Urocortin: a mechanism for the sustained activation of the HPA axis in the late-gestation ovine fetus?

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Urocortin: a mechanism for the sustained activation of the HPA axis in the late-gestation ovine fetus? Am J Physiol Endocrinol Metab 283: E165–E171, 2002. First published March 19, 2002; 10.1152/ajpendo.00497.2001.—We hypothesized that urocortin might be produced in the pituitary of the late-gestation ovine fetus in a manner that could contribute to the regulation of ACTH output. We used in situ hybridization and immunohistochemistry to identify urocortin mRNA and protein in late-gestation fetal pituitary tissue. Levels of urocortin mRNA rose during late gestation and were associated temporally with rising concentrations of pituitary proopiomelanocortin (POMC) mRNA. Urocortin was localized both to cells expressing ACTH and to non-ACTH cells by use of dual immunofluorescence histochemistry. Transfection of pituitary cultures with urocortin antisense probe reduced ACTH output, whereas added urocortin stimulated ACTH output from cultured pituitary cells. Cortisol infusion for 96 h in chronically catheterized late-gestation fetal sheep significantly stimulated levels of pituitary urocortin mRNA. We conclude that urocortin is expressed in the ovine fetal pituitary and localizes with, and can stimulate output of, ACTH. Regulation of urocortin by cortisol suggests a mechanism to override negative feedback and sustain feedforward of fetal hypothalamic-pituitary-adrenal function, leading to birth.

Urocortin: adrenocorticotrophic hormone; parturition; hypothalamic-pituitary-adrenal axis

MATERIALS AND METHODS

Animals and Tissue Collection

Pregnant mixed-breed ewes of known gestational age (GA) were used in these studies. The experiments were performed...
according to protocols approved by the Animal Care Committee of the University of Toronto, in accordance with the guidelines of the Canadian Council for Animal Care. For ontogeny studies of urocortin mRNA and protein expression, pituitaries were obtained from fetuses between 110 and 143 days GA (term = 147 days) from noninstrumented animals (n = 4–7 fetuses per group). To examine the effect of cortisol on urocortin mRNA expression in the pituitary, pituitaries were collected from chronically catheterized sheep fetuses that had received an intrafetal infusion of either cortisol (5 μg/min) or saline for 12 or 96 h (n = 4 per group) between 125 and 129 days GA. The details of the infusion protocol have been published previously (18). For tissue collection, animals were euthanized with an overdose of Euthanyl (MTC Pharmaceuticals, Cambridge, ON, Canada), and the fetal pituitary was rapidly dissected out. Pituitaries were either slowly frozen on dry ice and stored at −80°C until processed for in situ hybridization or fixed by immersion in Bouin’s solution at 4°C overnight for urocortin immunohistochemistry. Fixed tissues were washed in PBS and embedded in paraffin.

**Immunohistochemistry**

Immunohistochemical detection of urocortin was performed on 5-μm sections of fetal pituitaries. Tissue sections were deparaffinized in xylene, rehydrated, and washed in PBS. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% hydrogen peroxide (in methanol) for 30 min. The sections were then incubated with 10% normal goat serum and 1% BSA, and then with the primary antibody (1:2,000) overnight at 4°C. The urocortin antibody was a polyclonal antibody raised against the COOH terminus of the human urocortin peptide. Details of the antibody production and characteristics have been reported elsewhere (10). Sections were then washed in PBS, and immunostaining was identified by the avidin-biotin-peroxidase technique with the Vectastain kit (Vector Laboratories, Burlingame, CA), with diaminobenzidine as the chromogen. Tissue sections were then counterstained with Carazzi’s hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Control sections were incubated with primary antibody that had been preabsorbed with human urocortin (Bachem, King of Prussia, PA).

**In Situ Hybridization**

Frozen fetal pituitary glands (110–111 days GA, n = 4; 125–135 days GA, n = 7; 140–143 days GA, n = 5), cortisol treated (12 and 96 h, n = 4/group) or saline treated (12 and 96 h, n = 4/group), were cut in coronal sections (12 μm) on a cryostat (Jung CM 300, Leica Instruments, Nussloch, Germany), freeze-thaw mounted onto slides coated with poly-l-lysine (Sigma Chemical, St. Louis, MO), and air dried. Slides were then postfixed in 4% paraformaldehyde (pH 7.4, 4°C, 5 min), rinsed twice in PBS (pH 7.4, 1 min), dehydrated in an ascending ethanol series, and stored in 95% ethanol at 4°C until analysis by in situ hybridization.

The in situ hybridization technique used here has been described in detail previously (24, 35). Briefly, a 42-mer oligonucleotide probe complementary to bases 17–58 of the partial ovine urocortin gene (6) was labeled using terminal deoxynucleotidyl transferase (Pharmacia Biotech, Baie d’Urfe, PQ, Canada) and [α-35S]dATP (NEN Du Pont Canada, Mississauga, ON, Canada). To assess the relationship between POMC and urocortin during late gestation, pituitary sections from animals at 110–111 days GA, (n = 4), 125–135 days GA, (n = 7), and 140–143 days GA (n = 5) were also hybridized with a labeled 45-mer oligonucleotide probe complementary to bases 504–549 of ovine POMC (3). The sections were hybridized overnight in a moist chamber (42°C) with the radiolabeled probes. After hybridization, the sections were washed and exposed to autoradiographic film (Biomax, Kodak, Rochester, NY). The autoradiographic films were developed using standard methods. Linearity was established by simultaneous exposure of the film to 14C standards, and a control 45-mer nonsensical sequence oligonucleotide probe was included to assess nonspecific hybridization. The autoradiograms were then analyzed using computerized image analysis software (Imaging Research, St. Catharines, ON, Canada). The relative optical density of pituitary urocortin mRNA was assessed using a minimum of 12 sections for each animal. To identify the distribution of urocortin mRNA, some sections were dehydrated in ascending ethanol series, air dried, coated with Ilford K5 photoemulsion (Ilford, Mobberley, UK), and exposed at 4°C. The photoemulsion was developed, fixed, and mounted with Permount.

**Dual Immunofluorescence**

Dual immunostaining was performed to determine whether urocortin and ACTH were colocalized in the same cells. Tissue sections were rehydrated in serial dilutions of alcohol (100, 90, 70, and 50%) and washed in PBS. Nonspecific binding of antibodies was blocked with 1% BSA in PBS for 2 h at room temperature. The samples were then incubated with the primary antibodies rabbit anti-human urocortin (1:1,000) and mouse anti-human ACTH (1:100; Dako, Carpinteria, CA) and placed in a 1% BSA solution containing 0.3% Triton X-100. Tissue sections were incubated overnight (18–24 h) with the primary antibodies at 4°C. After the incubation period, the sections were washed three times in 0.1 M PBS. The secondary antibodies were added, and all was incubated at 37°C for 45 min. The secondary antibodies used were a fluorescein-conjugated sheep anti-mouse IgG used at 1:50 dilution (Amersham Pharmacia Biotech) and a CY3-conjugated sheep anti-rabbit IgG used at a 1:1,000 dilution in a 1% BSA sample. Slices were washed again in PBS and then dehydrated in serial dilutions of alcohol (50, 70, 90, and 100%). Anti-fading reagent (p-phenylenediamine, 1 mg/ml, 50% glycerol, 50% PBS) was added to the tissue sections, and coverslips were applied before analysis.

Tissue sections were analyzed under a fluorescent Optiphot-2 microscope (Nikon) by use of a green filter to visualize FITC and a red filter to visualize CY3. A Sensicam 128 bit cooled imaging camera (Cooke) was used to take a digital photograph of the section using Sensocontrol 4.02 software (Cooke), and this was visualized on a computer. The images were then exported into CorelDraw (Corel, Eastman Kodak) and superimposed to obtain the localization pattern of urocortin with ACTH.

**Tissue Culture**

Pregnant mixed-breed ewes of known GA were used in these studies. At 134–136 days GA (n = 5), the animals were euthanized with an overdose of Euthanyl (MTC Pharmaceuticals). The fetal pituitary was rapidly dissected out and immediately placed in Dulbeco’s phosphate-buffered saline (DPBS) supplemented with 1.35 g/l glucose and 0.1% BSA (DPBS+, pH 7.4, Gibco-BRL, Grand Island, NY) for tissue culture preparation. For these studies, only the anterior pituitary was used after dissecting it away from the intermediate and posterior pituitaries, as described previously (22). Tissue culture was performed according to a modified method of Wang et al. (33). Briefly, the anterior pituitary was chopped into blocks and incubated with 10 ml of DPBS+...
containing 0.5% trypsin at 37°C for 30 min under gentle shaking. Tissues were then incubated at 37°C for 30 min with DMEM (GIBCO-BRL) supplemented with FCS (10%), bovine holo-transferrin (5 mg/l), insulin (5 mg/l), and an antibiotic-antimycotic (containing 100,000 U of penicillin, 10 mg of streptomycin, and 25 μg of amphotericin B). The tissues were washed and shaken in calcium- and magnesium-free DPBS with 0.1% BSA and EDTA (0.75 g/l) (DPBS−, pH 7.4; GIBCO-BRL). The tissue fragments obtained were dispersed mechanically by passing them through a sterile, siliconized Pasteur pipette in 5 ml of DPBS−. The dispersed cells were then filtered. After centrifugation at 1,600 rpm for 10 min, the supernatant was discarded, and the pellet was resuspended with supplemented DMEM. The centrifugation was repeated. The viability of anterior pituitary cells was assessed before plating by trypsin blue exclusion and was generally >90%. The anterior pituitary cells were seeded in supplemented DMEM onto sterile poly-l-lysine-coated plates and incubated at 37°C in a water-saturated atmosphere of 95% air-5% CO2 before treatment.

Treatment of Fetal Anterior Pituitary Cells

Urocortin dose response. The cells from the anterior pituitary were plated at 100,000 cells/0.5 ml (8-well culture plates; Lab-Tek chamber slide, NUNC, Naperville IL) and incubated at 37°C for 3 days. The medium was changed at 48 h. On the 4th day (time 0 for the experiment), the medium was changed, and various concentrations (0, 10^−12, 10^−10, 10^−8, and 10^−6 M) of urocortin or ovine CRH (0, 10^−8, and 10^−6 M) were added in supplemented serum-free DMEM. At the end of the 24-h incubation period, the medium was collected and stored at −20°C until analysis. ACTH concentration in the media was determined with a commercially available radioimmunooassay kit (Diasorin, Stillwater, MN), which has been previously validated for use with fetal sheep plasma (18). The intra-assay coefficient of variation was 9%.

Antisense oligonucleotide treatment. The cells from the anterior pituitary were incubated at 37°C for 3 days at a density of 100,000 cells/ml (24-well culture plates; Corning Glass Works, Corning, NY). The medium was changed after 48 h. On the 4th day, cells were treated with fully phosphorothioated 18-mer oligonucleotides. The urocortin oligonucleotide was designed to be complementary to bases 114–131 of ovine urocortin (6). The antisense oligomer for bases 230–247 of ovine POMC was used as a positive control. To control for the nongenomic effects of the treatment, we used a random sense control (5′-CCT CAT TCT TGC GAA CAG-3′) oligomer. Antisense and sense oligonucleotides were used at a concentration of 5 μM and were transfected intracellularly by a cationic liposome-mediated process by using 1 μl of Lipofectin reagent (GIBCO-BRL). Treatments were performed for 6 h in serum-free DMEM, followed by 18 h in DMEM with 10% FCS for a total of 24 h. After 24 h, the medium was removed and replaced with DMEM supplemented with transferrin, insulin, and 10% FCS for 48 h. At the end of the 48-h incubation period, the medium was collected and stored at −20°C until analysis. Control cells received either no treatment or Lipofectin reagent with no oligonucleotide.

Statistical Analysis

Pituitary mRNA levels, reported as relative optical density, were subjected to a one-way ANOVA followed by Tukey’s pairwise test (α = 0.05) or Student’s t-test to determine the effect of 96 h of cortisol treatment. To examine the effect of urocortin on ACTH release in vitro, measurements of ACTH output were compared with those of the control group by Student’s t-test (α = 0.05). To assess the effect of antisense oligonucleotide treatment on ACTH output in vitro, data were converted to percentage of control and subjected to a one-way ANOVA, followed by Bonferonni’s multiple comparison vs. the control group (α = 0.05). Results are expressed as means ± SE.

RESULTS

Localization of Urocortin Protein and mRNA

Immunoreactive (ir)-urocortin was identified by immunohistochemical staining in the pars distalis of the fetal pituitary (Fig. 1). The ir-urocortin expression was stronger in the lateral aspect of the anterior pituitary. Negative controls incubated with the preabsorbed primary antibody did not show any specific staining. At all GAs, cells expressing ir-urocortin were identified in close proximity to cells expressing ir-ACTH (Fig. 2), and some cells express both ir-ACTH and urocortin. However, ir-urocortin expression in the anterior pituitary was not limited to areas where strong ir-ACTH staining was identified.

Urocortin mRNA expression was present in the fetal pars distalis at all ages studied (Fig. 3). Urocortin mRNA expression in the pituitary significantly increased from 110–111 days GA to 125–135 days GA (P < 0.05), and it remained at similar levels until term (Fig. 3). The rise in urocortin mRNA expression in the fetal pituitary preceded the rise in POMC mRNA in the inferior aspect of the pars distalis. A clear regional localization of urocortin protein and mRNA was observed in the fetal pituitary. In the anterior pituitary, urocortin mRNA and protein were present in a subset of cells, with strongest expression in the lateral aspects of the anterior pituitary. In the intermediate part of the anterior pituitary, urocortin mRNA and protein expression was weaker, and in the posterior pituitary, expression was absent.

Fig. 1. Identification of urocortin protein in ovine fetal pituitary. A: immunohistochemical identification of urocortin protein in the fetal ovine pituitary. B: negative control with preabsorbed primary antibody. Magnification bars (A and B), 12.5 μm.
distribution of urocortin mRNA expression in the pituitary was not identified, although urocortin mRNA appeared to be higher in the lateral aspects of the pars distalis, similar to the distribution of ir-urocortin.

**Urocortin Effect on Pituitary ACTH Output**

Urocortin treatment increased ir-ACTH output from cultured pars distalis cells (Fig. 4). ACTH output after treatment with $10^{-8}$ and $10^{-6}$ M urocortin was significantly elevated with respect to basal output. The stimulatory effect of $10^{-8}$ and $10^{-6}$ M urocortin on ACTH output was not significantly different from the effect of CRH at equimolar concentrations ($10^{-8}$ M urocortin 394.8 ± 97%, $10^{-8}$ M output (o)CRH 298.2 ± 104%; $10^{-6}$ M urocortin 349.8 ± 86%, $10^{-6}$ M oCRH 397.0 ± 82%).

Incubation of dispersed anterior fetal pituitary cells with a phosphorothioated antisense oligonucleotide complementary to urocortin mRNA significantly ($P < 0.001$) decreased basal ir-ACTH output (Fig. 5) to ~40% of control output. A similar effect was found when the POMC mRNA antisense oligonucleotide was
used. This reduction in ir-ACTH output was not observed when the cells were incubated with either a random sense oligomer, representing a nonspecific response, or Lipofectin reagent alone. Moreover, there was no effect of the urocortin antisense treatment in the absence of Lipofectin (data not shown).

Regulation of Urocortin mRNA Expression by Cortisol

Intrafetal cortisol administration for 12 h did not alter pituitary urocortin mRNA expression (Fig. 6). However, 96 h of cortisol infusion to the fetus significantly increased urocortin mRNA expression compared with that in the saline control group. This rise in urocortin mRNA expression after 96 h of cortisol administration was accompanied by a significant increase in fetal plasma ACTH and cortisol concentrations, which have been reported previously (18).

DISCUSSION

This study demonstrates for the first time that urocortin is synthesized in the fetal sheep pituitary and may be an important ACTH-stimulatory factor in this species. It has previously been thought that, in the fetal sheep, pituitary ACTH secretion is primarily regulated by the hypothalamic releasing factors CRH and arginine vasopressin (4, 23). However, more recently, studies in other species have suggested that urocortin may also be an important regulator of pituitary ACTH output (1, 12, 28). In the present study, we have shown that treatment of dispersed anterior pituitary cells with urocortin increased ACTH output in a dose-related manner, and at equimolar concentrations the stimulatory effects of urocortin and CRH were not significantly different. Because urocortin and CRH have a similar affinity for the CRH R1 receptor (14), and CRH R1 receptor mRNA has been identified in the ovine fetal pituitary throughout gestation (15), the data from the present study are consistent with an ACTH-stimulatory effect of urocortin, mediated via the CRH R1 receptor. Urocortin has also been identified as a potent ACTH secretagogue in rats (1, 12, 28), but studies using rat pituitary cells have shown that, at equimolar doses, urocortin is more potent than CRH (1, 12). Because the studies in rats used pituitary cells isolated from adult animals, we are unable to determine whether the apparent difference in the stimulatory ability of urocortin with respect to CRH in the fetal sheep is a species difference or a characteristic of fetal pituitary cells. However, these results suggest that, in the fetal sheep, as in other mammals, urocortin is an ACTH-stimulatory factor. Although we have shown that exogenous urocortin stimulates ACTH output from pituitary cells in vitro, the distribution of urocortin in the fetal sheep has not been fully characterized.

In the rat, urocortin mRNA and protein have been identified in the hypothalamus (2, 20, 25, 27), suggesting that urocortin acts as a conventional hypothalamic-releasing peptide to stimulate ACTH release from the pituitary. However, in humans, the localization of uro-
UROCORTIN IN FETAL SHEEP

Corticotropin is not consistent with urocortin acting as a hypothalamic-releasing factor. In the human, there is no evidence for urocortin mRNA or protein expression in either the paraventricular nucleus of the hypothalamus or the pituitary stalk. Similarly, there is no evidence of urocortin mRNA expression in the hypothalamus of the adult sheep. However, high levels of urocortin mRNA expression have been identified in the pituitary glands of humans and rats, suggesting that urocortin may not act solely as a traditional hypothalamic releasing peptide, but rather that urocortin produced locally in the pituitary might regulate ACTH output in a paracrine/autocrine manner.

In addition to urocortin, the anterior pituitary has recently been shown to synthesize a wide variety of peptides conventionally considered to be hypothalamic-releasing factors, including thyrotropin-releasing hormone, gonadotropin-releasing hormone, growth hormone-releasing hormone, somatostatin, and CRH. In rats, dispersed anterior pituitary cells synthesize and secrete CRH, and incubation of these cells with an antibody directed against CRH significantly decreased ACTH output by the corticotrophs. In the present study, the identification of both urocortin protein and mRNA in the pituitary demonstrates that urocortin is synthesized in the fetal sheep pituitary, and it suggests that this urocortin may be able to act in a paracrine manner to regulate ACTH output.

When dispersed fetal anterior pituitary cells were transfected with an 18-mer oligonucleotide complementary to ovine urocortin mRNA, ACTH output from these cells was significantly reduced compared with that from controls. This reduction was similar to the inhibition of ACTH output seen when the translation of ACTH was inhibited by treatment of the cells with an antisense oligonucleotide complementary to the ACTH precursor POMC. These results suggest that, in the fetal sheep pituitary, the regulation of ACTH output may be under tonic regulation from urocortin synthesized in the pituitary and acting in a local paracrine manner. These results are in contrast to a previous study in rats, in which immunoneutralization of endogenous urocortin with a urocortin antibody administered in vivo did not alter basal ACTH concentrations, suggesting that CRH, and not urocortin, is the endogenous regulator of ACTH secretion. However, the basal plasma ACTH concentrations in CRH knockout mice are not significantly different from those in the wild-type animals, suggesting that plasma ACTH levels are not solely regulated by CRH, which may be indicative of a role for urocortin in the control of basal ACTH output.

We have shown that urocortin is present and synthesized locally in the fetal sheep pituitary and that, in the ovine fetus, urocortin is an ACTH secretagogue. Furthermore, blocking the local pituitary production of urocortin significantly reduces ACTH output by fetal sheep pituitary cells. These data suggest that urocortin synthesized in the pituitary might act in a paracrine manner to regulate ACTH output in the late-gestation ovine fetus and might explain, in part, how fetal sheep plasma ACTH concentrations are maintained in the absence of hypothalamic input. Moreover, we have shown that intrafetal cortisol administration significantly increased urocortin mRNA expression in the pituitary, and this may contribute to the progressive increase in immunoreactive ACTH concentration of these animals. We speculate that fetal pituitary urocortin is an additional contributor to the prepartum activation of the fetal HPA axis, ensuring concurrent increases in fetal circulating concentrations of ACTH and cortisol and resulting in birth.

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