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COORDINATE REGULATION OF CORTICOTROPIN RELEASING FACTOR RECEPTOR I AND ARGININE VASOPRESSIN RECEPTOR V3 MESSENGER RIBONUCLEIC ACIDS IN THE ANTERIOR PITUITARY OF ENDOTOXEMIC STEERS

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Abstract

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The two primary hypothalamic peptides that regulate secretion of adrenocorticotropic hormone (ACTH) in cattle are corticotropin releasing factor (CRF) and arginine vasopressin (AVP). Regulation of pituitary responsiveness to CRF and AVP represents a potential mechanism to control the endocrine response to stress. Actions of CRF and AVP at the anterior pituitary (AP) are mediated by CRF receptor 1 (CRFR1) and AVP receptor 3 (V3), respectively. My objective was to determine the effect of LPS-induced endotoxemia on CRFR1 and V3 receptor mRNA in the AP. Holstein steers (n = 20) were injected with 200 ng/kg bacterial lipopolysaccharide (LPS). AP were collected at 0, 2, 4, 12 and 24 h following LPS administration. All animals injected with LPS responded with an increase in body temperature and circulating plasma concentrations of ACTH and cortisol characteristic of endotoxemia. Relative levels of AP CRFR1 and V3 mRNAs decreased at 2, 4, and 12 h following LPS administration ($P < 0.05$) and returned to basal levels by 24 h. CRFR1 mRNA was unaltered in the cerebellum. Likewise, expression of pro-opiomelanocortin (POMC) mRNA in the AP was not altered. Based on the finding that CRFR1 and V3 receptor mRNA were down regulated during LPS-induced endotoxemia, I conclude that this regulation represents a potential mechanism to control pituitary responsiveness to CRF and AVP.

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Key to Symbols or Abbreviations

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AP	Anterior pituitary
AtT-20	Mouse pituitary tumor cell line
AVP	Arginine vasopressin
bp	Base pair
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary cell line
CNS	Central nervous system
CRF	Corticotropin releasing factor
CRFR1	Corticotropin releasing factor receptor 1
CRFR2	Corticotropin releasing factor receptor 2
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine-tetraacetic acid
g-protein	Guanine nucleotide binding protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCR	Glucocorticoid receptor
GLM	General linear model
GRE	Glucocorticoid response element
HPA	Hypothalamic-pituitary-adrenal

i.p.	Intraperitoneal
i.v.	Intravenous
kb	Kilobase
LLCPK-1	Porcine kidney tumor cell line
LPS	Lipopolysaccharide
MCR	Mineralcorticoid receptor
mRNA	Messenger ribonucleic acid
NK	Natural killer cell
PC	Prohormone convertase
PC-12	Rat pheochromocytoma cell line
PKA	Protein kinase A (cAMP dependent)
PKC	Protein kinase C (calcium dependent)
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAS	Statistical Analysis System
s.c.	Subcutaneous
SON	Supraoptic nucleus
UCN	Urocortin
UCN II	Urocortin II
UV	Ultraviolet

V1	Arginine vasopressin receptor subtype 1 (V1a)
V2	Arginine vasopressin receptor subtype 2
V3	Arginine vasopressin receptor subtype 3 (V1b)

Chapter 1

Review of Literature

Introduction

Stress is any actual or perceived threat to an organism, a failure of expectations or a loss of control (Levine & Ursin, 1991). Coordinated physiological reactions maintain homeostasis in response to stress. Adaptive responses that counteract effects of aversive stimuli are believed critical for survival (Chrousos & Gold, 1992). Physiological responses associated with stress compromise vegetative mechanisms such as growth and reproduction (Moberg, 1991; Wilson et al., 1972) to increase the energy to cope with a stressful situation (Gelfand et al., 1984). Some consequences of exposure to stressors at inopportune times and (or) for prolonged periods, however, can compromise health and well being. For example, stress at parturition, weaning and during transportation predisposes animals to disease (Kelley, 1980; Kelley, 1985; Minton, 1994; Tarrant et al., 1992). Stress is a major factor contributing to the etiology of several economically significant disease conditions in livestock, such as bovine respiratory disease, which cost over 624 million dollars in 1995 (Lekeux, 1995). Negative effects associated with stress do not apply only to livestock. Human exposure to psychological stressors such as guilt and grief, also increase the risk of infections, neuropsychiatric disorders or other disease conditions (Cacioppo, 1994; Cassileth & Drossman, 1993; Raikkonen et al., 1996). A thorough understanding of the mechanisms that regulate the responses to stress may lead to development of new methods to prevent stress associated disease, which would make a substantial contribution to both veterinary and human medicine.

Although stress cannot be avoided, minimizing its negative effects should reduce the incidence of stress-associated disease. Concerns regarding prophylactic use of

antibiotics in livestock operations (Witte, 1998) to control stress-associated incidences of infectious disease necessitate the development of non-traditional approaches to enhance animal health and reduce the deleterious effects of stress associated with routine management practices. Inhibiting specific endocrine, neural or neuroendocrine mechanisms associated with the stress response may reduce the negative effects of stress on livestock. Human health applications of such a strategy are already being pursued. Studies in rodents indicate that non-peptide corticotropin releasing factor (CRF) receptor antagonists show great promise for treatment of anxiety and other stress-related disorders in people (Lundkvist et al., 1996; Schulz et al., 1996).

This review will discuss a major component of the endocrine responses to stress. Information presented will focus on specific pathways implicated in mediating the hypothalamic-pituitary-adrenal (HPA) axis response to stress. Discussion will also focus on some of the stress hormones that may have an impact on immunocompetence and susceptibility to infectious disease. I will then attempt to explain several mechanisms by which products of the HPA axis autoregulate the magnitude and duration of HPA axis activity. Several stress paradigms are utilized to study the endocrine response to stress. This review will also discuss the lipopolysaccharide (LPS)-induced endotoxemia model and the HPA axis response associated with this model.

increase in intracellular calcium and presumably an increase in PKC activity (Ventura et al., 1999). These distinct signaling pathways mediate the biological actions of CRF and AVP on the pituitary.

Changes in pituitary CRF and AVP receptor numbers are critical determinants of pituitary corticotrope responsiveness to hypothalamic stimulation, and hence help regulate the magnitude and duration of the endocrine response to stress (Hauger et al., 1990; Koch & Lutz, 1985; Wynn et al., 1985). Although the mechanisms that regulate pituitary responsiveness to CRF and AVP are not completely understood, data from studies in rats indicate that AP CRFR1 mRNA and V3 receptor mRNA are differentially regulated. For example, CRFR1 mRNA in cultured rat pituitary cells is decreased in response to glucocorticoids (Pozzoli et al., 1996), whereas, V3 receptor mRNA is increased after treating rats with dexamethasone (Rabadan Diehl et al., 1997a). Potential coordinate regulation of pituitary CRF and AVP receptors during LPS-induced endotoxemia has not been investigated. Such information is critical to elucidation of the mechanisms that control the endocrine response to stress, particularly in species such as cattle, where CRF and AVP both have potent ACTH releasing capacities, (Schwartz & Vale, 1988).

The primary goal of this study was to examine the effect of LPS administration on AP CRFR1 and V3 receptor mRNAs in cattle. CRF and AVP are two primary mediators of HPA axis activity in cattle. The AP receptor for CRF, the single most potent hypothalamic neuropeptide that stimulates HPA axis activity in rodents is down regulated

The endocrine response to stress

Corticotropin releasing factor (CRF)

Hypothalamic regulation of hormone secretion during stress was established as early as the 1950's, eventually leading to the isolation and subsequent synthesis of corticotropin releasing factor (CRF) in 1981 (Vale et al., 1981). Since then, there has been an explosion of investigations in laboratory species into the biological actions of CRF. In most mammals, CRF is the major hypophysiotropic hormone controlling activity of the HPA axis, a primary component of the endocrine response to stress (Owens & Nemeroff, 1991). Several studies demonstrate that CRF mRNA increases in the hypothalamus following various stress paradigms (Harbuz & Lightman, 1989; Suda et al., 1988; Timofeeva & Richard, 1997). Stress also causes a release of CRF from parvocellular neurons of the paraventricular nucleus (PVN) into the external zone of the median eminence. Several mechanisms are implicated in activating the CRF neurons in the PVN during a stressful situation. Axotomy of the hepatic branch of the vagus nerve blocks the increase in CRF mRNA expressed in the PVN following LPS administration (Sergeev & Akmaev, 2000). Results from brainstem hemisection experiments also indicate that baseline levels and immobilization-induced increases in CRF mRNA in the PVN depend on ipsilaterally ascending medullary tract catecholaminergic cells (Pacak, 2000). Proximal hypothalamic regions such as the dorsal medial hypothalamus are also implicated in activating CRF neurons in the PVN. Site-specific inhibition of the dorsal medial hypothalamus prevents stress-induced increases in heart rate, blood pressure and plasma ACTH, whereas stimulation of the dorsal medial hypothalamus increases these

parameters similar to what is observed during a stress response (Bailey & Dimicco, 2001).

Stress-induced release of CRF is demonstrated in several experiments where CRF concentrations in the hypothalamus are measured at different times after exposure to various stressors. Hypothalamic CRF concentrations decrease upon exposure of animals to stress, likely due to release into the portal circulation (Chappell et al., 1986; Engler et al., 1988; Engler et al., 1989; Plotsky & Vale, 1984). CRF concentrations in the hypothalamus increase approximately 24 hours after restraint stress. The protein synthesis inhibitor, anisomycin, blocks this increase in hypothalamic CRF concentrations indicating that the increase in CRF is an active replenishment of used CRF stores (Haas & George, 1988).

The hypothalamic-pituitary-adrenal (HPA) axis

CRF is a major regulator of activity of the HPA axis. CRF secreting parvocellular neurons of the PVN terminate in the external zone of the median eminence. The median eminence contains many fenestrated capillaries that readily exchange products with the surrounding environment. Hormones secreted into this region enter the capillaries of the portal circulation and are transported to the anterior pituitary gland (AP) through the portal circulation (Battaglia et al., 1998; Guillaume et al., 1989). The small volume of blood in the portal circulation allows hypothalamic neurohormones to be transported to the AP in relatively high concentrations. The portal vessels diverge into capillaries at the AP presenting the AP with signals from the hypothalamus encoded within the

neurohormones. CRF and other hypothalamic neurohormones that potentiate the actions of CRF are among the products transported to the AP (Engler et al., 1989). AP corticotropes cleave pro-opiomelanocortin (POMC) peptides upon CRF stimulation to liberate adrenocorticotropic hormone (ACTH), β-endorphin and other POMC derived peptides (Raymond et al., 1979; Vlaskovska & Knepel, 1984; Yasuda & Yasuda, 1986).

The CRF-induced increase in circulating ACTH promotes increased synthesis and secretion of glucocorticoids (cortisol in man and livestock, corticosterone in rodents) from the zona fasciculata region of the adrenal gland (Owens & Nemeroff, 1991). In addition to metabolic (Stalmans & Laloux, 1979) and immunomodulatory actions (Cohn, 1991; Jefferies, 1991), the increased circulating glucocorticoids negatively feed back at the hypothalamus (Sawchenko, 1987), pituitary (Keller Wood & Dallman, 1984), and other regions of the brain (Jacobson & Sapolsky, 1991) limiting the magnitude and duration of the endocrine response to stress (Owens & Nemeroff, 1991).

The negative feedback effects of glucocorticoids are mediated through two types of cytoplasmic steroid hormone receptors, glucocorticoid receptors (GCR) and mineralcorticoid receptors (MCR). GCR distribution is ubiquitous throughout the brain with high concentrations in the cerebral cortex, hippocampus, thalamus, paraventricular and supraoptic nucleus, as well as the AP (Reul & de Kloet, 1985). MCR expression is most prominent in specific regions of the hippocampus with limited expression in other regions of the brain (Reul & de Kloet, 1985). The MCR binds natural glucocorticoids with a much greater affinity than the GCR, however synthetic glucocorticoids, such as

dexamethasone bind only to the GCR (Reul et al., 2000). The activated receptors dimerize and translocate to the nucleus. Upon entering the nucleus the steroid-receptor complex binds to glucocorticoid response elements (GRE) to promote or inhibit transcription of glucocorticoid responsive genes (Malkoski et al., 1997).

Plasma ACTH and glucocorticoid concentrations increase in response to anxiety, fear, pain, restraint, changes in temperature, parturition, endotoxemia, and other stressful visual or acoustic stimuli. The broad distribution of CRF (Sawchenko et al., 1993) and its receptors (Primus et al., 1997; Sanchez et al., 1999) in the central nervous system (CNS) and the pronounced regulation of CRF during stress responses and neuropsychiatric disorders such as depression (Weiss et al., 1994) or Alzheimer's disease (Behan et al., 1995) have led scientists to believe that CRF may help mediate anxiety and fear as well as learning and memory (Croiset et al., 2000; Radulovic et al., 1999). Exaggerated responses of the HPA axis to stressors are also associated with disorders such as depression, obesity and Alzheimer's disease (Raber, 1998). These studies and others suggest that the etiology of these neuroendocrine disorders stems from a dysfunction in regulation of the HPA axis or an over-activity of hypothalamic neurons (Stenzel Poore et al., 1996). Circulating hormones associated with HPA axis activity provide a quantitative measure of the magnitude of a stressor or severity of an endocrine dysfunction.

Regulation of hypothalamic CRF release

Hypothalamic CRF release is regulated directly by glucocorticoids or indirectly by other hypothalamic and extra-hypothalamic regions that may be affected by glucocorticoids. The high concentrations of GCR in the PVN (Reul & de Kloet, 1985) suggest a potential route by which glucocorticoids secreted in response to stress or through daily circadian rhythms regulate hypothalamic CRF release. Studies utilizing a mouse pituitary tumor cell line (AtT-20 cells) show the ability of dexamethasone to reduce cAMP stimulated CRF gene expression (Van et al., 1990). Internal deletion of the GRE from the CRF promoter also results in decreased glucocorticoid-dependent repression of CRF promoter activity (Malkoski & Dorin, 1999). However, transcription factors that normally mediate hypothalamic CRF gene expression may not be expressed in the AtT-20 cells. Thus, this may not be a representative model for CRF gene regulation in the hypothalamus. Studies utilizing the PC12 rat pheochromocytoma cell line provide a more accurate depiction of CRF regulation in a neuronal cell. PC12 cells secrete CRF in a dose- and time-dependant manner in response to nicotine (Venihaki et al., 1997). Calcium ion influx in response to nicotine stimulation is inhibited by glucocorticoid treatment (Qiu et al., 1998). Glucocorticoids also inhibit expression of the G-protein alpha subunit, which plays a critical role in mediating downstream accumulation of cAMP and cellular activation (Li & Jope, 1997). Assuming a G-protein linked second messenger system and calcium ion influx are responsible for activation and subsequent secretion of CRF in PC12 cells, these studies provide evidence that glucocorticoids inhibit CRF secretion from a neuronal cell line.

Studies done *in vivo* also show that CRF mRNA in the PVN decreases following dexamethasone (Beyer et al., 1988) or corticosterone (Albeck et al., 1994) administration. Furthermore, removing the effects of glucocorticoids by adrenalectomy increases CRF mRNA in the PVN, an effect that is dose dependently reversed by glucocorticoid replacement (Imaki et al., 1991; Lightman & Young 3rd, 1989). In addition to the increase in CRF mRNA, CRF immunoreactivity in the PVN also decreases in adrenalectomized animals, an effect that is also reversed with glucocorticoid treatment (Sawchenko, 1987). In some studies however, no change in CRF mRNA in the PVN following adrenalectomy is observed (Albeck et al., 1994). The above described effects of glucocorticoid treatment and adrenalectomy on CRF mRNA are specific to the PVN (Swanson & Simmons, 1989).

CRF immunoreactivity in the portal circulation provides a quantitative measure of the amount of CRF released into the median eminence. Studies revealed an inhibitory effect of glucocorticoids on CRF levels in the pituitary portal circulation. Dexamethasone inhibits CRF release into the portal circulation associated with hemorrhage (Plotsky & Vale, 1984) and corticosterone suppresses CRF release associated with hypotension (Plotsky et al., 1986). Furthermore, adrenalectomy increases basal CRF secretion 2.2 fold (Plotsky & Sawchenko, 1987). These studies revealed that the changes observed in CRF mRNA and peptide expression following exposure to or after removal of the effects of glucocorticoids translate into decreases in circulating CRF concentrations that may have physiologically relevant consequences.

Some theories implicate the hippocampus in mediation of glucocorticoid negative feedback effects on CRF neuronal activity in the PVN. Based on lesion and electrical stimulation studies, it is thought that the hippocampus, a primary site of MCR expression, has an overall inhibitory effect on HPA axis activity (Jacobson & Sapolsky, 1991). Dorsal hippocampectomy and fornix transection (eliminates effects of the hippocampus on the PVN) result in increased basal HPA axis activity (Herman et al., 1989). Lesions in areas that may mediate hippocampal inhibition of the HPA axis also result in a prolonged glucocorticoid response to stress (Herman et al., 1995).

The differential effects of glucocorticoids in specific regions of the brain may be mediated by actions of site-specific steroid-metabolizing enzymes (Seckl et al., 1993), a topic which is beyond the scope of this review. Recent studies also show glucocorticoid inhibition of specific prohormone convertase (PC) mRNA expression in specific hypothalamic nuclei. Regulation of PC expression affects rates at which pro-forms of neurohormones, such as CRF, are cleaved into their biologically active forms (Dong et al., 1997). These studies suggest an inhibitory effect of glucocorticoids at the post-translational processing stage.

Regulation of ACTH production and release

In addition to regulation that may occur at the level of the hypothalamus to control subsequent synthesis and secretion of glucocorticoids, the AP also presents a site at which regulation occurs to control adrenocortical function. Studies are in agreement regarding the inhibitory effect of glucocorticoids on CRF-stimulated ACTH production

(Dayanithi & Antoni, 1989; Horiba et al., 1993). However glucocorticoid pretreatment does not affect the peak ACTH response to arginine vasopressin (AVP) or oxytocin stimulation, but it does reduce the duration of the response to either of these hormones (Oki et al., 1991). Other studies further demonstrate that glucocorticoids reduce ACTH secretion in response to AVP, angiotensin II and norepinephrine stimulation (Abou Samra et al., 1986). An interesting observation, however, is that pretreatment with glucocorticoids is necessary for inhibition of CRF-stimulated ACTH secretion, whereas simultaneous treatment of AP cells with AVP and glucocorticoids results in inhibition similar to that observed after pretreatment for 30 minutes (Shipston et al., 1996). These studies reveal that different hormones activate AP corticotropes through distinct signaling pathways. These mechanisms subsequently become integrated to produce an ACTH secretory response. However, the different mechanisms by which glucocorticoids regulate the actions of specific hormones that stimulate ACTH secretion are not completely understood.

The effects of glucocorticoids on CRF-stimulated cAMP production depend on the duration of exposure to glucocorticoids. Pre-incubation with dexamethasone for one hour significantly attenuates CRF-stimulated cAMP production (Bilezikjian & Vale, 1983; Sobel, 1985). However, pretreatment of AP cells with dexamethasone for 18 hours had no effect on CRF stimulated cAMP production, but did decrease ACTH release (Giguere et al., 1982). More recent studies have determined that glucocorticoids inhibit ACTH secretion by inducing expression of intracellular proteins that bind and regulate

membrane Ca^{2+} channels and prevent depolarization (Lim et al., 1998; Woods et al., 1994).

Role of CRF in integrating the stress response

A growing body of evidence indicates that CRF triggers many components of the stress response through mechanisms independent of the pituitary-adrenal axis. Such responses (described below) are not blocked by hypophysectomy, indicating that CRF-stimulated ACTH and glucocorticoid secretion are not required for the response. In the CNS, CRF plays a role in mediating the autonomic response to stress. Parvocellular neurons of the paraventricular nucleus terminate not only in the median eminence, but also in regions of the brainstem and spinal cord thought to mediate autonomic nervous system activation (Carrasco et al., 2001; Pyner & Coote, 2000). Studies suggest a role for CRF in mediating the decreases in gastric emptying and the increases in colonic motility associated with sympathetic activation through this pathway (Martinez et al., 1998; Martinez et al., 1997; Williams et al., 1987).

Upon sympathetic activation, CRF neurons are also responsible for activation of the sympatho-adrenomedullary axis (Matsumoto et al., 1998). The splanchnic nerves provide a pathway for sympathetic preganglionic cholinergic fibers to innervate the adrenal gland. Upon stimulation of the splanchnic nerves, these fibers synapse on chromaffin cells in the adrenal medulla and stimulate secretion of epinephrine and norepinephrine (Bornstein et al., 1990). Cell bodies located in the adrenal medulla are stimulated by epinephrine and norepinephrine and thereby provide an intrinsic source of

adrenal cortical innervation. These inter-neurons directly stimulate synthesis and release of glucocorticoids. Catecholamines secreted from the adrenal medulla also stimulate CRF production in the adrenal, which in turn stimulates adrenal ACTH production, and finally synthesis and secretion of glucocorticoids (Andreis et al., 1992; Andreis et al., 1991). Dispersed adrenocortical cells do not respond to CRF treatment, whereas adrenal slices including the cortex and medulla secrete ACTH and cortisol in response to CRF (Andreis et al., 1992). These results indicate that the high affinity CRF binding sites in the medulla play a role in mediating local regulation of glucocorticoid production in the adrenals. Mineralcorticoids such as aldosterone are also produced in response to CRF or AVP administration (Pradier et al., 1986).

In addition to its role in the autonomic nervous system, CRF centrally mediates behavioral responses to stress. Central infusion of a non-selective CRF receptor antagonist and intraperitoneal injection of a selective CRFR1 non-peptide antagonist are effective in reversing stress-induced suppression of appetite and increases in locomotor activity (Hotta et al., 1999). Several studies demonstrate that CRF in other regions of the brain, besides the PVN, mediates behavioral responses to diverse stressors. This allows an organism to activate not only the pituitary adrenal axis, but also the behavioral responses to stress (Koob et al., 1994).

HPA axis regulation of the immune system

One of the classical negative effects of stress that compromises animal health is glucocorticoid-induced immunosuppression (Pruett et al., 1999). Studies show a positive

correlation between elevated circulating levels of glucocorticoids and immunosuppression parameters (Anderson et al., 1999). However, there is also evidence suggesting that low levels of glucocorticoids have stimulatory effects on the immune system (Stanulis et al., 1997). Basal HPA axis activity may be important to maintain health, whereas an increase in HPA axis activity may play a role in limiting immune and inflammatory responses. The effects of different concentrations of circulating glucocorticoids may be mediated by the regional distribution of GCR and MCR. However, hyper-activity of the HPA axis for prolonged periods, or at inopportune times, increases an animal's susceptibility to infectious diseases such as respiratory disease (Lekeux, 1995). On the other hand, an insufficiency in the HPA axis response leads to an exaggerated inflammatory response similar to arthritis (Cutolo et al., 2000).

It would be naïve to assume that glucocorticoids are the only hormones involved in stress-induced immunosuppression (Dohms & Metz, 1991; Monjan & Collector, 1977). Some studies actually show a failure of adrenalectomy to reduce the immunosuppressive effects of stress (Jain et al., 1991). This suggests involvement of other hormones, besides those secreted from the adrenal gland, in mediating the immunosuppressive effects of stress. Immunocompetence is a balance achieved from integration of effects of factors secreted from specific cells of the immune system, hypothalamic neurohormones and hormones secreted from other endocrine glands (Cunnick et al., 1990; Dunn, 1988; Hassig et al., 1996).

CRF, directly and indirectly, plays a role in regulation of immune function and inflammation in humans and other animals. A functional CRF response system is present in leukocytes, suggestive of a potential paracrine and autocrine mechanism of action (Stephanou et al., 1990; Tsagarakis & Grossman, 1994). The typically low levels of circulating CRF also indicate that actions of CRF in the periphery are not mediated through endocrine mechanisms. Specific binding of CRF to lymphocytes increases intracellular cAMP (Audhya et al., 1991). Thus, CRF activates lymphocyte second messenger systems that may mediate immunomodulation. Similar to the response of corticotropes, glucocorticoids also inhibit CRF-stimulated ACTH secretion in leukocytes (Hendricks & Mashaly, 1998; Smith et al., 1986).

CRF also has direct analgesic and anti-inflammatory actions. Intradermal CRF injections into an affected site inhibit extravasation stimulated by saphenous nerve stimulation and vascular permeability stimulated by formaldehyde inhalation (Wei & Kiang, 1989; Wei et al., 1986). CRF administered i.v. or intradermally prior to, or after thermal injury, or exposure to concentrated acids, inhibits the acute inflammatory response. Simultaneous administration of a CRF antagonist abolishes these actions (Kiang & Wei, 1987; Tian & Wei, 1989; Wei et al., 1988). Conducting these studies using hypophysectomized and adrenalectomized rats eliminates the possibility that the above responses are secondary effects of ACTH or glucocorticoids. Studies also suggest that CRF acts directly on endothelial cells lining the vasculature. Treatment of bovine aortic endothelial cells with CRF inhibits interleukin-stimulated prostaglandin production (Fleisher Berkovich et al., 1998). These studies support a potential direct role of CRF in

the local regulation of inflammation. However, the effects of CRF produced locally at sites of inflammation may not have the same effect as locally or i.v. administered CRF. Studies utilizing a CRF antiserum demonstrate a proinflammatory role for CRF in contrast to its indirect immunosuppressive effects (Karalis et al., 1997; Karalis et al., 1991). However, utilization of a CRF antiserum results in an impaired HPA axis response (Turnbull & Rivier, 1996). This may explain the proinflammatory effects witnessed in the studies mentioned above.

In addition to the direct effects of CRF and the suppressive effects of glucocorticoids on leukocytes, CRF in the CNS also promotes immunosuppression. CRF administered centrally decreases natural killer (NK) cell cytotoxicity via sympathetic activation of the adrenal gland (Irwin et al., 1988). Similar effects on NK cell cytotoxicity occur using foot-shock stress rather than exogenous CRF. These effects are inhibited by central administration of a CRF antiserum (Irwin et al., 1990). Furthermore, the inability to generate a proper CRF response, as in the arthritis susceptible Lewis strain of rats, contributes to inadequate immune and inflammatory regulation similar to what is seen in cancer and autoimmune diseases (Sternberg et al., 1989a; Sternberg et al., 1989b).

CRF Receptors

The biological actions of CRF and CRF-related ligands are mediated by specific CRF receptors. Two CRF receptor subtypes (CRFR1 and CRFR2) sharing 70% amino acid sequence identity have been identified (Chang et al., 1993; Lovenberg et al., 1995). In rodents, high levels of CRFR1 are present in the AP, cerebral and cerebellar cortices,

hippocampal gyrus, as well as amygdaloid, thalamic, hypothalamic and brainstem nuclei (Van Pett et al., 2000). CRFR2 is more limited in expression and distribution than CRFR1. The highest levels of CRFR2 expression are in the choroid plexus, raphe and lateral septal nuclei, hippocampus, and posterior pituitary (Van Pett et al., 2000). Three splice variants of CRFR2 (CRFR2α, CRFR2β and CRFR2γ) also exist (Kostich et al., 1998; Lovenberg et al., 1995; Perrin et al., 1995). CRF-R2α and CRFR2γ are expressed mainly in the brain, whereas the other variant, CRFR2β, is found not only in the CNS, but also in cardiac and skeletal muscle, epididymis, and the gastrointestinal tract (Perrin & Vale, 1999).

The CRF receptor subtypes also display distinct affinities for CRF and CRF-related ligands. Urocortin (UCN) displays a 45% sequence identity with CRF and has a forty-fold higher affinity for CRFR2 than does CRF (Asaba et al., 1998; Vaughan et al., 1995). Furthermore UCN stimulates a ten-fold higher production of cAMP upon binding to CRFR2 than does CRF (Vaughan et al., 1995). UCN is primarily expressed in the brain in neurons that project to areas expressing CRFR2, suggesting a potential role in mediating CRFR2 systems. Central UCN neurons indeed do project to the lateral septal nucleus and other areas expressing CRFR2 (Vaughan et al., 1995). However, the lack of a pervasive relationship of UCN-immunoreactive projections with some CRFR2-expressing targets, such as the posterior pituitary, also supports the potential existence of additional endogenous CRF-related ligands in the mammalian brain (Bittencourt et al., 1999). More recently, Urocortin II (UCN II) has been characterized. UCN II binds selectively to CRFR2 and displays no apparent affinity for CRFR1 (Reyes et al., 2001).

Recent studies show that the CRF receptor subtypes exhibit anti-parallel regulation made possible by their distinct localization patterns and ligand affinities (Skelton et al., 2000).

CRF receptors are classical guanine nucleotide binding (G) protein-linked, seven transmembrane domain receptors positively coupled to adenylate cyclase (Chang et al., 1993). Increases in intracellular cAMP help mediate CRF-stimulated ACTH secretion. Stimulation of *in vitro* AP systems with CRF leads to an approximately 4-fold increase in cAMP within 2 minutes and a 15-fold increase by 30 minutes (Aguilera et al., 1983). Furthermore, cAMP dependent protein kinase A (PKA) inhibitors block CRF-stimulated ACTH secretion and POMC gene expression in AP cells (Reisine et al., 1985). Calmodulin inhibitors also inhibit CRF-stimulated ACTH release (Murakami et al., 1985a), indicating a role for intracellular Ca^{2+} in this process. Furthermore, Ca^{2+} channel alpha subunit mRNA expression increases in the rat AP following cold stress or *in vitro* following CRF treatment (Xie et al., 1999). Transfection of Chinese hamster ovary (CHO) cells with CRFR1 or CRFR2 and stimulation with a potent CRFR1 and CRFR2 agonist, sauvagine, reveal that signaling pathways downstream from CRFR1 and CRFR2 are similar (Rossant et al., 1999). However, CRF-stimulated porcine kidney cells (LLCPK-1) transfected with CRFR1 exhibit a greater cAMP response than cells transfected with CRFR2 (Nabhan et al., 1995). This response may be attributable to the lower affinity of CRF for CRFR2 than CRFR1.

Regulation of CRFR1

Subsequent sections will focus on mechanisms that regulate CRF receptors. Since CRFR1 is the primary CRF receptor in the AP (Van Pett et al., 2000), binding studies described are in essence measuring CRFR1 binding. Regulation of CRFR2 is beyond the scope of this review. CRF is the primary hypothalamic peptide that mediates the activity of the HPA axis. CRF mediates its effects through binding to AP CRFR1 (Smith et al., 1998). Desensitization of the AP to hypothalamic stimulation is a phenomenon that limits the magnitude and duration of hormone secretion from the AP and subsequent stimulation of the adrenal gland. Desensitization occurs in several ways. Membrane bound receptors that mediate the actions of hypothalamic neurohormones may decrease in numbers to limit the response to stimuli. Second messenger systems may uncouple from receptors preventing activation of cascades downstream from the ligand-receptor complex. Intracellular proteins can also be expressed that may affect signaling by regulating the activity of membrane ion channels, kinases, phosphatases, or phosphodiesterases. A decrease in transcription rate or stability of mRNA may also limit the abundance of mRNA encoding CRFR1 or other proteins necessary for AP ACTH secretion. The following section will discuss the above mechanisms whereby the AP corticotrope becomes desensitized to hypothalamic stimulation.

Role of glucocorticoids in CRFR1 regulation

Glucocorticoids are potent regulators of CRFR1 mRNA. Studies *in vivo* and *in vitro* both demonstrate an inhibitory effect of glucocorticoids on CRFR1 mRNA. Dexamethasone or corticosterone administration to rats causes a significant decrease in

AP CRFR1 mRNA levels 2-10 hours after injection, returning to basal levels by 18 hours (Luo et al., 1995; Ochedalski et al., 1998). Rat AP cells in culture respond in a similar fashion with a decrease in CRFR1 mRNA following dexamethasone treatment (Pozzoli et al., 1996).

Additional studies demonstrate that glucocorticoid treatments that decrease CRFR1 mRNA (as described above) also decrease AP responsiveness to CRF. AP cells from fallow deer pretreated with dexamethasone exhibit an attenuated ACTH response to CRF stimulation (Willard et al., 1995). Furthermore, implanting corticosterone pellets in rats inhibits ACTH responses to CRF treatments *in vitro* as well as the ACTH response to restraint (Young et al., 1995). Studies that show the ability of glucocorticoids to inhibit ACTH secretion stimulated by CRF or stress further substantiate the role of elevated glucocorticoids in limiting the duration of an endocrine response to stress.

To further substantiate the physiological relevance of mRNA down regulation studies, it is important to also discuss studies that examined the effect of glucocorticoids on numbers of CRF binding sites on AP corticotropes. In addition to the effects of glucocorticoids on CRFR1 mRNA, chronic administration of glucocorticoids causes a dose-dependent decrease in CRF receptor numbers (Hauger et al., 1987). Acute glucocorticoid treatment for one hour also causes a decrease in binding of biotinylated CRF to corticotropes (Childs & Unabia, 1990). A similar decrease in CRF receptor numbers in the AP is also observed in chronically stressed animals that exhibit elevated circulating glucocorticoid concentrations (Hauger et al., 1988). The down regulation of

AP CRF binding sites may be a result of the negative feedback effects of glucocorticoids on the expression and subsequent translation of CRFR1 mRNA, or a stimulatory effect of glucocorticoids on expression of proteins that promote internalization of CRFR1.

Adrenalectomy studies also provide valuable information on the role of glucocorticoids in regulation of CRFR1 and subsequent HPA axis regulation. There are several hallmark effects associated with removing the influence of glucocorticoids by adrenalectomy. Adrenalectomized rats do not exhibit attenuated CRF-stimulated ACTH responses following 1 hour of low intensity electroshock, whereas intact animals do. However, intact and adrenalectomized animals both exhibit attenuated CRF-stimulated ACTH responses following moderate intensity foot-shock (Rivier & Vale, 1987). These studies suggest that glucocorticoids are partly responsible for the desensitization of the AP to CRF stimulation. They also demonstrate that desensitization occurs through other mechanisms in the absence of glucocorticoids. Similar to the effects of increased glucocorticoid concentrations, adrenalectomy also decreases AP CRFR1 binding sites. This effect is reversed by glucocorticoid replacement (Aguilera et al., 1986; Holmes et al., 1987; Wynn et al., 1983; Wynn et al., 1984). This phenomenon may be attributable to ligand-induced down regulation of the receptors due to removal of the inhibitory effects of glucocorticoids on hypothalamic secretion of CRF.

Role of CRF in CRFR1 regulation

Chronic stress, endocrine dysfunctions or relief from glucocorticoid feedback by adrenalectomy results in continuous AP exposure to CRF. These paradigms reduce

subsequent ACTH responses to stimuli that typically activate the HPA axis (Evans et al., 1985; Rivier & Vale, 1983; Rivier & Vale, 1985). Corticotrope desensitization to CRF stimulation occurs by uncoupling of adenylate cyclase from the CRF receptor, internalization of the receptor and a decrease in binding sites available for CRF, or ultimately by a decrease in transcription and or translation of CRFR1 mRNA.

Upon binding, the CRF-CRFR1 complex is rapidly internalized into the corticotrope (Childs et al., 1986). Studies using biotinylated or iodinated CRF have investigated the fate of the internalized ligand-receptor complex (Leroux & Pelletier, 1984). The complex is rapidly internalized into granules where the ligand is removed from the receptor. Receptors then end up either in primary lysozomes where they are degraded or in secretory granules where they could possibly be recycled back to the plasma membrane (Childs et al., 1986).

Following adrenalectomy, inhibition of CRF neurons by glucocorticoids is removed causing an increase in hypothalamic CRF synthesis and release into the median eminence (Healy, 1992). Receptors for CRF in the AP decrease by 70% post adrenalectomy. This effect is reversed with glucocorticoid replacement (De Souza et al., 1985; Wynn et al., 1985). Increased AP exposure to CRF is thought to mediate the decrease in CRFR1 observed following adrenalectomy. Hypothalamic deafferentation or lesions in the PVN reduce the magnitude of CRFR1 down regulation observed following adrenalectomy (Rabadan Diehl et al., 1997b). Furthermore, chronic subcutaneous CRF infusion (100 ng/min) to rats mimics the acute effects of adrenalectomy on AP CRFR1

concentrations (Wynn et al., 1988), but this effect is transient and the CRFR1 numbers return to basal levels by 4 hours (Ochedalski et al., 1998). What is most intriguing however, is the apparent interaction between CRF and glucocorticoids. Simultaneous infusion of doses of CRF and glucocorticoids that individually have no effect on CRF binding increases CRF binding sites (Ochedalski et al., 1998). This suggests an interaction between glucocorticoids and hypothalamic factors such as CRF in regulating the abundance of CRFR1 in the AP. These studies further solidify the role of the hypothalamus in regulating AP responsiveness to CRF.

Studies conducted *in vitro* using pretreatments of CRF followed by stimulation with CRF or other factors known to stimulate AP ACTH secretion help clarify the effects of hypothalamic CRF on AP responsiveness. Following a 3-hour pretreatment with CRF, corticotrope responsiveness to subsequent CRF stimulation decreases but responsiveness to forskolin is unaffected (Reisine & Hoffman, 1983). Also, pretreatment with CRF did not cause desensitization of corticotropes to the stimulatory effects of catecholamines, indicating desensitization mechanisms do not affect β-adrenergic receptors (Hoffman et al., 1985; Reisine & Hoffman, 1983). These studies also show no change in intracellular ACTH levels, indicating that desensitization does not occur due to a depletion of ACTH. Similar results are observed utilizing AP cells obtained from adrenalectomized rats. These cells display a decreased ability to generate cAMP and secrete ACTH in response to CRF stimulation. Similar to the response of cells pretreated with CRF, cells from adrenalectomized animals respond normally to stimulatory factors besides CRF (Wynn et al., 1988). This indicates desensitization following adrenalectomy occurs through a

decrease in CRFR1 numbers or an uncoupling of adenylate cyclase. Desensitization through an attenuation of the stimulated rate of cAMP production is similar to what is seen with other G-protein linked receptors (Hausdorff et al., 1990).

In addition to down regulation of CRF binding sites, CRF stimulation decreases the abundance of CRFR1 mRNA further limiting the ability of the corticotrope to replenish CRF receptors and subsequent sensitivity to CRF stimulation. Treating AP cells with CRF, chronic infusion of CRF and adrenalectomy all result in a decrease in AP CRFR1 mRNA (Pozzoli et al., 1996; Sakai et al., 1996). What is most interesting however, is the apparent interaction between CRF and glucocorticoids. The transient nature of the down regulation of CRFR1 mRNA observed after CRF administration appears to be a direct effect of CRF, rather than an exhaustion of the effects of CRF. Similar effects to those observed after CRF infusion are observed after simultaneous infusion of CRF with a dose of glucocorticoids that on its own results in a more prolonged down regulation of CRFR1 mRNA (Ochedalski et al., 1998).

Arginine vasopressin (AVP)

While CRF is well known for its role in regulation of the pituitary adrenal axis, other neurohormones, such as AVP, also play a key role. AVP is produced and secreted primarily by magnocellular neurons of the supraoptic nucleus (SON) and PVN terminating in the posterior pituitary. It is this source that is responsible for actions of AVP in the periphery (Taniguchi et al., 1988). However, AVP is also co-secreted with some parvocellular CRF neurons terminating in the median eminence (Makara, 1992). AVP secreted into the median eminence reaches the AP through the pituitary portal

circulation rather than being secreted into the general circulation, which is the fate of magnocellular neuron-secreted AVP. It is widely accepted that AVP of parvocellular origin is responsible for HPA axis activation (Hauger & Aguilera, 1993). However, there is some controversy regarding the fate of AVP of magnocellular origin. Comparisons of median eminence and parvocellular and magnocellular neuron peptide concentrations following chemical depletion of parvocellular neurons and subsequent potassium ion stimulation reveal two sources of AVP secretions into the median eminence. Only one of these sources contains CRF as well as AVP (Holmes et al., 1986). Since all AVP of parvocellular origin is co-expressed in CRF neurons, these neurons would have to selectively release AVP-containing secretory granules, or else some of the AVP secreted into the median eminence is of magnocellular origin. These studies further demonstrate that PVN lesions eliminate the increases in CRF associated with potassium ion stimulation but do not affect the AVP response (Holmes et al., 1986). Furthermore, lesions of the PVN do not reduce basal concentrations of AVP in the pituitary portal circulation or prevent increases associated with adrenalectomy (Antoni et al., 1990). These studies further implicate involvement of AVP of magnocellular origin in regulation of the HPA axis.

Arginine vasopressin plays a permissive role in CRF stimulation of the HPA axis. The extent to which AVP stimulates ACTH secretion depends on the species in question (Castro, 1993; Familari et al., 1989; Gillies et al., 1980; Kemppainen et al., 1992; Meller et al., 1991; Schwartz et al., 1994; Tonon et al., 1986). Rodents display a 10-fold increase in plasma ACTH concentrations in response to i.p. injection of 1 microgram

CRF compared to an 8-fold increase in response to the same dose of AVP (Spinedi & Negro Vilar, 1983). However, *in vitro*, CRF treatment elicits an approximately 4-fold greater ACTH response than similar concentrations of AVP (Spinedi & Negro Vilar, 1983). The differences observed *in vivo* are likely due to the synergistic actions of AVP with endogenous CRF. In contrast to rodents, it is suggested that AVP is the primary mediator of AP ACTH secretion in sheep (Familari et al., 1989; Liu et al., 1990), although there are discrepancies in the literature (McFarlane et al., 1995). Our preliminary data indicates that CRF and AVP are both potent stimulators of ACTH secretion *in vivo* and *in vitro* in cattle (Qahwash, et al., unpublished).

In most cases, CRF and AVP act synergistically to elicit a more robust ACTH response than either hormone could produce on its own (Murakami et al., 1984; Turkelson et al., 1982). Detailed kinetic studies examined the responses to CRF and AVP treatments in a microperifusion system and revealed different properties for the ACTH secretory responses to each hormone (Watanabe & Orth, 1987). The distinct secretory properties suggest each hormone activates AP corticotropes through distinct mechanisms. AVP mediates its actions through stimulation of protein kinase C (PKC) leading to an influx of calcium from intracellular stores (Bilezikjian et al., 1987; Murakami et al., 1985b; Raymond et al., 1985). However, it is not completely understood how CRF and AVP signaling pathways are integrated to produce the synergistic ACTH secretory response (Koch & Lutz Bucher, 1991).

AVP receptors

AVP mediates its actions on AP corticotropes by binding to specific AVP receptors. Autoradiographic studies revealed specific binding sites for AVP on AP corticotropes, distinct from those for CRF (Du Pasquier et al., 1991; Koch & Lutz, 1985). These studies provide the basis for the cloning and characterization of the AP AVP receptor cDNA (Saito et al., 1995). The AP AVP receptor is one of three distinct AVP receptor subtypes and is referred to in the literature as either the V3 or V1b receptor. The AP AVP receptor will hereafter be referred to as the V3 receptor. The V3 receptor is distinct from other AVP receptor subtypes (V1 and V2) in affinity for AVP and its second messenger cascade (Birnbaumer, 2000). Similar to the CRF system, AP responsiveness to AVP stimulation, abundance of V3 receptor mRNA, and number of AVP binding sites are all sensitive to glucocorticoids and stress (Aguilera et al., 1994; Rabadan Diehl et al., 1995; Rabadan Diehl et al., 1997a).

The AP ACTH response to AVP stimulation and AVP binding to AP membranes increases following chronic stress paradigms, such as 14 day repeated immobilization or i.p. hypertonic saline injections (Aguilera et al., 1994). AP V3 receptor mRNA also increases following similar chronic stress paradigms (Rabadan Diehl et al., 1995). However, 60-hour water deprivation or supplementing drinking water with 2% NaCl results in a decrease in AP V3 receptor mRNA (Rabadan Diehl et al., 1995).

Changes in AP AVP receptors and mRNA following acute stress are also dependent on the stress paradigm. Abundance of V3 receptor mRNA increases four

hours after one-hour immobilization and is accompanied by an increase in AVP binding (Rabadan Diehl et al., 1995). However, four hours following a single i.p. hypertonic saline injection, V3 receptor mRNA is decreased but AVP binding is increased (Rabadan Diehl et al., 1995). This effect may be a result of the transient increase in circulating AVP associated with the i.p. hypertonic saline injection.

Glucocorticoids also regulate AP AVP receptors. Similar to its effects on CRFR1 mRNA, adrenalectomy causes a transient down regulation of V3 receptor mRNA. Levels are decreased markedly by 18 hours after adrenalectomy and return to basal levels within four days (Rabadan Diehl et al., 1997a). Replacing basal glucocorticoid concentrations with dexamethasone prevents these changes. Furthermore, administration of dexamethasone for 7 days increases V3 receptor mRNA (Rabadan-Diehl & Aguilera, 1998). However, increases in V3 receptor mRNA after glucocorticoid administration are accompanied by decreases in AVP binding (Aguilera & Rabadan Diehl, 2000). These findings indicate that glucocorticoids may also have post-transcriptional effects on V3 receptors. The above described effects of glucocorticoids on V3 receptor mRNA and AVP binding are likely attributable to direct effects of glucocorticoids. Removing the influence of the hypothalamus by PVN lesions or hypothalamic anterolateral cuts (ALC) did not prevent the decreases in V3 receptor mRNA associated with adrenalectomy (Rabadan Diehl et al., 1997a). These findings eliminate the possibility of ligand-induced down regulation of V3 receptor mRNA following adrenalectomy. However, adrenalectomy of di/di Brattleboro rats (lack hypothalamic AVP) results in sustained decreases in V3 receptor mRNA suggesting a potential role for hypothalamic AVP in the

recovery from, rather than the down regulation of, V3 receptor mRNA following adrenalectomy. To my knowledge, *in vitro* studies have not been conducted to examine the effects of AVP treatments on V3 receptor mRNA in AP cells. Furthermore, exposure of di/di Brattleboro rats to stress paradigms that result in increases in V3 receptor mRNA, such as acute i.p. hypertonic saline injections or water deprivation would prove useful in determining the potential role of AVP in increasing V3 receptor mRNA.

AVP regulation of AP CRF receptors

AVP effects on CRF-stimulated ACTH secretion have been clearly established (Gibbs, 1986). Coordinate regulation of AP CRF receptors by CRF and AVP has also been examined. Similar to CRF, AVP decreases CRFR1 mRNA and receptor numbers (Hoffman et al., 1985; Pozzoli et al., 1996). Treatment of AP cells with AVP causes a sustained down regulation of CRFR1 mRNA in contrast to the transient decrease observed following treatment with CRF (Sakai et al., 1996). However, these results are not consistent with a similar study that demonstrates a sustained decrease in CRFR1 mRNA following treatment with the same dose of CRF (Pozzoli et al., 1996). Regardless, these studies suggest that AVP stimulation decreases CRFR1 mRNA, potentially through effects on transcription and (or) mRNA stability.

In addition to its negative effects on AP CRFR1 mRNA, AVP also has negative effects on AP CRF receptors. Similar to the synergistic effect of CRF and AVP on ACTH secretion (Gibbs, 1986), CRF receptor down regulation after treatment with a combination of both hormones is greater than that observed after treatment with either

hormone alone (Holmes et al., 1987). Infusion of CRF, AVP or a combination of both hormones demonstrates that either peptide decreases CRF receptors. However, only the combination of CRF and AVP causes decreases similar in magnitude to those observed after adrenalectomy (Holmes et al., 1987). Adrenalectomy in di/di Brattleboro rats results in less down regulation of CRF receptors than observed following adrenalectomy of control rats. Infusion of AVP into adrenalectomized di/di Brattleboro rats enhanced the down regulation of CRF receptors to levels comparable to adrenalectomized control rats (Holmes et al., 1987). Furthermore, pretreatment with AVP has an inhibitory effect on the AP ACTH response to CRF treatment (Hoffman et al., 1985). Collectively, these findings support an important role of other hypothalamic factors, such as AVP, in regulation of AP corticotrope responses during stress.

Stress Models

Our understanding of the stress response and mechanisms involved in AP desensitization is far from complete. Our focus in forthcoming experiments (described in Chapter 2) is on regulation of AP receptors for the primary hypothalamic peptides (CRF and AVP) that stimulate ACTH secretion in cattle. Several stress models are commonly used to study the regulation of components of the HPA axis during and following a stress response. Endotoxin treatment, restraint, food deprivation, foot-shock, and i.p. injection of hypertonic saline all cause pronounced activation of the HPA axis (Aubry et al., 1997; Makino et al., 1995; Rabadan Diehl et al., 1996; Timofeeva & Richard, 1997). Many of these models are not validated or appropriate for use in cattle. However, lipopolysaccharide (LPS)-induced endotoxemia is a well-characterized model that has

been previously utilized in cattle (Andersen et al., 1996; Griel et al., 1975; Kahl et al., 2000).

LPS is a component of the cell wall of gram-negative bacteria that elicits an immune reaction when introduced into an animal. The classical response of animals to LPS injection has been recently reviewed by McCann and Kimura (McCann et al., 2000). Briefly, the immune system secretes proinflammatory cytokines in response to an LPS challenge (Rivest et al., 2000). Cytokines mediate the communication between the immune and neuroendocrine systems resulting in activation of the HPA axis (Kakucska et al., 1993; Rivier et al., 1989). LPS administration increases CRF and AVP mRNA in hypothalamic nuclei (Rivest & Laflamme, 1995). CRF and AVP secretion into the pituitary portal circulation also increases, thereby initiating activation of the HPA axis (Dadoun et al., 1998). Administration of CRF antiserum or CRF receptor antagonists to endotoxin treated rats inhibits ACTH responses (Aubry et al., 1997). These studies demonstrate that the ACTH response to LPS-induced endotoxemia is primarily mediated by CRF. The robust and predictable nature of the hypothalamic, AP and adrenocortical response to LPS makes it a useful model system for studies of regulation of CRF and AVP receptors during a stress response in cattle.

Summary

Activation of the HPA axis is a primary component of the response to stress (Fig. 1). Hormones secreted in association with HPA axis activation function to provide an animal with the energy necessary to cope with a stressful situation. However, exposure

to stress at inopportune times or for prolonged periods can predispose an animal to infectious disease. CRF, the primary mediator of HPA axis activity, in addition to having direct immunomodulatory actions, suppresses the immune system through stimulation of AP ACTH secretion and subsequent adrenocortical glucocorticoid production. The immunosuppressive effects of glucocorticoids have been well documented in the literature.

In addition to CRF, other hypothalamic neurohormones, such as AVP, stimulate AP production of ACTH. The actions of CRF and AVP occur by binding to specific AP corticotrope membrane bound receptors. Abundance of mRNA encoding for these receptors and the numbers of receptors expressed are hormonally regulated. CRF and AVP have inhibitory effects on CRFR1 and V3 receptor numbers and mRNA. In addition, glucocorticoids, the end product of HPA axis activation, also have regulatory effects on CRF and AVP receptor numbers and mRNA. Regulation of AP receptors for hypothalamic neurohormones ultimately helps control AP responsiveness to subsequent hypothalamic stimuli and represents a potentially important mechanism by which the magnitude and duration of the endocrine response to stress may be regulated.

Our focus is to understand the regulation of the stress response in cattle. As stated previously exposure to stress increases susceptibility of cattle to infectious diseases such as bovine respiratory disease (LeKeux, 1995). Most of the information described previously in this review is derived from studies utilizing rodents. Little information is available describing the specific mechanisms that mediate the endocrine response to

stress in cattle. Furthermore, the actions of specific hormones in the HPA axis vary somewhat between species (Spinedi & Negro Vilar, 1983; Familari et al., 1989; Liu et al., 1990). Our preliminary data (Qahwash, et al., unpublished) indicates that both CRF and AVP are potent stimulators of ACTH secretion *in vitro* and *in* vivo in cattle. I believe that pituitary desensitization to CRF and AVP represents a potential mechanism to control the stress response in cattle. However, little is known regarding regulation of AP receptors for CRF and AVP in species other than rodents. In Chapter 2 experiments designed to examine the regulation of AP CRF and AVP receptors in cattle following LPS-induced endotoxemia are described. Based on the observed ACTH response to CRF and AVP in cattle, I hypothesize that AP CRFR1 and V3 receptor mRNA will decrease following LPS administration.

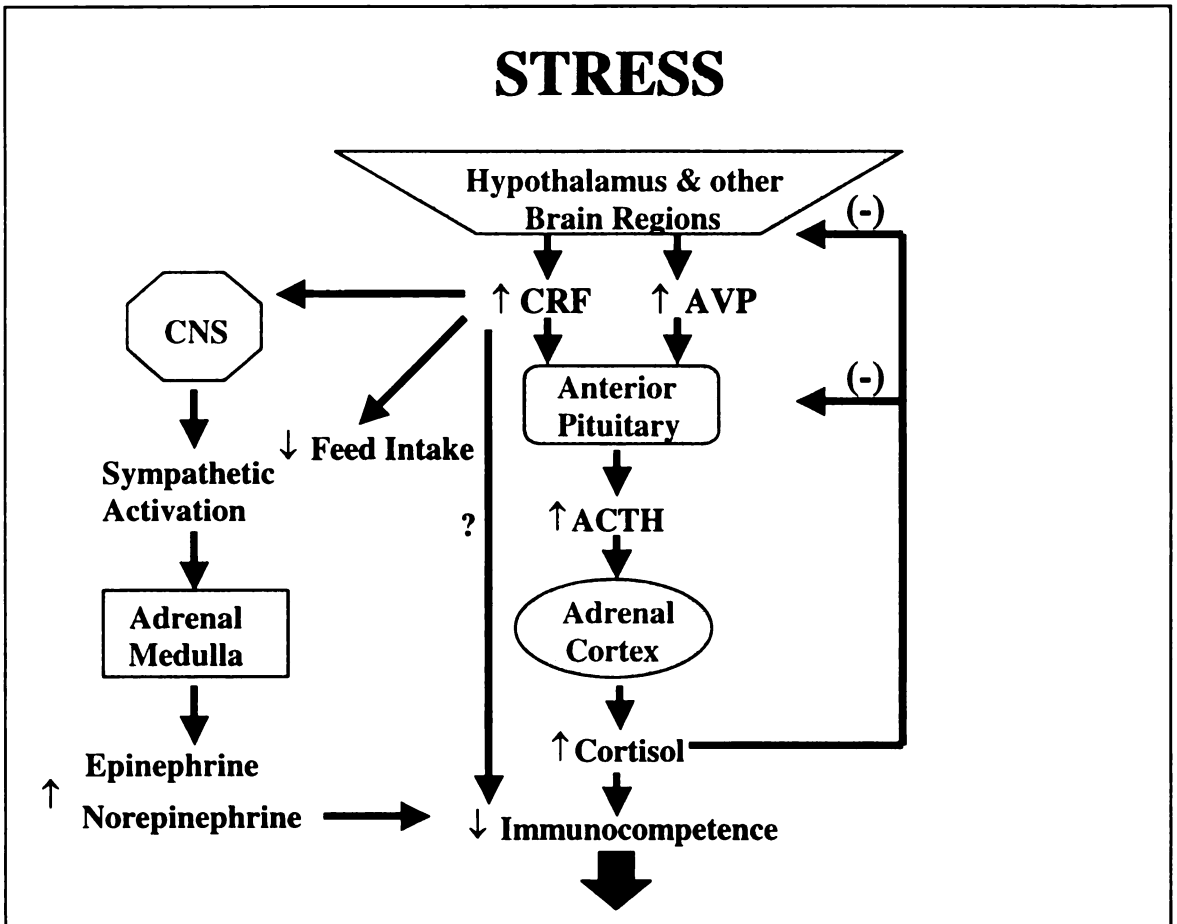

Figure 1. The response to stress.

Chapter 2

Coordinate regulation of corticotropin releasing factor receptor 1 and arginine vasopressin receptor V3 messenger ribonucleic acid in the anterior pituitary of endotoxemic steers

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. Activation of the hypothalamic-pituitary-adrenocortical (HPA) axis is a hallmark adaptive response to stress (Minton, 1994).

In response to stress, parvocellular neurons in the paraventricular region of the hypothalamus, terminating in the median eminence, release factors that stimulate anterior pituitary (AP) secretion of adrenocorticotropic hormone (ACTH) (Antoni, 1986). The two primary hypothalamic releasing factors that regulate basal and stress-induced secretion of ACTH are corticotropin releasing factor (CRF) and arginine vasopressin (AVP) (Makara, 1992). In most instances, CRF is the primary hypophysiotropic factor controlling ACTH secretion and the role of AVP is more species dependent (Chan et al., 1982). In cattle, AVP also has significant ACTH releasing activity independent of CRF (Schwartz & Vale, 1988). Coordinate regulation of hypothalamic synthesis and secretion of CRF and AVP, and of pituitary responsiveness to these releasing factors, is critical for regulation of the endocrine response to stress.

The biological actions of CRF and AVP on the AP are each mediated by binding to specific receptors coupled to G-protein linked signaling pathways. Binding of CRF to CRFR1 activates adenylate cyclase and increases intracellular cAMP concentrations (De Souza, 1995). Binding of AVP to the V3 receptor causes a phospholipase C mediated

following LPS administration. However the effect of LPS administration on AP receptors for AVP, a primary mediator of HPA axis activity in cattle, has not been examined. My hypothesis is that LPS administration will decrease AP CRFR1 and V3 receptor mRNA.

LPS, a component of the outer membrane of gram-negative bacteria, stimulates a profound inflammatory response and activation of the HPA axis (Rivest et al., 2000; Takemura et al., 1997; Tilders et al., 1994). This provides a powerful tool to study physiological regulation of CRF and AVP receptors by endocrine and inflammatory stimuli. In the present study we determined relative abundance of CRFR1 and V3 receptor mRNA in the bovine AP prior to and at several time points after LPS administration. Tissue specificity of the effects of LPS administration on CRFR1 mRNA was determined. Abundance of AP POMC mRNA was also assessed to determine the effects of LPS administration on other components of the pituitary ACTH producing machinery. Plasma ACTH and cortisol concentrations were also measured and correlated with changes in CRFR1 and V3 receptor mRNA abundance.

Materials and Methods

Animals

All experiments were approved by the Committee on Animal Use and Care at Michigan State University (Approval# 10/98-141-00). Holstein steers (n = 20) ranging between 250 and 350 lbs. were used in this experiment. Animals were maintained on a 16:8 light:dark cycle in environmentally controlled chambers at the Michigan State

University Dairy Cattle Teaching and Research Center. Animals were fed a pellet diet (18% crude protein and 19.6% acid detergent fiber, Land O'Lakes, Indianapolis, IN) ad libitum between 1000 and 1200 daily with unlimited access to water and allowed to acclimate to chambers for a minimum of 14 days before initiation of experiments. At least 48 hours before initiation of experiments, animals were weighed for dose calculations and jugular catheters inserted for treatment administration and blood collection.

Treatments

One mg of bacterial lipopolysaccharide (LPS; E. 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 i.v. 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 minute intervals for one hour before administration of treatments (time 0), 15 minute intervals for one hour after treatment administration, 30 minute intervals for hours 2-6 after administration of treatments, then hourly for hours 3-9 and every 3 hours for hours 10-18. Final samples were collected at 24 hours. Blood was collected into chilled polypropylene tubes containing 1.4 mg EDTA/ml and centrifuged immediately at approximately 1,500 X g for

5 minutes. Plasma was aliquoted into chilled microcentrifuge tubes and stored at –20 C. Rectal temperatures were taken hourly from one hour prior through nine hours after treatment administration to monitor the pyrogenic response to systemic LPS challenge.

ACTH Assay

A double antibody human ACTH radioimmunoassay (RIA; DiaSorin, Inc., Stillwater, MN) validated for bovine ACTH was used to measure ACTH concentrations. 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 minutes 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. Sensitivity of the assay, determined as the value two standard deviations away from the mean of ten replicates of the zero standard, was approximately 10 pg/ml. Inter and intra-assay coefficients of variation were calculated from a 1:2 dilution of the ACTH high pool run in each assay and twelve times in a single assay. The inter and intra assay coefficients of variation were 8.9 % and 8.0 % respectively.

Cortisol assay

Cortisol was assayed using a double antibody RIA (Diagnostics Products, Inc., Los Angeles, CA). The cortisol assay was validated as described above for ACTH. A high pool of cortisol was obtained by drawing blood 30 minutes after bolus injection of

250 μg bovine CRF. Sensitivity of the assay was determined at 0.02 μg/dl and inter and intra assay coefficients of variation were 4.3 % and 3.7 % respectively.

Tissue collection

Steers were euthanised with an i.v. injection of 80 mg/kg sodium pentobarbital (Sigma, St. Louis, MO) at 0, 2, 4, 12, or 24 hours after LPS administration (n = 4 per time point). Pituitary glands were removed and AP were dissected from posterior and neurointermediate lobes, frozen in liquid nitrogen, and stored at –80 C until RNA isolation. A portion of the cerebellum was also collected from each animal and processed as described above.

Cloning of bovine CRFR1, V3 and POMC cDNAs

Primers derived from the reported sequence of the human and rat CRFR1 cDNAs were used to amplify a 481 bp partial bovine CRFR1 cDNA. Primers derived from the reported sequence of the human and rat V3 receptor cDNAs were used to amplify a 626 bp partial bovine V3 receptor cDNA. Primers derived from the reported sequence of the bovine pro-opiomelanocortin (POMC) cDNA were used to amplify a 297 bp partial bovine POMC cDNA. All cDNAs were amplified from bovine AP total RNA using the reverse transcriptase-polymerase chain reaction (RT-PCR). The PCR products were ligated into the PGem-T Easy vector. Dye terminator and dye primer sequencing was 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 receptor cDNAs, respectively (Chang et al., 1993; Lolait et al., 1995).

Probe labeling, northern blot and dot blot analyses

Total RNA was isolated using the TRIZOL® Reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Pools of total RNA were made from each time point and 25 μg of each pool were subjected to electrophoresis through 1% agarose-formaldehyde gels. RNA was then capillary transferred to nylon membranes (BioRad, Richmond, CA) and UV crosslinked. For dot blot analysis, 5 μg of total RNA isolated from each individual sample was spotted onto nylon membranes in duplicate using a dot blot apparatus (BioRad, Richmond, CA). Membranes were allowed to air dry and then UV crosslinked.

Probes were labeled using PCR with α-[^{32}P] dCTP (New England Nuclear, Boston, MA). Each PCR mix contained 1X PCR buffer, 2.5 mM $MgCl_2$, 1.55 μM dNTPs (-dCTP), 1.5 units of Taq polymerase (Life Technologies, Inc., Gaithersburg, MD), 0.25 μM of each primer, 5 μl α-[^{32}P] dCTP and 100 pg of template. PCR conditions were as follows: 95 C 5 min, 40 cycles of [94 C 30 s, 54 C 1 min, 72 C 1.5 min], followed by a final 10 minute extension at 72 C.

Membranes were prehybridized at 42 C overnight in 50% formamide, 5X SSC, 5X Denhardts, 50 mM $NaPO_4$, 0.1% SDS, and 250 μg sperm DNA/ml prehybridization buffer. Then, hybridizations took place in 50% formamide, 5X SSC, 1X Denhardts, 20

mM $NaPO_4$, 0.1% SDS, 10% dextran sulfate, and 100 μg sperm DNA/ml hybridization buffer containing $1e^6$ cpm/ml of ^{32}P-labeled cDNA. Hybridizations were at 42 C for 18 hours. Membranes were washed in Wash I (1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate) for 20 min at 42 C followed by one 20 min wash in Wash II (0.1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate) at 42 C and one 20 min wash in Wash II at 47 C. Membranes were then exposed to a phosphoimager screen (BioRad, Richmond, CA) and subjected to densitometry. Size of mRNA transcripts detected was determined based on relative migration of RNA molecular weight markers. Membranes were then stripped and probed as described above with a ^{32}P-labeled ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA for normalization purposes.

Statistical analysis

Differences in CRFR1 mRNA (adjusted relative to levels of GAPDH mRNA) were determined by 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 by ANOVA using the mixed procedure of SAS. Hormone data were log transformed to achieve uniform variation within each group. Differences between means, when the F-test was significant ($P < 0.05$), were adjusted for multiple comparisons using the Dunnett method. Correlations between plasma hormone concentrations and mRNA were made using the correlation (CORR) procedure of SAS.

Results

Effects of LPS on plasma ACTH and cortisol concentrations and body temperature

LPS administration to steers (200 ng/kg, iv) induced significant increases ($P < 0.05$) in plasma concentrations of ACTH at 2 and 4 hours (Fig. 2) and cortisol at 2, 4 and 12 hours (Fig. 3) after LPS administration compared to 0 hour (pre-LPS) hormone concentrations. Body temperature was also significantly increased ($P < 0.05$) at 2 and 4 hours after LPS administration (Fig. 4). Body temperatures and ACTH and cortisol concentrations in diluent-treated control animals did not change over the course of the 24 hour sampling period (data not shown).

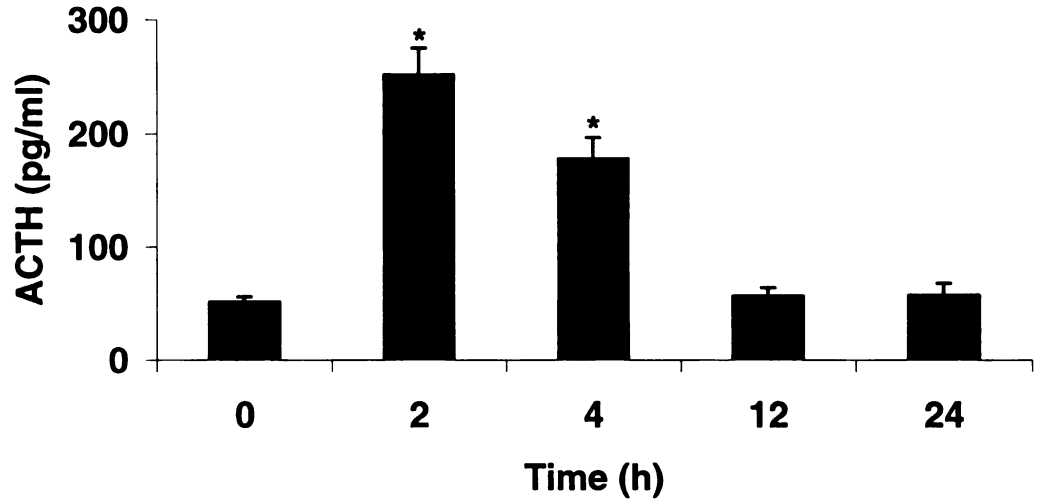

Figure 2. Effect of LPS administration on plasma ACTH concentrations in Holstein steers. Blood was drawn through indwelling jugular catheters inserted 48 hours before experimentation. Blood was collected from all animals up until respective time of tissue collection (n = 4 per time point). Time 0 = time of LPS administration (* $P < 0.05$ versus 0 hour).

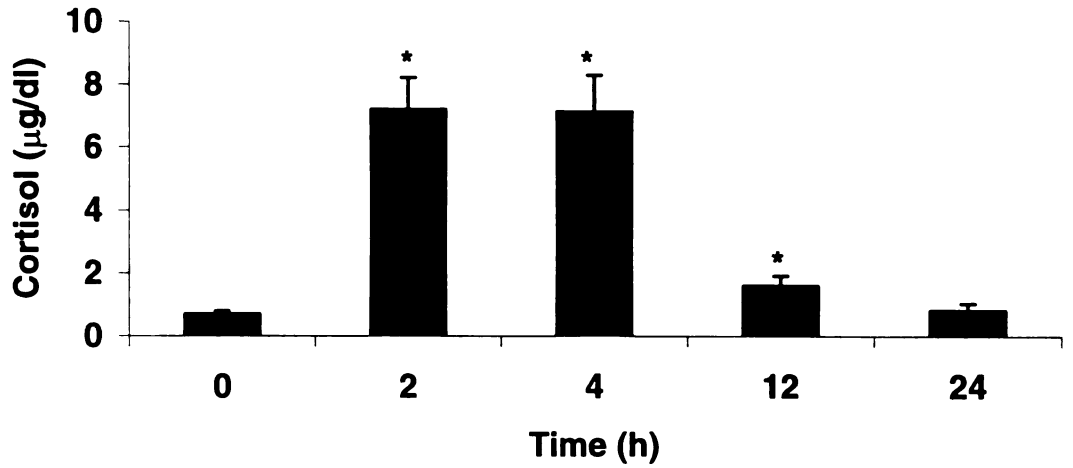

Figure 3. Effect of LPS administration on plasma cortisol concentrations in Holstein steers. Blood was drawn through indwelling jugular catheters inserted 48 hours before experimentation. Blood was collected from all animals up until respective time of tissue collection (n = 4 per time point). Time 0 = time of LPS administration (* $P < 0.05$ versus 0 hour).

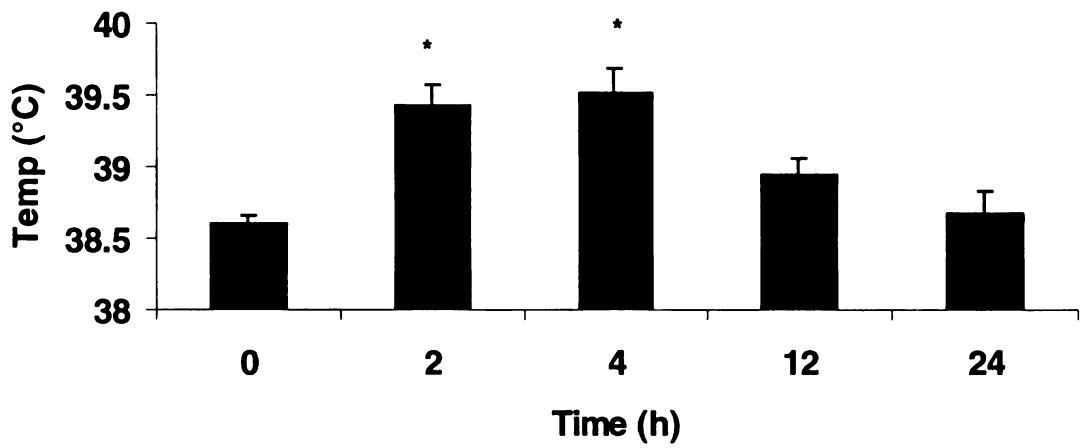

Figure 4. Effect of LPS administration on rectal temperatures in Holstein steers (n = 4 per time point). Note the significant increase in rectal temperatures by 2 hours after LPS administration. Time 0 = time of LPS administration (* $P < 0.05$ versus 0 hour).

Regulation of CRFR1 mRNA in the AP and cerebellum of endotoxemic steers

Northern analysis of bovine AP 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. 5). AP CRFR1 mRNA decreased following LPS administration. Relative levels of CRFR1 mRNA were significantly decreased ($P < 0.05$) at 2, 4 and 12 hours following LPS administration and returned to baseline by 24 hours (Fig. 6). Relative levels of pituitary CRFR1 mRNA were negatively correlated with plasma ACTH and cortisol concentrations ($r = -0.66$ and -0.78, respectively).

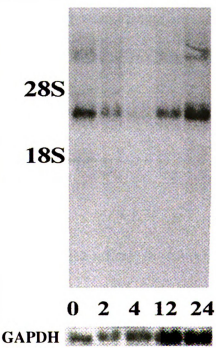

Figure 5. Northern blot analysis of CRFR1 mRNA in bovine AP collected at 0, 2, 4, 12, and 24 hours after LPS administration. Note hybridization to a major 2.5 kb and a minor 8.0 kb transcript.

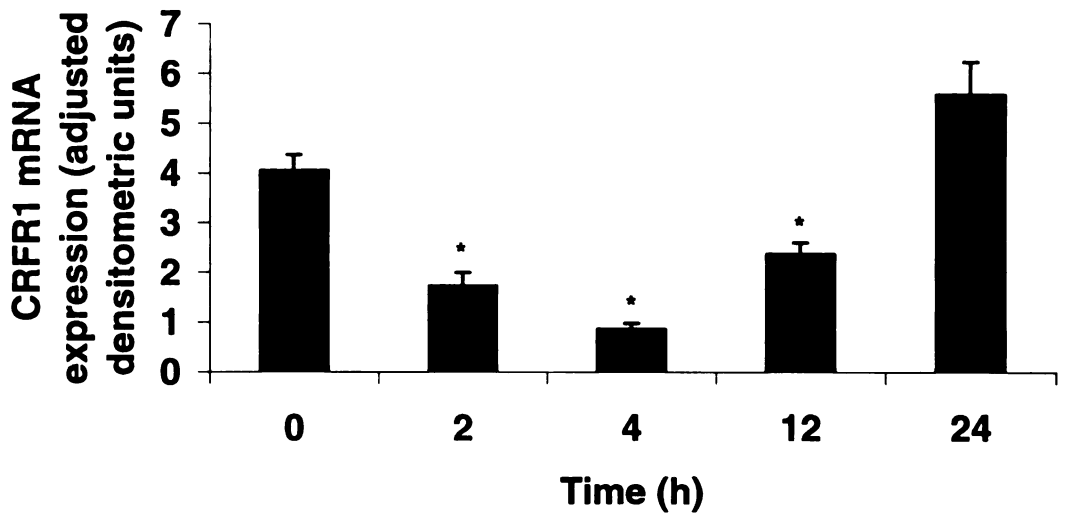

Figure 6. Quantification of AP CRFR1 mRNA at 0, 2, 4, 12 and 24 hours after LPS administration (n = 4 animals per time-point). CRFR1 mRNA levels were adjusted relative to levels of GAPDH mRNA (adjusted densitometric units). Note significant decrease in CRFR1 mRNA by 2 hours after LPS administration (* $P < 0.05$ versus 0 hour).

CRFR1 mRNA levels were also measured in cerebellar tissue collected from the same animals to determine if CRFR1 mRNA is coordinately regulated in other brain regions following LPS administration. Number and size of CRFR1 transcripts were not different than that observed in the AP (Fig. 7). No significant changes ($P > 0.05$) in cerebellum CRFR1 mRNA following LPS administration were detected (Fig. 8).

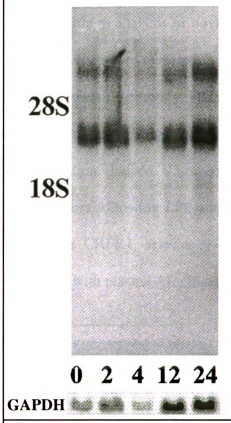

Figure 7. Northern blot analysis of CRFR1 mRNA in bovine cerebellum collected at 0, 2, 4, 12, and 24 hours after LPS administration.

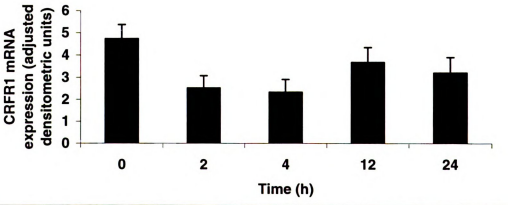

Figure 8. Quantification of cerebellum CRFR1 mRNA at 0, 2, 4, 12 and 24 hours after LPS administration (n = 4 animals per time-point). CRFR1 mRNA levels were adjusted relative to levels of GAPDH mRNA (adjusted densitometric units). Note no effect of LPS administration on CRFR1 mRNA in the cerebellum was observed ($P > 0.05$ versus 0 hour).

Regulation of V3 receptor mRNA in the AP of endotoxemic steers

Northern analysis of bovine AP RNA collected at 0, 2, 4, 12, and 24 hours after LPS administration revealed a single 7.0 kb V3 receptor mRNA species (Fig. 9). Relative levels of pituitary V3 receptor mRNA were also measured to determine if V3 receptor mRNA changes in a similar fashion as CRFR1 mRNA following LPS administration. Indeed, V3 receptor mRNA was significantly decreased ($P < 0.05$) at 2, 4 and 12 hours following LPS administration, and returned to baseline by 24 hours (Fig. 10). Like CRFR1, relative levels of AP V3 receptor mRNA were also negatively correlated with plasma ACTH and cortisol ($r = -0.65$ and -0.78, respectively).

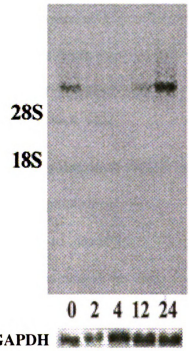

Figure 9. Northern blot analysis of V3 receptor mRNA in bovine AP collected at 0, 2, 4, 12, and 24 hours after LPS administration. Note hybridization to a 7.0 kb transcript.

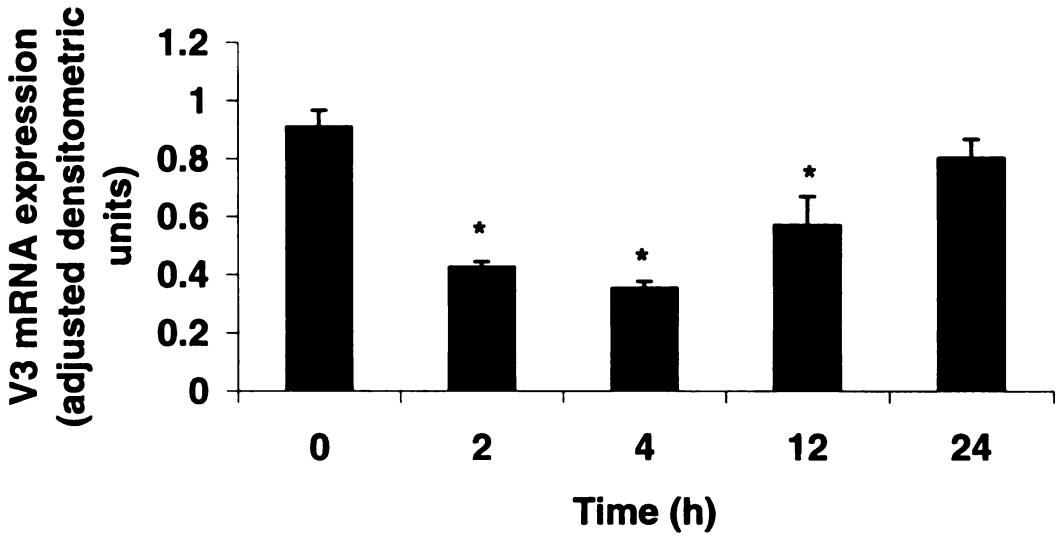

Figure 10. Quantification of AP V3 receptor mRNA at 0, 2, 4, 12 and 24 hours after LPS administration (n = 4 animals per time-point). V3 receptor mRNA levels were adjusted relative to levels of GAPDH mRNA (adjusted densitometric units). Note significant decrease in V3 receptor mRNA by 2 hours after LPS administration (* $P < 0.05$ versus 0 hour).

Regulation of POMC mRNA in the AP of endotoxemic steers

Regulation of pituitary POMC mRNA following endotoxin administration was examined to determine if the LPS-induced decrease in mRNA for CRF and AVP receptors is shared by other components of the pituitary ACTH producing machinery. Northern analysis revealed a prominent 1 kb transcript for POMC in the AP (Fig. 11). In contrast to the expression pattern observed in the pituitary for CRFR1 and V3 receptor mRNAs, no significant changes ($P > 0.05$) in POMC mRNA were detected (Fig. 12).

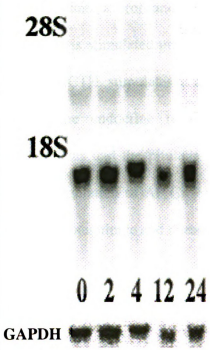

Figure 11. Northern blot analysis of POMC mRNA in bovine AP collected at 0, 2, 4, 12, and 24 hours after LPS administration. Note hybridization to a 1.0 kb transcript.

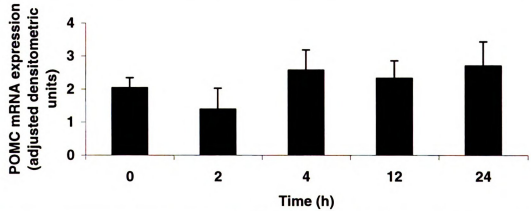

Figure 12. Quantification of AP POMC mRNA at 0, 2, 4, 12 and 24 hours after LPS administration (n = 4 animals per time-point). POMC mRNA levels were adjusted relative to levels of GAPDH mRNA (adjusted densitometric units). No effect of LPS administration on POMC mRNA in the AP was observed ($P > 0.05$ versus 0 hour).

Discussion

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, elicits a profound endocrine response. In response to LPS, pituitary ACTH production is stimulated primarily by hypothalamic CRF and AVP (Aubry et al., 1997). In turn, ACTH stimulates adrenal synthesis and secretion of glucocorticoids, which regulate the endocrine response through negative feedback at the level of the hypothalamus and the pituitary (Birnberg et al., 1983; Kretz et al., 1999; Sakai et al., 1996). Regulation of AP CRF and AVP receptors represents a potential mechanism to control the magnitude and duration of the endocrine response to stress.

As an initial step towards understanding the regulation of AP CRFR1 and V3 receptors during a stress response, I determined the effect of LPS-induced endotoxemia on CRFR1 and V3 receptor mRNA in the bovine AP. Comparisons were made with the effects of LPS-induced endotoxemia on CRFR1 mRNA in the cerebellum, and with effects on AP POMC mRNA in order to assess specificity of the regulatory response for CRFR1 and V3 receptor mRNA observed in the AP.

In the present studies, AP CRFR1 and V3 receptor mRNA levels were coordinately down regulated in response to LPS administration. Down regulation of pituitary CRFR1 mRNA following LPS administration has also been demonstrated in rats (Aubry et al., 1997). The mechanisms responsible for down regulation of 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, hypothalamic

releasing factors, and (or) glucocorticoids. The potential role of CRF in regulation of CRFR1 mRNA during inflammatory stress is not completely understood. Treatment of rat AP cells with CRF decreases relative levels of CRFR1 mRNA *in vitro* (Sakai et al., 1996). However immunoneutralization of CRF does not attenuate LPS-induced down regulation of pituitary CRFR1 mRNA *in vivo* (Aubry et al., 1997). This suggests that down regulation of CRFR1 mRNA during LPS-induced endotoxemia is not solely a result of the actions of CRF. CRFR1 mRNA in rat pituitary cells *in vitro* is decreased following treatment with LPS or the proinflammatory cytokine IL-1β (Aubry et al., 1997). These results suggest that the pituitary responds directly to inflammatory mediators with a decrease in CRFR1 mRNA. More investigation will be required to dissect the precise mechanisms responsible for CRFR1 mRNA down regulation during LPS-induced systemic inflammatory stress in cattle.

Messenger RNA for CRFR1 is widely distributed throughout the CNS (De Souza, 1987). Roles for CRF in the stress response independent of HPA axis activation have been established (De Souza, 1995). Therefore, CRFR1 may be differentially regulated in specific brain regions in response to stress. In the present studies, AP CRFR1 mRNA was significantly reduced following LPS administration, but levels of CRFR1 mRNA in the cerebellum were not affected. Tissue specific regulation of CRFR1 mRNA in the CNS in response to stress has been observed previously in rats. In response to chronic stress, hippocampal CRFR1 mRNA increased, whereas CRFR1 mRNA in the cerebral cortex decreased (Iredale et al., 1996). Likewise, CRFR1 mRNA increased in the PVN but decreased in the pituitary of LPS-treated rats (Rivest et al., 1995). Our results support

the hypothesis that tissue specific regulation of CRFR1 mRNA in the AP occurs during LPS-induced endotoxemia in cattle.

To my knowledge, the effect of LPS-induced endotoxemia on V3 receptor mRNA has not been reported. However, evidence in the literature suggests that pituitary V3 receptor mRNA is subject to feedback regulation in other stress paradigms in rats. For example, increases in V3 receptor mRNA are observed following 4 hours of immobilization stress or following chronic stress paradigms (Rabadan Diehl et al., 1995). Glucocorticoid administration also increases V3 receptor mRNA (Aguilera & Rabadan Diehl, 2000). This is in contrast to the pronounced inhibitory effect of glucocorticoids on CRFR1 mRNA in the AP and PVN (Makino et al., 1995). This suggests that glucocorticoids do not have the same regulatory effect on V3 receptor mRNA as observed for components of the CRF system. In addition, levels of V3 receptor mRNA decreased after removal of glucocorticoids by adrenalectomy. This effect is reversed by glucocorticoid replacement, but not by PVN lesions (Rabadan Diehl et al., 1997a). These findings indicate that the decrease in V3 receptor mRNA is not AVP induced. However, AVP cannot independently activate the rodent HPA axis as well as occurs in the bovine system. Thus, it could be hypothesized that the AP AVP receptor V3 mRNA will be regulated in a similar fashion as CRFR1. In the present study, AP V3 receptor mRNA levels were significantly decreased following LPS administration in cattle, despite the pronounced increase in circulating cortisol. Elucidation of the specific mechanisms responsible for the decrease in pituitary V3 receptor mRNA during systemic inflammatory stress in cattle will require further investigation.

In contrast to the regulation observed for CRFR1 and V3 receptor mRNA, POMC mRNA in the bovine AP did not change in response to LPS-induced endotoxemia. These results were surprising because CRF and glucocorticoids are potent regulators of ACTH secretion and POMC mRNA expression in rats. Pituitary POMC mRNA increases in rats following adrenalectomy, an effect that is reversed by glucocorticoid replacement (Rabadan Diehl et al., 1996). A significant increase in POMC mRNA is also detected following treatment of mouse pituitary tumor AtT-20 cell lines with CRF (Ruzicka & Akil, 1995). Pituitary POMC mRNA in rats is increased in response to LPS administration (Li et al., 1999). Our results indicate that similar regulatory mechanisms to those observed for CRFR1 mRNA and V3 receptor mRNA do not control AP expression of POMC mRNA during LPS-induced endotoxemia in cattle. The experimental timeline utilized in the present studies may not have been frequent enough to detect a change in POMC mRNA and mechanisms that control ACTH secretion during LPS-induced endotoxemia in cattle may not affect POMC mRNA. Thus, regulation of POMC mRNA expression does not appear to be a primary mechanism for control of the endocrine response to LPS-induced endotoxemia in cattle. Mechanisms that mediate ACTH secretion after LPS administration require further investigation.

In summary, systemic LPS administration caused a decrease in CRFR1 and V3 receptor mRNA in the bovine AP. The decrease in mRNA was negatively correlated with concentrations of circulating ACTH and cortisol. I conclude that the down regulation of CRFR1 and V3 receptor mRNA following LPS administration may

represent a mechanism that decreases AP responsiveness to CRF and AVP, thereby limiting the magnitude and duration of the endocrine response to LPS-induced endotoxemia. More investigation is necessary to determine the specific cellular and molecular mechanisms responsible for the LPS-induced decrease in CRFR1 and V3 receptor mRNA and to determine if such changes are reflected by changes in AP CRF and AVP binding sites which may impact the magnitude and duration of the pituitary-adrenal response during LPS-induced endotoxemia.

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