Endocrine responses of ovariectomized ewes to i.c.v. infusion of urocortin

B J Holmberg, C D Morrison and D H Keisler

Department of Animal Sciences, University of Missouri, Columbia, Missouri 65211, USA

(Requests for offprints should be addressed to D H Keisler; Email: keislerd@missouri.edu)

Abstract

Urocortin is a novel corticotropin-releasing factor-like peptide, first isolated from the rat midbrain, which has anorexigenic properties, possibly associated with its involvement in the stress axis. Urocortin has been implicated in blood pressure regulation, ACTH release and feed intake, but its role as an integral component of the reproduction–nutrition axis has not been examined. The present experiment was designed to determine the effects of i.c.v. infusion of urocortin on feed intake and endocrine profiles of LH, GH, IGF-I, cortisol and leptin in ovariectomized ewes. Ewes were fitted with two latero-cerebroventricular cannulae and urocortin was continuously infused in a linearly increasing manner from 0.001 µg/h on day 0, to a maximum of 31.6 µg/h on day 5. Blood samples were collected via jugular catheters at 10 min intervals for 4 h on day 1, 3 and 5, and assayed by RIA for LH, GH, IGF-I, cortisol and leptin. All ewes were allowed free access to feed and water, and feed intake was recorded daily. Urocortin-infused ewes responded with a

significant decrease in feed intake beginning on day 1 ($P<0.02$) and were aphagic for the remainder of the experiment. Serum concentrations of LH were elevated in individual samples from urocortin-treated compared with saline-treated ewes on day 3 (treatment $\times$ day $\times$ sample, $P=0.05$), but were not different on day 1 or 5. Mean serum concentrations of GH increased ($P<0.04$) over days with urocortin treatment, although concentrations of IGF-I were not influenced by treatment ($P>0.5$). Serum concentrations of cortisol were markedly increased by urocortin treatment ($P<0.001$). Leptin tended to be influenced by treatment and day ($P=0.08$), with leptin levels tending to be elevated in urocortin-treated vs saline-treated ewes on day 5 ($P=0.08$). The ability of urocortin to decrease feed intake while increasing LH, GH, cortisol and leptin provides evidence that urocortin is not only an integral component of the stress axis, but possibly of the nutrition–reproduction axis in sheep.

Journal of Endocrinology (2001) **171**, 517–524

Introduction

Urocortin is a novel anorexigenic peptide with homology to corticotropin-releasing factor (CRF)-like proteins including CRF, urotensin I and sauvagine (Vaughan *et al.* 1995). Urocortin was first isolated from the Edinger–Westphal nucleus of the rat midbrain (Vaughan *et al.* 1995), and has since been detected in other animals including humans (Donaldson *et al.* 1995), Syrian hamsters (Robinson *et al.* 1999) and sheep (Cepoi *et al.* 1999). The physiological functions of urocortin and CRF are similar, most likely due to the cross-reactivity of these peptides for CRF receptors. i.c.v. infusion of CRF into rats activated the stress axis resulting in increased sympathetic activity, increased arousal, increased locomotor activity, decreased exploratory behavior, decreased parasympathetic outflow, and decreased appetite behavior (Spina *et al.* 1996). Similarly, i.v. or i.c.v. infusion of urocortin into rats resulted in dose-related decreases in meal sizes and frequency of food intake, and increased locomotor activity,

but had no effect on exploratory behavior (Spina *et al.* 1996). In lean and *ob/ob* mice, urocortin dose-dependently decreased feed intake, body weight gain and gastric emptying. Urocortin was more potent than CRF, cholecystokinin-8 or leptin at producing these effects (Asakawa *et al.* 1999). In addition, due to its sympathomimetic actions, i.v. infusion of urocortin produced prolonged hypotension, and i.c.v. infusion also resulted in transient hypertension in the rat (Spina *et al.* 1996). Other cardiovascular changes that accompanied i.v. delivery of urocortin in the ovine included increased cardiac contractility, increased aortic flow, increased mean arterial pressure, increased heart rate, increased cardiac output and increased coronary blood flow, but no change in central venous pressure, total peripheral resistance or stroke volume (Parkes *et al.* 1997). Apart from the cardiovascular effects, ewes also exhibited a decrease in feed intake (Parkes *et al.* 1997). Collectively, the physiological effects of CRF and urocortin appear to overlap, with urocortin having more potent anorectic and cardiovascular effects.

Figure 1 Feed intake among urocortin- and saline-infused ewes over days. Intake decreased (* $P < 0.02$, ** $P < 0.001$) in urocortin-treated ewes by day 1, and urocortin-treated ewes were aphagic by day 3.

The objective of the present study was to determine the effect of i.c.v. infusion of urocortin on food intake and pituitary and adrenal hormone release, as well as compen-

satory or synergistic effects on concentrations of leptin and insulin-like growth factor (IGF-I). We hypothesized that urocortin would decrease both food intake and serum concentrations of luteinizing hormone (LH) and increase serum concentrations of growth hormone (GH) through modulation of the hypothalamic-pituitary-adrenal axis.

Materials and Methods

Cross-bred ewes, approximately 8 months of age and weighing 42 ± 5 kg, were randomly assigned to saline control ($n=4$) or urocortin ($n=4$) treatment groups. Ewes were housed in individual pens under ambient conditions with free access to water and a commercially blended pellet diet containing 16% crude protein beginning 2 weeks prior to the commencement of the study. According to procedures approved by the University of Missouri Animal Care and Use Committee, all ewes were bilaterally ovariectomized through a midventral celiotomy. After

Figure 2 Serum concentrations of LH in urocortin- and saline-infused ewes over days. Samples were taken at 10 min intervals on day 1, 3 and 5. Means for the sampling day are plotted after the individual samples. Asterisks indicate a significant difference between individual points in time. For means over days, means with different superscripts are different ($P < 0.05$).

Figure 3 Serum concentrations of GH in urocortin- and saline-infused ewes over days. Samples were taken at 10 min intervals on day 1, 3 and 5. Means for the sampling day are plotted after the individual samples. For means over days, means with different superscripts are different ($P<0.05$). A day effect of GH was also present ($P<0.055$).

2 weeks of post-operative care, ewes were fitted with two lateral cerebroventricular cannulae. Under 1–3% halothane anesthesia, a 5 cm diameter skin flap was removed over the bregma, the periosteum was removed, and two 1.5 mm holes were drilled 1 cm caudal and 1 cm lateral to the bregma. Cannulae were then inserted to a depth of 35 mm, at which point retrograde flow of cerebrospinal fluid occurred and was used as a positive marker for correct placement into the laterocerebroventricles. Protective caps were then attached to the skull with dental acrylic to prevent movement of the cannulae. Ewes were allowed 1 week of recovery before silastic tubing was connected between the i.c.v. cannula and a computer-driven pump system designed to deliver the infusate (saline or rat urocortin (kindly provided by W Vale, Salk Institute, La Jolla, CA, USA) solubilized in saline). Coding regions of ovine and rat urocortin are 84% homologous and the ovine peptide encoded is predicted to be identical to the rat peptide (Cepoi *et al.* 1999). On day 0, the infusion was initiated and the infusate was continuously delivered in a linearly increasing manner. The infusion pump was programmed to deliver vehicle or urocortin beginning at

0.001 $\mu\text{g}(\mu\text{l})/\text{h}$, and then increase 0.3 $\mu\text{g}/\text{h}$ until a rate of 31.6 $\mu\text{g}/\text{h}$ was attained on day 5. This linearly increasing method of delivery was utilized to ensure that animals were exposed to subphysiological through physiological dosages of urocortin over time. Neither physiological nor circulating concentrations of urocortin have previously been reported for the ovine, although dosages of urocortin that are capable of evoking an effect in the ovine have been reported. Thus the manner of urocortin delivery in this study allowed an investigation of urocortin-evoked effects over a continuous range of subphysiological through physiological dosages with intermittent blood sampling.

To assess feed intake, the amount of feed consumed by each ewe was measured at 24 h intervals. Each morning, the amount of feed remaining in each animal's bin was weighed and recorded as an estimate of previous 24 h food intake and 4200 g fresh feed were replaced.

To facilitate repeated blood sample collection, ewes were fitted with indwelling jugular catheters on day -1. On day 1, 3 and 5, blood samples (4 ml) were collected every 10 min for 4 h. After collection of samples, blood

Figure 4 Serum concentrations of cortisol in urocortin- and saline-infused ewes over days. Samples were taken at 10 min intervals on day 1, 3 and 5. Means for the sampling day are plotted after the individual samples. Asterisks indicate a significant difference between individual points in time. For means over days, means with different superscripts are different ($P < 0.05$).

was allowed to clot at 4 °C overnight, and serum was harvested the following day. Serum concentrations of LH (McShane *et al.* 1993), GH (Powell & Keisler 1995), leptin (Delavaud *et al.* 2000) and IGF-I (Lamberson *et al.* 1995) were assessed using RIA procedures reported previously for use in our laboratory, and using a commercial kit for cortisol (Diagnostic Systems Laboratories, Webster, TX, USA). Intra-assay and inter-assay coefficients of variation for LH were 7 ± 1 and $5 \pm 2\%$, and for GH were 4 ± 3 and $7 \pm 1\%$. Intra-assay coefficients of variation for cortisol, leptin and IGF-I were 4 ± 1 , 3 ± 1 and $7 \pm 1\%$ respectively.

At the completion of the experiment, ewes were killed in accordance with procedures approved by the United States Department of Agriculture. Placement of i.c.v. cannulae were confirmed by injection of India ink through the catheter and immediate removal and examination of the diencephalon and telencephalon for stain residue. Stain was located in the lateral cerebroventricles of all animals, confirming delivery of infusate into the laterocerebroventricular system.

Values for LH and GH were subjected to the CLUSTER method of pulse analysis (Veldhuis & Johnson 1986) for determination of number and amplitude of pulses per 4 h sampling period. All hormonal and feed-intake data were analyzed by repeated measures ANOVA using the General Linear Models procedures of SAS. All variables were tested using a statistical model that included treatment, ewe, sample and day. The error term ewe (treatment) was used to test for the effect of treatment, while ewe(treatment $\times$ day) was used to test for the effect of day and treatment $\times$ day. Differences between means were determined using the LSMEANS procedure of SAS with the PDIF option (SAS 1985). Probability values of $P < 0.05$ are considered significant.

Results

On day 0, feed intake did not differ between saline- and urocortin-infused ewes; however, within 24 h of starting the infusions (i.e. day 1), the urocortin-treated ewes

Figure 5 Serum concentrations of leptin in urocortin- and saline-infused ewes over days. Samples were taken at 10 min intervals on treatment day 1, 3 and 5. Means for the sampling day are plotted after the individual samples. Asterisks indicate a significant difference between individual points in time ($P < 0.08$).

responded with a decrease ($P < 0.02$) in feed intake relative to the saline-treated ewes. Feed intake continued to decrease in urocortin-treated ewes ($P < 0.001$), and remained suppressed in urocortin-treated ewes for the duration of the study (Fig. 1).

Mean serum concentrations of LH were influenced by a treatment $\times$ day $\times$ sample interaction ($P = 0.05$). On day 3, individual sample means were higher ($P < 0.05$) in urocortin- vs saline-treated ewes, although no difference between sample means was detected on day 1 or 5 (Fig. 2). No differences were detected in LH pulse frequencies or amplitudes per sampling interval among treatment groups ($P > 0.8$ and $P > 0.7$ respectively).

Mean serum concentrations of GH increased ($P < 0.04$) in urocortin-infused ewes compared with saline-infused ewes (Fig. 3), with the highest concentration occurring on day 5. A significant effect of day was also detected ($P < 0.05$), with GH increasing in a stepwise manner over time. The number of GH pulses per sampling interval increased over days ($P < 0.01$), but was not significantly different from saline-treated ewes on day 1 or 3 ($P > 0.5$). Amplitude of GH pulses increased over days among

urocortin-infused ewes and values were significantly greater than in saline-treated ewes on day 3 and 5 ($P < 0.01$).

Serum concentrations of cortisol were significantly greater ($P < 0.001$) among urocortin- vs saline-treated ewes only on day 5; notably long after urocortin's effects on reducing feed intake were observed (Fig. 4).

Due to the similarity in leptin and urocortin in their anorexigenic properties, serum concentrations of leptin were assessed to determine if i.c.v. infusion of urocortin affected circulating concentrations of leptin. Serum leptin tended to be influenced by treatment and day (treatment $\times$ day, $P = 0.08$), with serum leptin concentrations on day 5 tending to be greater in urocortin- than saline-treated ewes ($P < 0.08$, Fig. 5).

IGF-I was assayed to determine if the increase in serum GH in urocortin-treated ewes was due to the lack of inhibitory feedback by IGF-I on GH-releasing hormone neurons or pituitary somatotrophs. IGF-I did not differ between urocortin- and saline-treated ewes (Fig. 6, $P > 0.5$). There was a day effect, in which IGF-I increased over days within treatments ($P < 0.008$).

Figure 6 Serum concentrations of IGF-I in urocortin- and saline-infused ewes over days. There was no main effect of treatment ($P > 0.1$), although a day effect was present ($P < 0.01$). Means with different superscripts are different ($P < 0.05$).

Discussion

The present study examined the effect of centrally administered (via i.c.v. infusion) urocortin on LH, GH, IGF-I, cortisol, leptin and feed intake, as measures of urocortin's involvement in the reproduction–nutrition–stress axes of ewes. Among urocortin-treated ewes, feed intake declined on day 1 and was negligible by day 3, illustrating that an i.c.v. dose of 12.4 µg or less of urocortin was capable of decreasing feed intake, while 25 µg or less completely abolished feed intake in the ewe. Of notable significance, the decrease in feed intake in the urocortin-treated ewes occurred prior to any changes in measured circulating hormone concentrations. Alterations in hormone levels did not differ until day 3, providing evidence that a low dose of urocortin can suppress feed intake without significantly affecting the secretion of these hormones. Chronic feed restriction alone negatively affects the release of LH and GH (reviewed by Keisler & Lucy 1996). Acute feed restriction also resulted in decreased LH secretion, but

concomitantly increased serum concentrations of GH due most likely to decreased negative feedback from lower circulating IGF-I (Foster *et al.* 1989, Hodgkinson *et al.* 1991).

Despite the similarities between CRF, CRF-like peptides and urocortin, they all have individually unique actions. We observed that in ewes infused i.c.v. with urocortin, LH was increased on day 3 but not day 5. Cortisol negatively affects the hypothalamic–pituitary axis resulting in a decrease in LH release but not its synthesis (Li & Wagner 1983). Therefore, unlike cortisol, urocortin appears to have a stimulatory effect on the secretion of LH. As previously noted, the increase in LH on day 3 was attenuated by day 5. Interestingly, cortisol levels also increased during day 3 to day 5, and therefore may be the stimulus responsible for the attenuation in secretion of LH.

The cross-reactivity of urocortin and CRF for the CRF family of receptors may be responsible for their similar bioactivity. Both peptides bind to CRF-binding proteins, as well as CRF receptors 1, 2a and 2b (Behan *et al.* 1996),

and induce adrenocorticotropin release from the anterior pituitary through the activation of CRF-R1 (Asaba *et al.* 1998). Although both peptides bind CRF receptors, urocortin preferentially binds to CRF-R2a>CRF-R2b>CRF-R1, while CRF has highest affinity for CRF-R1 (Donaldson *et al.* 1995). The distribution of these receptors corresponds to the location of peptide, suggesting that urocortin is the endogenous ligand for CRF-R2a. CRF-R2a are primarily located peripherally in the duodenum, skeletal muscle and perivascular cells of the heart (Perrin *et al.* 1995), and centrally in the lateral septal nuclei and ventromedial hypothalamic nuclei (Lovenberg *et al.* 1995). Urocortin has been localized in the Edinger–Westphal nucleus, lateral superior olivary nucleus, lateral hypothalamus, supraoptic nucleus (Bittencourt *et al.* 1999) and pituitary (Oki *et al.* 1998). Neither CRF-R1 nor CRF mRNA is abundant in these areas and they tend to be absent in diencephalic nuclei, except for the paraventricular nucleus of the hypothalamus (Matthews *et al.* 1991). Therefore, the interaction of urocortin with CRF-R2a in the ventromedial and lateral hypothalamus (known ‘appetite centers’) may be responsible for the anorexia exhibited by animals treated with urocortin.

We hypothesized that GH would increase in urocortin-infused ewes due to lack of IGF-I negative feedback in response to the decrease in feed intake. GH was significantly elevated due to treatment, increasing in a stepwise manner over time in urocortin- but not saline-infused ewes. Since IGF-I concentrations were not different between treatment groups, the increase in GH over days may have been due to stimulation by urocortin and not due to a lack of IGF-I. Somatotrophs express CRF-R2 and stimulation of these receptors in human pituitary adenoma cells by urocortin resulted in an increase in GH secretion in 50% of cells (Murakami *et al.* 1997).

Cortisol, as well as other hormones such as insulin, stimulates the expression of leptin (Houseknecht *et al.* 1998). Both leptin and urocortin suppress appetite and also appear to regulate the expression of the other. *i.c.v.* infusion of urocortin tended to increase serum concentrations of leptin. This response may be a direct action of urocortin due to entry into the blood stream, or more likely is secondary to the increase in cortisol secretion.

In summary, urocortin is an anorexigenic peptide that may serve as an integral component within the nutrition, reproductive and/or stress axes of ewes. Utilizing physiological to perhaps pharmacological levels of urocortin, appetitive and endocrinological responses were elicited in ewes following central urocortin administration.

Acknowledgements

This is Missouri Agricultural Experimental Station Journal Series Number 13,079.

References

- Asaba K, Makino S & Hashimoto K 1998 Effect of urocortin on ACTH secretion from rat anterior pituitary *in vitro* and *in vivo*: comparison with corticotropin-releasing hormone. *Brain Research* **806** 95–103.
- Asakawa A, Inui A, Ueno N, Makino S, Fujino MA & Kasuga M 1999 Urocortin reduces food intake and gastric emptying in lean and *ob/ob* mice. *Gastroenterology* **116** 1287–1292.
- Behan DP, Khongsaly O, Ling N & DeSouza EB 1996 Urocortin interaction with corticotropin-releasing factor (CRF) binding protein (CRF-BP): a novel mechanism for elevating ‘free’ CRF levels in human brain. *Brain Research* **725** 263–267.
- Bittencourt JC, Vaughan J, Arias C, Rissman RA, Vale WW & Sawchenko PE 1999 Urocortin expression in the rat brain: evidence against a pervasive relationship of urocortin-containing projections with targets bearing type 2 CRF receptors. *Journal of Comparative Neurology* **415** 285–312.
- Cepoi D, Sutton S, Arias C, Sawchenko P & Vale WW 1999 Ovine genomic urocortin: cloning, pharmacologic characterization, and distribution of central mRNA. *Brain Research* **68** 109–118.
- Delavaud C, Bocquier F, Chilliard Y, Keisler DH, Gertler A & Kann G 2000 Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep. *Journal of Endocrinology* **165** 519–526.
- Donaldson CJ, Sutton S, Perrin MH, Corrigan AZ, Lewis KA, Rivier JE, Vaughan JM & Vale WW 1995 Cloning and characterization of human urocortin. *Endocrinology* **137** 2167–2170.
- Foster DL, Ebling FJ, Micka AF, Vannerson LA, Bucholtz DC, Wood RI, Suttie JM & Fenner DE 1989 Metabolic interfaces between growth and reproduction. I. Nutritional modulation of gonadotropin, prolactin, and growth hormone secretion in the growth-limited female lamb. *Endocrinology* **125** 342–350.
- Hodgkinson SC, Bass JJ & Gluckman PD 1991 Plasma IGF-I binding proteins in sheep: effect of recombinant growth hormone treatment and nutritional status. *Domestic Animal Endocrinology* **8** 343–351.
- Houseknecht KL, Baile CA, Matteri RL & Spurlock ME 1998 The biology of leptin: a review. *Journal of Animal Science* **76** 1405–1420.
- Keisler DH & Lucy MC 1996 Perception and interpretation of suboptimal nutrition by the reproductive system. *Journal of Animal Science* **74** (Suppl 3) 1–17.
- Lamberson WR, Safranski TJ, Bates RO, Keisler DH & Matteri RL 1995 Relationships of serum IGF-I concentrations to growth, composition and reproductive traits of swine. *Journal of Animal Science* **73** 3241–3245.
- Li PS & Wagner WS 1983 *In vivo* and *In vitro* studies on the effect of adrenocorticotrophic hormone or cortisol on the pituitary response to gonadotropin releasing hormone. *Biology of Reproduction* **29** 25–37.
- Lovenberg TW, Liaw CW, Grigoriadis DE, Clevenger W, Chalmers DT, DeSouza EB & Oltersdorf T 1995 Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. *PNAS* **92** 836–840.
- McShane TM, Petersen SL, McCrone S & Keisler DH 1993 Influence of food restriction on neuropeptide-Y, proopiomelanocortin and LHRH gene expression in sheep hypothalami. *Biology of Reproduction* **49** 831–839.
- Matthews SG, Heavens RP & Sirinathsinghji DJ 1991 Cellular localization of corticotropin releasing factor mRNA in the ovine brain. *Brain Research* **11** 171–176.
- Murakami Y, Mori T, Koshimura K, Kurosaki M, Hori T, Yanaihara N & Kato Y 1997 Stimulation by urocortin of growth hormone (GH) secretion in GH-producing human pituitary adenoma cells. *Endocrine Journal* **44** 627–629.
- Oki Y, Iwabuchi M, Masuzawa M, Watanabe F, Ozawa M, Iino K, Tominaga T & Yoshimi T 1998 Distribution and concentration of urocortin, and effect of adrenalectomy on its content in rat hypothalamus. *Life Sciences* **62** 807–812.

- Parkes DG, Vaughan J, Rivier J, Vale W & May CN 1997 Cardiac inotropic actions of urocortin in conscious sheep. *American Journal of Physiology* **272** H2115–H2122.
- Perrin M, Donaldson C, Chen R, Blount A, Berggren T, Bilezikjian L, Sawchenko P & Vale W 1995 Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. *PNAS* **92** 2969–2973.
- Powell M & Keisler DH 1995 A potential strategy for decreasing milk production in the ewe at weaning using a growth hormone release blocker. *Journal of Animal Science* **73** 1901–1905.
- Robinson BM, Tellam DJ, Smart D, Mohammad YN, Brennand J, Rivier JE & Lovejoy DA 1999 Cloning and characterization of corticotropin-releasing factor and urocortin in Syrian hamster (*Mesocricetus auratus*). *Peptides* **20** 1177–1185.
- SAS 1985 *SAS User's Guide: Statistics*. Cary, NC: SAS Institution Inc.
- Spina M, Merlo-Pich E, Chan RKW, Basso AM, Rivier J, Vale W & Koob GF 1996 Appetite suppressing effects of urocortin, a CRF-related neuropeptide. *Science* **273** 1561–1563.
- Vaughan J, Donaldson C, Brittencourt J, Perrin M, Lewis K, Sutton S, Chan R, Turnbull A, Lovejoy D, Rivier C, Rivier J, Sawchenko PE & Vale WW 1995 Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotrophin-releasing factor. *Nature* **378** 287–292.
- Veldhuis JD & Johnson ML 1986 Cluster analysis: a simple, versatile and robust algorithm for endocrine pulse detection. *American Journal of Physiology* **250** E486.

Received in final form 9 August 2001

Accepted 9 August 2001