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Bacterial Lipopolysaccharide-Induced Coordinate Downregulation of Arginine Vasopressin Receptor V3 and Corticotropin-Releasing Factor Receptor 1 Messenger Ribonucleic Acids in the Anterior Pituitary of Endotoxemic Steers

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AVP and CRF are potent stimulators of pituitary ACTH secretion in cattle. Actions of AVP and CRF at the anterior pituitary are mediated by AVP receptor V3 (V3) and CRF receptor 1 (CRFR1). The primary objective of these studies was to determine the effect of systemic inflammatory stress on V3 and CRFR1 mRNAs in the bovine anterior pituitary. Holstein steers ($n = 20$) were injected with 200 ng/kg bacterial lipopolysaccharide (LPS) and tissues collected 0, 2, 4, 12, and 24 h later. All animals responded to LPS administration with an increase in body temperature, plasma ACTH, and cortisol ($p < 0.05$). Abundance of anterior pituitary V3 mRNA was decreased at 2, 4, and 12 h following LPS administration ($p < 0.05$) and returned to basal by 24 h. A similar temporal regulation of pituitary CRFR1 mRNA ($p < 0.05$), but not pituitary pro-opiomelanocortin (POMC) mRNA, was observed following LPS administration. Similar downregulation of CRFR1 mRNA was not observed in other brain regions following LPS administration (cerebellum, hypothalamus). Our results indicate that V3 and CRFR1 mRNAs are coordinately downregulated in the anterior pituitary during systemic inflammatory stress. Decreased AVP and CRF receptor expression may help regulate the pituitary–adrenal response to stress.

Key Words: Arginine vasopressin receptor V3; corticotropin-releasing factor receptor 1; anterior pituitary; LPS; POMC; bovine.

Introduction

Maintenance of homeostasis in response to stress is critical for survival and optimal performance. Homeostasis is maintained through adaptational responses believed to counteract the effects of aversive stimuli. However, chronic exposure to stress or exposure to stress at inopportune times can have a pronounced negative impact on health and well-being in livestock species *(1–3)* and humans *(4,5)*. For example, stress is a major factor contributing to the etiology of several prominent disease conditions in cattle, such as bovine respiratory disease *(6)* and Johne's disease *(7)*. However, the mechanisms that control the response to stress in cattle are not understood.

In response to stress, parvocellular neurons in the paraventricular region of the hypothalamus (PVN), terminating in the median eminence, release factors that stimulate anterior pituitary secretion of ACTH *(8,9)*. The two primary hypothalamic releasing factors that regulate basal and stress-induced secretion of ACTH are CRF and AVP *(10)*. In most instances, CRF is the primary hypophysiotropic factor controlling ACTH secretion. The extent to which AVP stimulates ACTH secretion depends on the species in question *(11–15)*. In the bovine model system, AVP has significant ACTH-releasing activity independent of CRF *(16)*. Coordinate regulation of hypothalamic synthesis and secretion of CRF and AVP and of pituitary responsiveness to trophic hormone stimulation is critical for regulation of the endocrine response to stress.

The AVP receptor V3 (V3) and CRF receptor 1 (CRFR1) mediate actions of CRF and AVP at the level of the pituitary *(17,18)*. Changes in pituitary CRF and AVP receptors are critical determinants of pituitary corticotrope responsiveness to hypothalamic stimulation and hence help regulate the magnitude and duration of the endocrine response to stress *(19–21)*. Although the mechanisms that regulate pituitary responsiveness to CRF and AVP are not completely understood, data from studies in rats indicate that anterior pituitary V3 and CRFR1 mRNAs are differentially regulated. For example, CRFR1 mRNA in cultured rat pituitary cells is decreased in response to glucocorticoids *(22)*, whereas V3 mRNA is increased after treating rats with dexamethasone *(23)*. Potential coordinate regulation of pituitary CRF and AVP receptors during systemic inflammatory stress has not been investigated. Such information is critical to the elucidation of the mechanisms that control the endocrine response to stress, particularly in species such as cattle,

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where CRF and AVP both have potent ACTH-releasing capacities *(16)*.

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, stimulates a profound inflammatory response and activation of the HPA axis *(24–26)*. This provides a powerful tool for studying physiological regulation of CRF and AVP receptors by endocrine and inflammatory stimuli in cattle. We hypothesized that pituitary CRFR1 and V3 mRNAs are dynamically regulated during systemic inflammatory stress in cattle and in a tissue specific manner. In the present study, the relative abundance of V3 and CRFR1 mRNAs in the bovine anterior pituitary was examined prior to and at several time-points after LPS administration. Changes in V3 and CRFR1 mRNAs were correlated with LPS-induced changes in plasma ACTH and cortisol. The effect of LPS administration on the abundance of anterior pituitary mRNA for pro-opiomelanocortin (POMC) was also investigated to determine whether LPS-induced regulation of mRNA for other components of the pituitary ACTH-producing machinery is similar to that observed for CRFR1 and V3 mRNAs. To determine tissue specificity of the effects of LPS administration on CRFR1 mRNA, the relative abundance of CRFR1 mRNA in the hypothalamus and cerebellum during LPS-induced systemic inflammatory stress was also determined. Our studies established that V3 and CRFR1 mRNAs are coordinately downregulated in the bovine anterior pituitary during systemic inflammatory stress and suggest that dynamic regulation of V3 and CRFR1 expression may represent a potential mechanism to help regulate the pituitary–adrenal response to stress in cattle.

Results

Effects of LPS on Plasma ACTH and Cortisol Concentrations and Body Temperature

Lipopolysaccharide administration induced significant increases ($p < 0.05$) in plasma concentrations of ACTH at 2 and 4 h (Fig. 1) and cortisol at 2, 4, and 12 h (Fig. 2) after LPS administration compared to 0 h (pre-LPS) hormone concentrations. Body temperature was also significantly increased ($p < 0.05$) at 2 and 4 h after LPS administration (Fig. 3).

Expression of V3 mRNA in the Bovine Anterior Pituitary During Systemic Inflammatory Stress

In the present study, relative levels of anterior pituitary V3 mRNA were measured to determine if V3 expression is regulated during systemic inflammatory stress. Northern analysis of bovine anterior pituitary RNA collected at 0, 2, 4, 12, and 24 h after LPS administration revealed a single 7.0-kb V3 mRNA transcript (Fig. 4A). Expression of V3 mRNA was significantly decreased ($p < 0.05$) at 2, 4 and 12 h following LPS administration and returned to basal (pre-LPS) levels by 24 h (Fig. 4B). Relative levels of anterior pituitary V3 receptor mRNA were negatively correlated with plasma ACTH and cortisol ($r = -0.65$ and -0.78, respectively; $p < 0.05$).

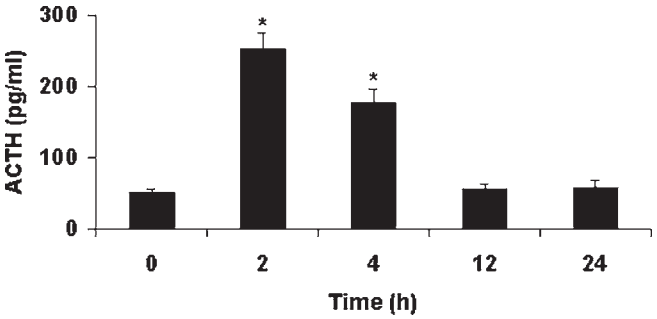

Fig. 1. Effect of LPS administration on plasma ACTH concentrations in Holstein steers. Blood was drawn through indwelling jugular catheters inserted 48 h before experimentation. Blood was collected from all animals up until the respective time of tissue collection ($n = 4$ per time-point). Time 0 = time of LPS administration. (Mean ± SEM; *$p < 0.05$ vs 0 h.)

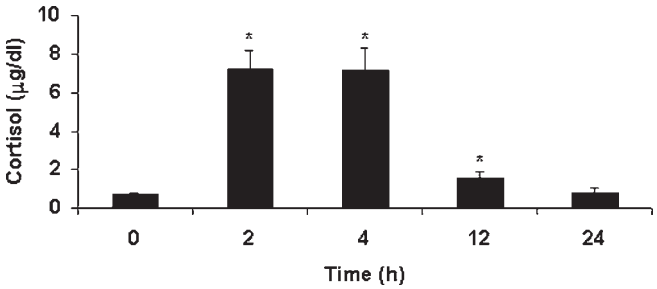

Fig. 2. Effect of LPS administration on plasma cortisol concentrations in Holstein steers. Blood was drawn through indwelling jugular catheters inserted 48 h before experimentation. Blood was collected from all animals up until the respective time of tissue collection ($n = 4$ per time-point). Time 0 = time of LPS administration. (Mean ± SEM; *$p < 0.05$ vs 0 h.)

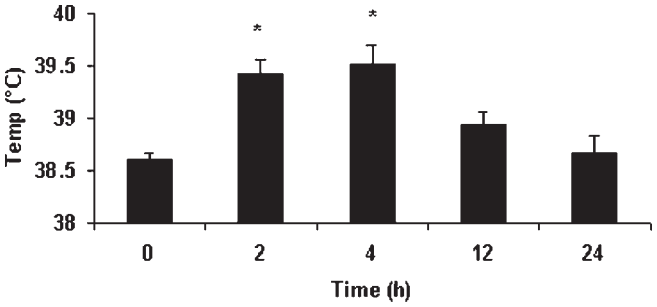

Fig. 3. Effect of LPS administration on body temperatures in Holstein steers ($n = 4$ per time-point). Note the significant increase in body temperatures by 2 h after LPS administration. Time 0 = time of LPS administration. (Mean ± SEM; *$p < 0.05$ vs 0 h.)

Expression of CRFR1 mRNA in the Bovine Anterior Pituitary During Systemic Inflammatory Stress

The effect of LPS administration on CRFR1 mRNA expression in the anterior pituitary was also investigated to determine whether the temporal regulation of CRFR1 and

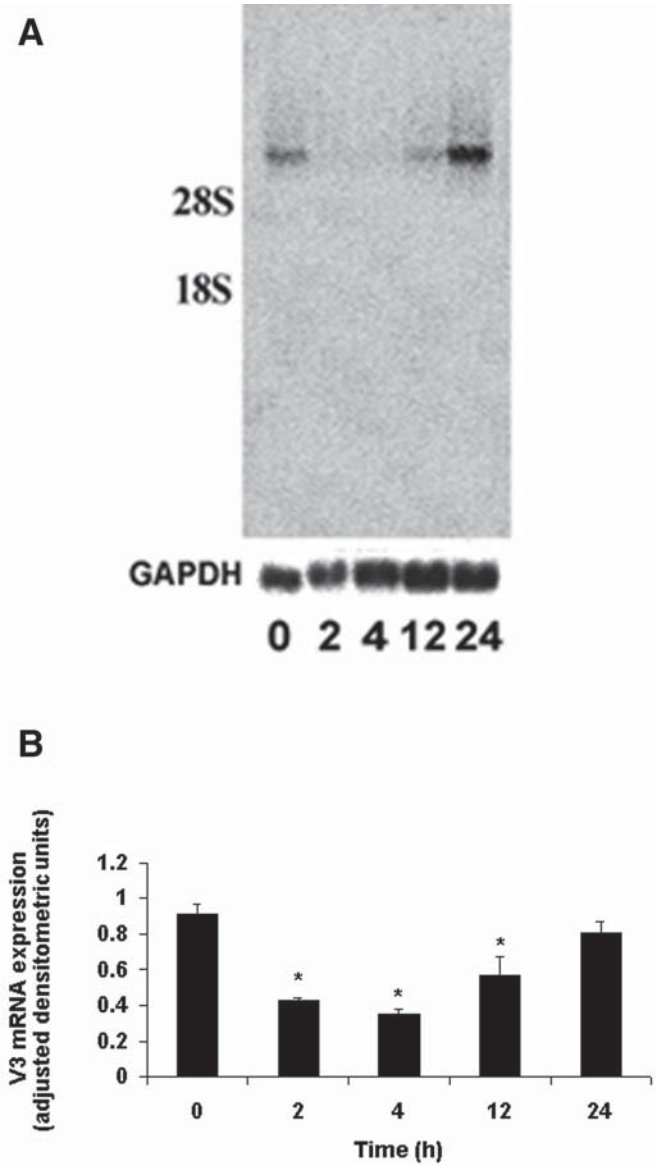

Fig. 4. Effect of systemic inflammatory stress on V3 mRNA abundance in the bovine anterior pituitary. (**A**) Northern blot analysis of V3 mRNA in bovine anterior pituitaries collected at 0, 2, 4, 12, and 24 h after LPS administration. Note hybridization to a 7.0-kb transcript. (**B**) Quantification of anterior pituitary V3 mRNA abundance at 0, 2, 4, 12, and 24 h after LPS administration ($n = 4$ animals per time-point). Pituitary V3 mRNA levels were adjusted relative to levels of GAPDH mRNA (adjusted densitometric units). Note significant decrease in V3 mRNA by 2 h after LPS administration. (Mean ± SEM; *$p < 0.05$ vs 0 h.)

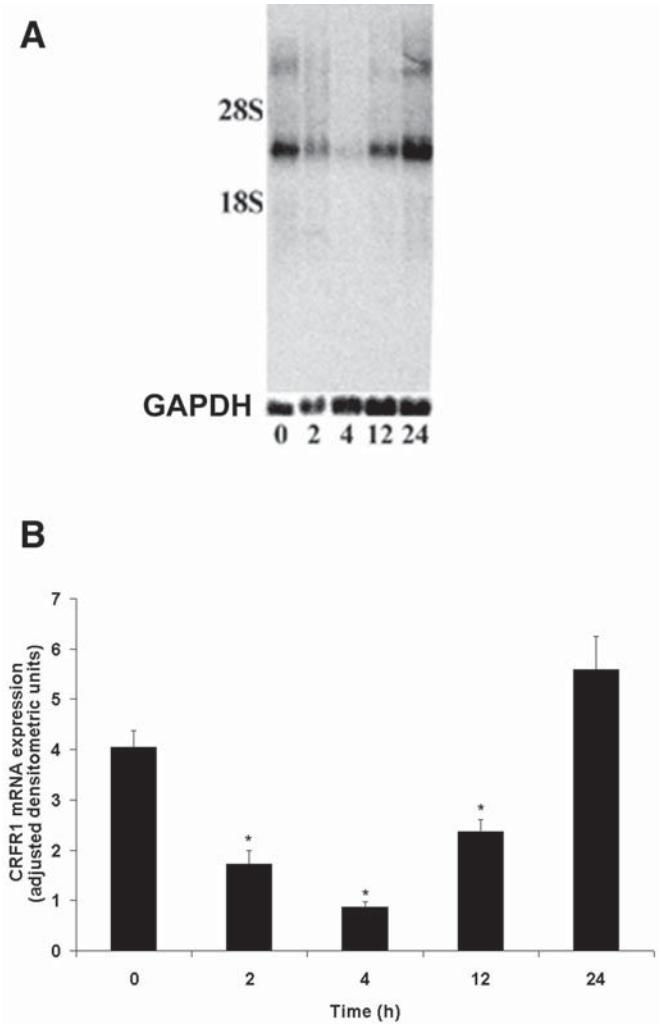

Fig. 5. Effect of systemic inflammatory stress on CRFR1 mRNA abundance in the bovine anterior pituitary. (**A**) Northern blot analysis of CRFR1 mRNA in bovine anterior pituitaries collected at 0, 2, 4, 12, and 24 h after LPS administration. Note hybridization to a major 2.5-kb and a minor 8.0-kb transcript. (**B**) Quantification of anterior pituitary CRFR1 mRNA abundance at 0, 2, 4, 12, and 24 h after LPS administration ($n = 4$ animals per time-point). CRFR1 mRNA levels were adjusted relative to levels of GAPDH mRNA (adjusted densitometric units). Note significant decrease in CRFR1 mRNA by 2 h after LPS administration. (Mean ± SEM; *$p < 0.05$ vs 0 h.)

V3 mRNAs during systemic inflammatory stress in cattle are similar. Northern analysis of bovine anterior pituitary RNA collected at 0, 2, 4, 12, and 24 h after LPS administration revealed two CRFR1 mRNA species. The bovine CRFR1 cDNA hybridized to a predominant 2.5-kb transcript and a second minor 8.0-kb transcript (Fig. 5A). Like V3 mRNA, anterior pituitary CRFR1 mRNA was also downregulated following LPS administration. Relative levels of CRFR1 mRNA were significantly decreased ($p < 0.05$) at 2, 4, and 12 h following LPS administration and returned to basal by 24 h (Fig. 5B). Relative levels of pituitary CRFR1 mRNA were also negatively correlated with plasma ACTH and cortisol concentrations ($r = –0.66$ and $–0.78$, respectively; $p < 0.05$).

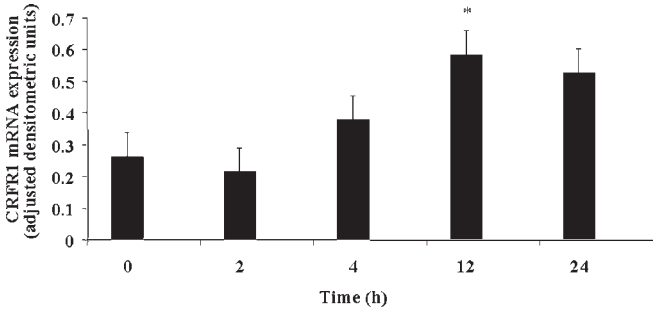

Fig. 6. Effect of systemic inflammatory stress on CRFR1 mRNA abundance in the bovine hypothalamus. Quantification of hypothalamic CRFR1 mRNA abundance at 0, 2, 4, 12, and 24 h after LPS administration ($n = 4$ animals per time-point). CRFR1 mRNA levels were adjusted relative to levels of GAPDH mRNA (adjusted densitometric units). Note significant increase in CRFR1 mRNA by 12 h after LPS administration. (Mean ± SEM; $*p < 0.05$ vs 0 h.)

Differential Regulation of CRFR1 mRNA in Other Bovine Brain Regions During Systemic Inflammatory Stress

CRFR1 mRNA levels were also measured in hypothalamic and cerebellar tissue collected from the same animals to determine if CRFR1 mRNA is coordinately downregulated in other brain regions following LPS administration. The number and size of CRFR1 mRNA transcripts in the cerebellum and hypothalamus were not different than that observed in the anterior pituitary (data not shown). Relative abundance of CRFR1 mRNA in the cerebellum was not affected by LPS administration ($p > 0.05$; data not shown). However, CRFR1 mRNA was significantly upregulated in the hypothalamus at 12 h following LPS administration (Fig. 6; $p < 0.05$).

Expression of POMC mRNA in the Bovine Anterior Pituitary During Systemic Inflammatory Stress

Regulation of pituitary POMC mRNA following LPS administration was examined to determine if other components of the pituitary ACTH producing machinery are also dynamically regulated in a similar fashion as V3 and CRFR1 mRNAs during systemic inflammatory stress in cattle. Northern analysis revealed a prominent 1-kb transcript for POMC in the bovine anterior pituitary (Fig. 7A). However, administration of LPS did not affect POMC mRNA abundance in the bovine anterior pituitary ($p > 0.05$; Fig. 7B).

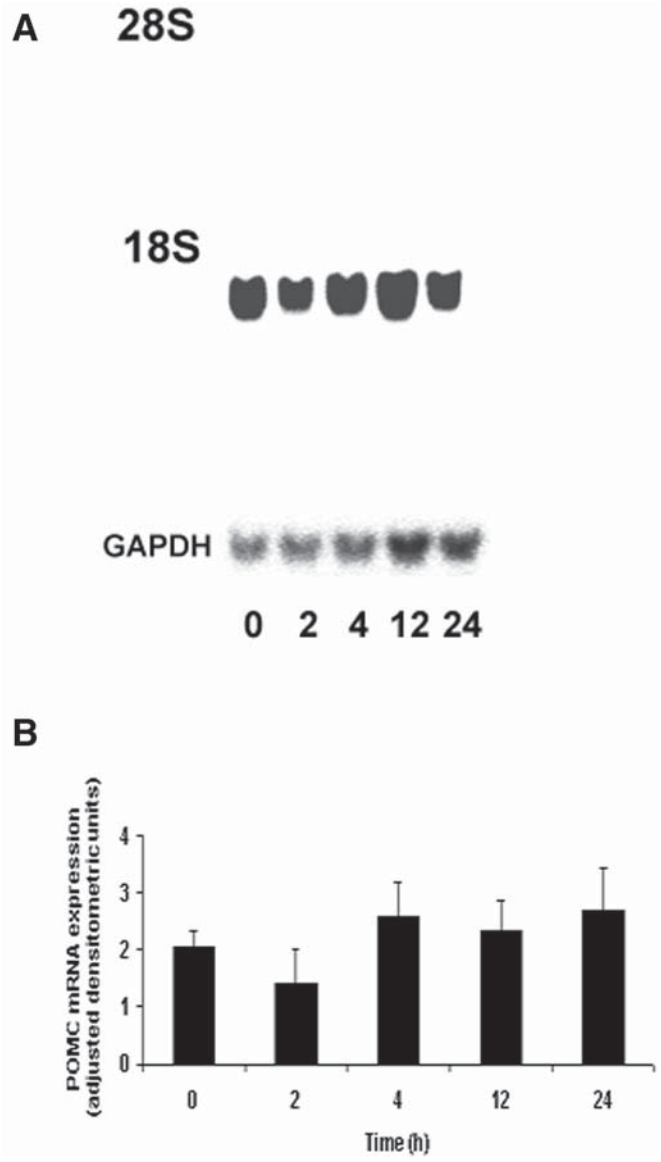

Fig. 7. Effect of systemic inflammatory stress on POMC mRNA abundance in the bovine anterior pituitary. (**A**) Northern blot analysis of POMC mRNA in bovine anterior pituitaries collected at 0, 2, 4, 12, and 24 h after LPS administration. Note hybridization to a 1.0-kb transcript. (**B**) Quantification of anterior pituitary POMC mRNA abundance at 0, 2, 4, 12, and 24 h after LPS administration ($n = 4$ animals per time-point). POMC mRNA levels were adjusted relative to levels of GAPDH mRNA (adjusted densitometric units). No effect of LPS administration on POMC mRNA in the anterior pituitary was observed. (Mean ± SEM; $p > 0.05$.)

Discussion

Lipopolysaccharide, a component of the cell wall of Gram-negative bacteria, elicits a profound activation of the hypothalamic-pituitary-adrenocortical (HPA) axis. In response to LPS, CRF and AVP stimulate pituitary ACTH secretion *(27)*. In turn, ACTH stimulates adrenal synthesis and secretion of glucocorticoids, which may regulate the endocrine response to inflammatory stress through negative feed-back at the level of the hypothalamus and the pituitary *(28–30)*. Regulation of anterior pituitary AVP and CRF receptors represents a potential mechanism to control the magnitude and duration of the endocrine response to stress.

As an initial step toward understanding the regulation of anterior pituitary AVP and CRF receptors during a stress response, the effects of LPS administration on V3 and CRFR1 mRNAs in the bovine anterior pituitary were determined. Comparisons were made with the effects of systemic inflam-

matory stress on CRFR1 mRNA in the hypothalamus and cerebellum and with effects on anterior pituitary POMC mRNA in order to assess specificity of the regulatory response for V3 and CRFR1 mRNAs observed in the anterior pituitary.

In the present study, anterior pituitary V3 mRNA was dramatically downregulated in response to LPS administration. To our knowledge, the effect of systemic inflammatory stress on anterior pituitary V3 mRNA has not been previously reported. However, evidence in the literature suggests that V3 mRNA in the pituitary is subject to feedback regulation during other stress paradigms in rats. For example, anterior pituitary V3 mRNA is increased following 4 h of immobilization stress or following chronic stress paradigms *(31)*. Conversely, levels of V3 mRNA are decreased following adrenalectomy, and the effect is reversed by glucocorticoid replacement *(23)*. However, anterior pituitary V3 mRNA is increased following glucocorticoid administration to adrenal intact rats *(32)*. In the present study, anterior pituitary V3 mRNA levels were significantly decreased following LPS administration in cattle, despite the pronounced increase in circulating cortisol. Such results may be reflective of species differences in glucocorticoid regulation of pituitary V3 mRNA and (or) dominant negative regulation of V3 mRNA through other mechanisms during systemic inflammatory stress in cattle.

In the present study, anterior pituitary V3 and CRFR1 mRNA levels were coordinately downregulated in response to LPS administration. To our knowledge, coordinate downregulation of pituitary V3 and CRFR1 mRNAs during a stress response has not been previously established. However, downregulation of pituitary CRFR1 mRNA following LPS administration has also been demonstrated in rats *(27)*. The mechanisms responsible for reductions in CRFR1 mRNA during inflammatory stress are not clear. Decreases in pituitary CRFR1 mRNA may be mediated by the actions of cytokines and other inflammatory mediators, CRF, AVP, and/or glucocorticoids. Treatment of rat anterior pituitary cells with CRF decreases relative levels of CRFR1 mRNA in vitro (*22*,*28*). However, immunoneutralization of CRF does not attenuate LPS-induced downregulation of pituitary CRFR1 mRNA in vivo *(27)*. These results suggest that downregulation of CRFR1 mRNA during LPS-induced endotoxemia is not solely a result of the actions of CRF. Messenger RNA for CRFR1 in rat pituitary cells is also decreased in vitro following treatment with LPS or the proinflammatory cytokine interleukin (IL)-1β *(27)*. These results suggest that the pituitary can respond directly to inflammatory stimuli with a decrease in CRFR1 mRNA. More investigation will be required to determine the precise mechanisms responsible for CRFR1 and V3 mRNA downregulation during LPS-induced systemic inflammatory stress in cattle. It is also possible that the coordinate downregulation of pituitary CRFR1 and V3 mRNAs observed is unique to the bovine model system utilized versus other rodent model systems studied to date. Coordinate downregulation of both pituitary CRFR1 and V3 mRNAs in the bovine model system is logical given the fact that both CRF and AVP are potent stimulators of pituitary ACTH secretion in cattle *(16)*, whereas effects of AVP are primarily synergistic in rodents and AVP has little CRF-independent ACTH-releasing activity *(10)*.

Messenger RNA for CRFR1 is widely distributed throughout the central nervous system *(33)*. Roles for CRF in the stress response independent of HPA axis activation have been established *(8,34)*. Therefore, CRFR1 may be differentially regulated in specific brain regions in response to stress. In the present studies, anterior pituitary CRFR1 mRNA was significantly reduced following LPS administration, but levels of CRFR1 mRNA in the cerebellum were not affected and expression in the hypothalamus was increased. Tissue-specific regulation of CRFR1 mRNA in the central nervous system in response to stress has been observed previously in rats. Hippocampal CRFR1 mRNA is increased, whereas CRFR1 mRNA in the frontal cortex is decreased in response to chronic stress *(35)*. Likewise, CRFR1 mRNA is increased in the PVN *(36)* but decreased in the pituitary *(27)* of LPS-treated rats. Our results support the hypothesis that tissue-specific regulation of CRFR1 mRNA in the anterior pituitary occurs during LPS-induced endotoxemia in cattle.

The relative abundance of POMC mRNA in the bovine anterior pituitary did not change in response to LPS administration. These results were in contrast to the dynamic regulation of anterior pituitary V3 and CRFR1 mRNAs observed in the present study and somewhat surprising, because pituitary POMC mRNA in rats is increased in response to LPS administration *(26,37)*. In addition, CRF and glucocorticoids are also potent regulators of ACTH secretion and POMC mRNA expression in rats. Pituitary POMC mRNA is increased in rats following adrenalectomy, and the effect is reversed by glucocorticoid replacement *(38–40)*. Messenger RNA for POMC is also increased following treatment of mouse pituitary tumor cells (AtT-20) with CRF *(41)*. Our results indicate that regulatory mechanisms similar to those observed for CRFR1 and V3 mRNAs do not control anterior pituitary expression of POMC mRNA during systemic inflammatory stress in cattle. Regulation of pituitary POMC mRNA expression does not appear to be a primary mechanism to help regulate the ACTH response to LPS-induced immune activation in cattle.

In summary, systemic LPS administration caused a decrease in V3 and CRFR1 mRNAs in the bovine anterior pituitary. The decreases in V3 and CRFR1 mRNAs were negatively correlated with concentrations of circulating ACTH and cortisol and were tissue-specific. Downregulation of V3 and CRFR1 mRNAs following LPS administration may represent a potential mechanism to help regulate anterior pituitary responsiveness to CRF and AVP and potentially help limit the magnitude and/or duration of the endocrine response to systemic inflammatory stress in cattle. Whether similar

regulation is observed in other species where AVP does not play a direct (CRF independent) role in mediating pituitary ACTH secretion is not yet known. Future studies will also be required to determine the specific cellular and molecular mechanisms responsible for the LPS-induced decrease in anterior pituitary V3 and CRFR1 mRNAs in cattle.

Materials and Methods

Animals

All experiments were approved by the All University Committee on Animal Use and Care at Michigan State University (Approval #10/98-141-00). Holstein steers (n = 20) ranging between 110 and 160 kg (approx 14–20 wk of age) were utilized. Animals were maintained on a 16:8 light:dark cycle in environmentally controlled rooms at the Michigan State University Dairy Cattle Teaching and Research Center. Animals were fed a pelleted diet (18% crude protein and 19.6% acid detergent fiber; Land O'Lakes, Indianapolis, IN) ad libitum between 1000 AM and 1200 PM daily with unlimited access to water and allowed to acclimate to rooms for a minimum of 14 d before initiation of experiments. At least 48 h before initiation of experiments, animals were weighed for dose calculations and jugular catheters were inserted for treatment administration and blood collection.

Treatments

One milligram of LPS (*Escherichia coli* B:055; Sigma, St. Louis, MO) was solubilized in 1 mL of sterile water and diluted in sterile 0.9% saline to a final concentration of 0.2 mg/mL. Treatments consisted of 0.2 μg/kg LPS (0.1–0.2 mL) or diluent (0.1 mL) administered intravenously at time 0. The dose of LPS used was selected based on results from preliminary dose-response experiments (data not shown).

Blood Sampling and Temperature Measurements

For measurement of plasma ACTH and cortisol concentrations, blood samples were collected at the following intervals until tissue collection; 30-min intervals for 1 h before administration of treatments (time 0), 15-min intervals for 1 h after treatment administration, 30-min intervals for h 2–6 after administration of treatments, then hourly for h 3–9 and every 3 h for h 10–18. Final samples were collected at 24 h. Blood was collected into chilled polypropylene tubes containing 1.4 mg EDTA/mL and centrifuged immediately at approx 1500*g* for 5 min. Plasma was aliquoted into chilled microcentrifuge tubes and stored at –20ºC. Rectal temperatures were taken hourly from 1 h prior to treatment through 9 h after treatment administration to monitor the pyrogenic response to systemic LPS challenge.

ACTH Assay

Plasma ACTH concentrations were measured using a double antibody human ACTH radioimmunoassay (RIA; DiaSorin, Inc., Stillwater, MN) validated for bovine ACTH. Bovine ACTH, a 39-amino-acid polypeptide, is identical to human ACTH in the first 24 amino acids. Therefore, a human assay that specifically recognizes the first 24 amino acids of the peptide can be utilized. For validation, a pool of bovine plasma was collected 15 min after bolus injection of 250 μg bovine CRF into a Holstein cow. Serial dilutions of this high ACTH pool in the assay's zero standard were parallel in binding properties to the assay's standard curve. The sensitivity of the assay, determined as the value 2 standard deviations away from the mean of 10 replicates of the zero standard, was approximately 10 pg/mL. Interassay and intra-assay coefficients of variation were calculated from a 1:2 dilution of the ACTH high-pool run in each assay and 12 times in a single assay. The interassay and intra-assay coefficients of variation were 8.9% and 8.0%, respectively.

Cortisol Assay

Cortisol was assayed using a Coat a Count RIA kit (Diagnostics Products, Inc., Los Angeles, CA). The cortisol assay was validated as described for ACTH. A high pool of cortisol was obtained by drawing blood 30 min after bolus injection of 250 μg bovine CRF. The sensitivity of the assay was determined at 0.02 μg/dL and interassay and intra-assay coefficients of variation were 4.3% and 3.7%, respectively.

Tissue Collection

Steers were euthanized with an intravenous injection of 80 mg/kg sodium pentobarbital (Sigma, St. Louis, MO) at 0, 2, 4, 12, or 24 h after LPS administration (n = 4 per timepoint). Pituitary glands were removed and the anterior lobes were dissected from posterior and neurointermediate lobes, frozen in liquid nitrogen, and stored at –80ºC until RNA isolation. Hypothalamic tissue and a portion of the cerebellum were also collected from each animal. For hypothalamic tissue collection, the entire hemihypothalamus (right side) from the optic chiasm (anterior) to the optic nerves (posterior) and from the third ventricle laterally to the hypothalamic sulci was dissected from each animal and processed as described earlier. All hypothalamic nuclei were represented in these samples, including the periventricular, paraventricular, arcuate, anterior hypothalamic, medial hypothalamic, posterior hypothalamic, dorsal hypothalamic, supra-chiasmatic and preoptic nuclei, and so forth. An approx 3-cm^3 portion of the cerebellum was then collected (from the anterior lobe) and processed as described earlier. Efforts were made to collect tissue from approximately the same region in all animals.

Cloning of Bovine V3, CRFR1, and POMC cDNAs

Primers derived from the reported sequence of the human and rat CRFR1 cDNAs were used to amplify a 461-bp bovine CRFR1 cDNA (Genbank accession #AF420212). Primers derived from the reported sequence of the human and rat V3 receptor cDNAs were used to amplify a 627-bp bovine V3 cDNA (Genbank accession #AF420213). Primers derived

from the reported sequence of the bovine POMC cDNA (Genbank accession #M38606) were used to amplify a 297-bp bovine POMC cDNA. All cDNAs were amplified from bovine anterior pituitary total RNA using reverse transcription–polymerase chain reaction (RT-PCR) procedures. The PCR products were ligated into the PGEM-T Easy vector (Promega, Madison, WI). Dye terminator and dye primer sequencing were used to obtain the nucleotide sequence of the respective cDNAs. The sequences were then compared to those previously published in Genbank. Sequence analysis confirmed the identity of the bovine POMC cDNA. The nucleotide sequence of the partial bovine CRFR1 and V3 receptor cDNAs were 90% and 86% similar to the rat CRFR1 and V3 cDNAs, respectively *(42,43)*.

Characterization of V3, CRFR1, and POMC mRNA Abundance

Total RNA was isolated using TRIZOL® Reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. For hypothalamic and cerebellar RNA isolation, the entire tissue samples were homogenized for RNA isolation to increase uniformity of isolated samples. To determine transcript size and number and to optimize specificity of hybridization conditions, pools of total RNA were made from each time-point and 25 μg of each pool was subjected to electrophoresis through 1% agarose–formaldehyde gels. RNA was then capillary transferred to nylon membranes (Bio-Rad, Richmond, CA) and ultraviolet (UV) crosslinked. For quantitation of V3, CRFR1, and POMC mRNA abundance, 5 μg of total RNA isolated from each individual sample was spotted onto nylon membranes in duplicate using a dot blot apparatus (Bio-Rad, Richmond, CA). Membranes were allowed to air dry and then UV crosslinked.

Northern and dot blot analysis was then carried out using ^{32}P-labeled bovine V3, bovine CRFR1, bovine POMC, or ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probes generated by PCR. GAPDH was used for normalization purposes. Probes were labeled using PCR with α-[^{32}P] dCTP (New England Nuclear, Boston, MA). Each PCR mix contained 1X PCR buffer, 2.5 m*M* $MgCl_2$, 1.55 μ*M* dNTPs (minus dCTP), 1.5 units of *Taq* polymerase (Life Technologies, Inc., Gaithersburg, MD), 0.25 μ*M* of each primer, 5 μL α-[^{32}P] dCTP, and 100 pg of template. PCR conditions were as follows: 95ºC for 5 min, 40 cycles of 94ºC, 30 s, 54ºC, 1 min, 72ºC, 1.5 min, followed by a final 10-min extension at 72ºC.

Membranes were incubated at 42ºC overnight in prehybridization buffer (50% formamide, 5X SSC [saline–sodium citrate buffer; single strength is 0.15 *M* NaCl and 0.015 *M* sodium citrate, pH 7.0]), 5X Denhardt's (single strength is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin [BSA]), 50 m*M* $NaPO_4$, 0.1% sodium dodecyl sulfate (SDS), and 250 μg sperm DNA/mL prehybridization buffer). Then, hybridizations took place in fresh buffer (50% formamide, 5X SSC, 1X Denhardt's, 20 m*M* $NaPO_4$, 0.1% SDS, 10% dextran sulfate, and 100 μg sperm DNA/mL hybridization buffer) containing 1×10^6 cpm/mL of appropriate ^{32}P-labeled cDNA. Hybridizations were at 42ºC for 18 h. Membranes were washed in 1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate for 20 min at 42ºC, one 20-min wash in 0.1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 42ºC, one 20-min wash at 47ºC, and one 20-min wash at 55ºC. Following washing, filters were exposed to a PhosphoImager cassette. After exposure (12–48 h), the cassette was scanned using a PhosphoImager (Bio-Rad, Hercules, CA). After Northern analyses, size of RNA transcripts was determined based on relative migration of RNA molecular-weight markers (Roche, Indianapolis, IN). After hybridization for V3, CRFR1, or POMC, the membranes were then stripped and reprobed with the ^{32}P GAPDH cDNA. Preliminary experiments demonstrated that GAPDH mRNA abundance in tissues of interest was not affected by LPS administration ($p > 0.05$; data not shown). Relative densitometric units for V3, CRFR1 and POMC were quantitated and adjusted relative to GAPDH mRNA expression using Molecular Analyst Version 1.5 software (Bio-Rad, Hercules, CA). Preliminary Northern blot experiments demonstrated that hybridization and washing conditions for V3, CRFR1, and POMC used in subsequent dot blot analyses were specific and yielded hybridization to distinct transcripts of consistent size and number. Preliminary experiments also demonstrated that an increase in hybridization intensity was detected following hybridization of each cDNA to increasing amounts of sample RNA (1–10 μg).

Statistical Analyses

Differences in V3, CRFR1 and POMC mRNA (adjusted relative to levels of GAPDH mRNA) were determined by analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS with time relative to LPS administration as the main effect. Differences in mean hormone concentrations were determined using the mixed procedure (PROC MIXED) of SAS *(44)*. Hormone data were log-transformed to achieve uniform variation within each group. When the *F*-test was significant ($p < 0.05$), differences between mean hormone concentrations or mRNA levels were adjusted using the Dunnett method, with comparisons made to 0 h (pre-LPS time-point). Correlations between plasma hormone concentrations and V3 and CRFR1 mRNA abundances were made using the correlation (CORR) procedure of SAS.

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