) were wrapped in a flexible wire mesh and kept for 90 min between 1300 and 1500 in an isolated room (1, 21). Rats were killed in the adjacent room immediately after immobilization, and their pituitaries were collected for RNA expression analysis. Nonstressed control rats were housed in a separate room from the stressed rats and were otherwise treated the same way.

miRNA identification. The miRBase (www.mirbase.org) and TargetScan (www.targetscan.org) database were searched for miRNAs predicted to bind to the LH β-subunit 3'-UTR.

In situ hybridization and immunohistochemistry. Digoxigenin-labeled LNA probe against mmu-miR-325-3p was purchased from Exiqon (Denmark). Oligonucleotides were annealed and purified and then immobilized on a nylon membrane and then hybridized with 3'-DIG-labeled miRCURY LNA miRNA detection probe against mmu-miR-325-3p (Exiqon) at 45°C for 14 h. Signals were detected using a DIG luminescent detection kit according to the manufacturer’s instructions (Roche Diagnostics). Sections were incubated in 10% normal goat serum for 20 min and then incubated in 0.3% H2O2 for 30 min at room temperature, and the sections were incubated in 10% normal goat serum for 20 min and then incubated for 60 min at room temperature with an antibody to LH β-subunit 3'-subunit and GAPDH, as previously reported (21). To normalize each sample for RNA content, GAPDH, a housekeeping gene, or U6 small nuclear RNA (Clontech) were used for miRNA and miRNA expression analyses, respectively. Diluted normal rat pituitary cDNA and the second-derivative method were used as the standard and for calculating Ct values, respectively (24).

Northern blotting of miRNA. Total RNA from transfectedants was separated by electrophoresis in 15% acrylamide gel containing 8 M urea. RNA was then electrically transferred to nylon membranes and fixed by UV cross-linking, prehybridized with DIG Easy Hyb solution (Roche Diagnostics) containing 10 μg/ml yeast tRNA at 45°C for 1 h, and then hybridized with 3'-DIG-labeled miRCURY LNA miRNA detection probe against mmu-miR-325-3p (Exiqon) at 45°C for 14 h. Signals were detected with a DIG luminescent detection kit according to the manufacturer’s instructions (Roche Diagnostics).

3'-UTR assay. Synthetic oligonucleotide for LH β-subunit 3'-UTR, positioned from translational termination codon together with Nhel and XhoI linker at its 5’ and 3’ extends, and its antisense oligonucleotide were synthesized by Invitrogen (Carlsbad, CA). Oligonucleotides were annealed, digested with Nhel and XhoI, and then the Nhel-XhoI fragment was subcloned into Nhel and XhoI sites of pmir-Glo plasmid [Promega, Madison, WI; pmir-Glo-LHβ-3'-UTR (WT); see Fig. 5A] (30). We also generated a 3'-UTR variant that was not recognized by mmu-miR-325-3p [pmir-Glo-LHβ-3'-UTR (SC); see Fig. 5A], pmir-Glo-LHβ-3'-UTR (WT), pmir-Glo-LHβ-3'-UTR (SC), or pmir-Glo-mock plasmid and pBA-miR-325-3p or pBA-mock plasmid (2.5 μg of each plasmid amount) were cotransfected with Multitfectam (Promega) into HEK293 cells and seeded in 60-mm dishes. Cells were collected after 72 h and assayed using the dual-luciferase reporter assay system according to the manufacturer’s instructions (Promega).

Statistical analysis. Statistical analysis was performed by ANOVA followed by Tukey’s post hoc test using Prism 5.0 software (GraphPad Software, La Jolla, CA). Pituitary cell culture using the same experimental protocol was performed twice. For real-time RT-PCR data, all results were expressed as percentage of control values. Statistical significance was defined at the P < 0.05 level.
**RESULTS**

Ucn2 increases miRNA expression in monolayer cultured rat pituitary cells. A search of miRNA databases revealed three miRNAs, mo-miR-325-3p (MI0000596), mo-miR-370 (MI0003486), and mo-miR-742 (MI0006161), with sequences predicted for binding to positions 59–65, 34–40, and 66–71 of LH β-subunit 3'–UTR, respectively (Fig. 1A). miR-325-3p appears to be present in essentially all pituitary cells, including those that are immunopositive for LH by a double-labeling technique for combined in situ hybridization and immunohistochemical analysis (Fig. 1B). Treatment of monolayer cultured rat anterior pituitary cells with Ucn2 significantly increased miR-325-3p expression, whereas there was no change in miR-370 and miR-742 levels (data not shown). Treatment with Ucn2 for 1 h revealed that miR-325-3p expression was significantly increased at concentrations of 30 pM (1.75 ± 0.16-fold vs. control) and 100 pM (2.41 ± 0.15-fold vs. control) (Fig. 2A). A time course study revealed that miR-325-3p expression was significantly increased at 0.5, 1, and 2 h (1.79 ± 0.18, 2.41 ± 0.16, and 1.59 ± 0.12-fold vs. control, respectively) after treatment with Ucn2 at a concentration of 100 pM (Fig. 2B).

Immobilization stress increases mir-325-3p expression, and anti-Ucn2 IgG blocks the increase in the pituitary. Ninety-minute immobilization stress significantly increased miR-325-3p expression in the anterior pituitary of rats pretreated with NRS IgG (1.42 ± 0.12-fold vs. nonstressed rats pretreated with NRS IgG; Fig. 3), and pretreatment with anti-Ucn2 IgG blocked immobilization stress-induced increase in miR-325-3p expression in the anterior pituitary (Fig. 3). There was a significant interaction between IgG injection and immobilization exposure (F1,16 = 5.01, P = 0.040, n = 5).

**Fig. 1.** miRNA binding sites of LH β-subunit 3'–UTR sequence and expression of miR-325-3p in the anterior pituitary of rats. A: predicted miRNA binding sites are underlined. Transcriptional termination codon is shown in bold. B: typical picture of combination of in situ hybridization of miR-325-3p (purple) and immunohistochemistry of LH β-subunit (brown) in the anterior pituitary is shown. Double-positive cells are indicated by arrows. Scale bar, 50 μm.

**Fig. 2.** Effects of urocortin 2 (Ucn2) on miR-325-3p expression in rat monolayer cultured anterior pituitary cells. A: miR-325-3p expression in anterior pituitary cells treated with Ucn2 at various concentrations ranging from 1 to 100 pM for 1 h. B: time course for miR-325-3p expression in anterior pituitary cells treated with 100 pM Ucn2. Levels are shown as % of control. Eight wells were used for each treatment. cont, control. Data shown are means ± SE. *P < 0.05 vs. control.

**Fig. 3.** Effect of anti-Ucn2 IgG pretreatment on miR-325-3p expression in the anterior pituitary of immobilization-stressed rats. NRS IgG, normal rabbit serum IgG-injected rats; anti-Ucn2, anti-Ucn2 IgG-injected rats; IMO, 90-min immobilization stress exposed group. miRNA expression levels are shown as % of that in NRS IgG-injected nonstressed controls. For each experimental group, n = 5. *P < 0.05 vs. nonstressed controls (nonstress).
between transfectants and treatment in intracellular LH content and LH secretion.

**LH β-subunit 3′-UTR activity assay.** When intact LH β-subunit 3′-UTR-fused luciferase vector [pmir-Glo LHβ 3′-UTR (WT)] was cotransfected with miR-325-3p, its luciferase activity was significantly lower than that cotransfected with pBA-mock vector (75.9 ± 3.5% of mock transfectants; Fig. 5B). When the miR-325-3p binding site of LH β-subunit 3′-UTR was replaced with scramble mutation [pmir-Glo LHβ 3′-UTR (SC)], there was no difference in luciferase activity between pBA-miR325-3p coexpression and pBA-mock coexpression (Fig. 5B).

**DISCUSSION**

Our previous study has shown that Ucn2 suppresses LH secretion from monolayer cultured anterior pituitary cells of rats (23). In the present study, Ucn2 increased expression of miR-325-3p in the anterior pituitary cells of rats. The present study has also demonstrated that miR-325-3p overexpression decreases intracellular LH contents and suppresses basal LH secretion and LH β-subunit 3′-UTR activity, and that miR-325-3p knockdown blocks Ucn2-induced suppression of intracellular contents and secretion of LH. These results suggest that miRNA is involved in Ucn2-induced suppression of LH biosynthesis and secretion. Our previous study had also revealed that mRNA expression and secretion of Ucn2 in the pituitary are increased by CRF in vitro (20) and by immobilization stress in vivo (21), and that the increases of mRNA expression and secretion of Ucn2 in immobilization stress are blocked by anti-CRF IgG in vivo (21), suggesting that stress-induced CRF induces Ucn2 secretion. Furthermore, we showed that stress-induced suppression of LH secretion is blocked partially by anti-Ucn2 IgG in vivo (21). Considered together with these previous findings, the results of the present study suggest that stress-induced Ucn2 secretion causes an increase in miR-325-3p expression in the pituitary, which in turn suppresses LH β-subunit translation and secretion.

We tested the effect of intravenous administration of Ucn2 on LH secretion and miR-325-3p expression in the anterior pituitary of rats, and no significant changes in plasma LH or
miR-325-3p expression levels in the anterior pituitary were induced by peripheral administration of Ucn2 at doses of 5 and 15 μg (data not shown). Since Ucn2 suppresses and a CRF-R2 antagonist increases LH secretion in the monolayer culture of rat anterior pituitary cells (23), these results suggest that factors that block or attenuate the inhibitory effect of Ucn2 on LH secretion may be present in the peripheral circulation. CRF-binding protein (CRF-BP), which shows an intermediate affinity to Ucn2, would be proposed as one of factors (12). There is no study that shows CRF-BP in rat plasma; however, CRF-BP is likely to exist in peripheral blood of rats, because it has been reported that CRF-BP in human plasma binds to CRF (4) of rats, and CRF-BP is detected in human peripheral blood (9, 19). Therefore, Ucn2 administered intravenously may bind to CRF-BP during peripheral circulation before reaching the gonadotrophs of the pituitary and lose its inhibitory effect on gonadotrophs. In any case, the Ucn2 secreted by corticotrophs seems to act on gonadotrophs in a paracrine manner (23). Intracerebroventricular (icv) administration of Ucn2 reportedly suppresses LH secretion in rats, although the site of action of Ucn2 is unknown (17). Furthermore, icv administration of a selective CRF-R2 antagonist blocks restraint-, hypoglycemia-, or lipopolysaccharide-induced suppression of LH secretion in ovariectomized rats with estrogen replacement (16, 17). These findings suggest that CRF-R2 probably mediates stress-induced LH suppression at some site(s) other than the pituitary and that Ucn2 may play a role in the control of LH secretion under stress in the central nervous system.

Not only Ucn2 but also Ucn1, Ucn3, and CRF bind to CRF-R2. However, since there has been no report showing that the anterior pituitary cells express Ucn1 and Ucn3, it seems that they have no physiological role in the regulatory mechanism of LH secretion at the pituitary level. Furthermore, we have shown that CRF has no effect on LH secretion in the monolayer cultured anterior pituitary cells of rats (21). Therefore, CRF, an endogenous ligand to CRF-R1, does not seem to affect miR-325-3p expression, although 13% of gonadotrophs express CRF-R1 (31).

miR-325-3p is reportedly expressed in brain, spleen, and testis in rats (18). In addition to these tissues, we have found that miR-325-3p is expressed in the anterior pituitary and that it is, at least in part, expressed in LH cells in the present study. Therefore, the miR-325-3p expressed in LH cells seems to play a suppressive role in the regulatory mechanism of LH biosynthesis. Although pituitary cells other than LH cells also express miR-325-3p, the physiological significance of miR-325-3p in the cells is unknown. In general, one miRNA has various target genes, which may differ in each cell or tissue (14). Actually, miR-325-3p has more than 1,000 target genes detected using the Targetscan database. Further studies are needed to clarify the functions of miR-325-3p in other pituitary cells.

The present study has shown that overexpression and knockdown of miR-325-3p did not affect LH β-subunit mRNA expression, although the intracellular content and secretion of Ucn2 were influenced, suggesting that miR-325-3p inhibits translation but not transcription. These results are consistent with our previous in vitro study showing that Ucn2 suppresses LH secretion without changing LH β-subunit mRNA expression. By contrast, immobilization stress suppresses not only LH secretion but also LH β-subunit mRNA expression, and these changes are completely reversed by pretreatment with anti-CRF IgG, whereas anti-Ucn2 IgG significantly but partially blocked the immobilization-induced changes (21). Taking these findings into consideration, it seems that some factor other than the Ucn2/miR-325-3p system, which is induced by CRF, is involved in immobilization stress-induced suppression of LH β-subunit mRNA expression.

The human genome may encode thousands of miRNAs, which are abundantly expressed in many human cell types and may target about 60% of genes (3). These miRNAs target the 3′-UTR of miRNAs to inhibit translation. Recent studies have revealed that miRNAs have many physiological roles in endocrine and metabolic systems. Progesterone receptor translation and casein secretion are inhibited by miR-126-3p in mouse mammary epithelial cells (6). Toll-like receptor 4 is suppressed by miR-146a, and miR-146a reduces intracellular LDL cholesterol and secretions of IL-6, IL-8, chemokine ligand 2, and MMP-9 by inhibiting the toll-like receptor 4 signaling pathway (33). Since high glucose concentrations increase miR-410, miR-200a, and miR-130a expression, these may be involved in...
PKA-induced Wnt-merase II or III and primary miRNA is processed in the nucleus. It has not been reported. The current study provides new insight into understanding the function of miRNAs in regulating pituitary hormone biosynthesis.

Although miRNA transcription is controlled by RNA polymerase II or III and primary miRNA is processed in the nucleus (2, 7, 15), little is known about the signaling pathways that control its expression. It was reported that protein kinase A (PKA) affects the Wnt signaling pathway and that inhibition of PKA-induced Wnt-β catenin signaling is mediated by miRNA expression (13). We previously demonstrated that Ucn2 activates PKA and MAPK in PC12 cells (22). Unfortunately, H-89 and Rp-cAMPs, both PKA inhibitors, failed to block Ucn2-induced miR-325-3p expression, and miR-325-3p expression was unaffected by forskolin, a PKA activator (data not shown). Further studies are needed to clarify other intracellular signaling pathways leading to Ucn2-induced increases in miR-325-3p expression.

In summary, the present study suggests that miR-325-3p is involved in stress-induced suppression of LH secretion and that Ucn2 plays a role in increasing the expression of miR-325-3p. This is a newly identified pathway underlying regulation of LH secretion. These results may increase our understanding of the molecular mechanisms involved in development of stress-induced gonadal dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.N. conception and design of research; T.N. and A.M. performed experiments; T.N. analyzed data; T.N. prepared figures; T.N. wrote the first draft of the manuscript; T.N. edited and revised manuscript.

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