ROLE OF LEPTIN IN REGULATING THE BOVINE HYPOTHALAMIC-GONADOTROPIC AXIS

A Dissertation

by

MARCEL AMSTALDEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Physiology of Reproduction

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ABSTRACT

Role of Leptin in Regulating the Bovine Hypothalamic-Gonadotropic Axis.

(December 2003)

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The physiological mechanisms through which nutrition mediates its effects in controlling reproduction are not well characterized. Both neural and endocrine components have been implicated in the communication of nutritional status to the central nervous system. Leptin, a hormone synthesized and secreted mainly by adipocytes, is heavily involved in this communication network. The objectives of studies reported herein were 1) to determine the effects of short-term restriction of nutrients on circulating leptin, leptin gene expression in adipose tissue, and leptin receptor (LR) gene expression in the adenohypophysis of ovariectomized cows; and 2) to investigate the responsiveness of the hypothalamic-adenohypophyseal (AP) axis of fasted and non-fasted cattle to leptin. Studies demonstrated that circulating concentrations of leptin and leptin gene expression in subcutaneous adipose tissue are decreased by fasting. Although 2 to 3 days of fasting did not affect patterns of release of luteinizing hormone (LH), cerebroventricular infusions of leptin increased mean

circulating concentrations of LH in fasted, but not normal-fed cows, without affecting frequency or amplitude of pulses of LH. *In vitro* studies were conducted to determine whether the *in vivo* effects of leptin could be accounted for at the hypothalamic and/or AP levels. Leptin did not affect the release of gonadotropin-releasing hormone (GnRH) from hypothalamic-infundibular explants from either normal-fed or fasted cattle. Moreover, leptin did not affect the basal release of LH from bovine AP cells or AP explants from normal-fed cows. However, leptin induced a higher basal release of LH from AP explants of fasted cows and increased GnRH-stimulated release of LH from AP explants of normal-fed cows. Results demonstrate that leptin acts directly at the AP level to modulate the secretion of LH, and its effects are dependent upon nutritional status. Cellular mechanisms associated with the increased responsiveness of gonadotropes to leptin in fasted cows were investigated. Expression of LR and suppressor of cytokine signaling-3 (SOCS-3) in the adenohypophysis did not account for the increased responsiveness of fasted cows to leptin. Therefore, although leptin clearly stimulates the hypothalamic-gonadotropic axis in nutrient-restricted cattle, it is unclear why cattle maintained under neutral or positive energy balance are resistant to leptin.

DEDICATION

This manuscript is dedicated to the memory of my brother, Marcos, who has always been with me and to my daughter, Sophia, who has fulfilled my life.

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CHAPTER I

INTRODUCTION

Reproductive efficiency in food-producing animals is a key component of the livestock production system. Age at puberty, for example, is an essential process that determines future reproductive potential of beef cows (Schillo *et al.,* 1992). In mature beef cows, rebreeding within 80 to 90 days post-partum is crucial to maximize annual calf production (Williams, 1990). The identification, study, and understanding of factors that impact reproduction in cattle are important goals for long-term sustainability of the beef cattle industry. Among those factors, nutrition is one of the main components that influence reproduction in cattle and other mammals. Nutrients are primarily partitioned towards homeostatic body functions for maintenance (Wade *et al.,* 1996). Surplus nutrients are directed to growth and reproduction. As nutritional demands for tissue and milk synthesis increase, reproductive functions are compromised if no compensatory intake of nutrients is achieved. Therefore, puberty and resumption of postpartum estrous cyclicity can be delayed if inadequate nutrition is provided (Dunn and Kaltenbach, 1980; Day *et al.,* 1986a; Randel, 1990).

Availability of nutrients is perceived ultimately by centers that control energy homeostasis in the central nervous system (Keisler and Lucy, 1996; Williams, 1998), and

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This dissertation follows the style and format of *Reproduction*.

that information is relayed to hypothalamic centers that control reproduction. In this context, prolonged restriction of nutrients reduces the secretion of luteinizing hormone (LH), probably by decreasing gonadotropin-releasing hormone (GnRH) release from hypothalamic neurons into the pituitary portal system (Kile *et al.,* 1991; I'Anson *et al.,* 2000).

In monogastric species, acute changes in nutrient intake decrease mean circulating concentrations of LH (Cagampang *et al.,* 1990; Samuels and Kramer, 1996; Cameron and Nosbish, 1991). In contrast, short-term feed restriction has less distinct impacts on the reproductive axis of ruminants, despite clear effects on metabolism (McCann and Hansel, 1986; Kadokawa and Yamada, 1999; Nagatani *et al.,* 2000). However, recent observations with prepubertal heifers (Amstalden *et al.,* 2000; Maciel *et al.,* 2003a) and estradiol-implanted, castrated rams (Nagatani *et al.,* 2000) have demonstrated that short-term fasting can reduce secretion of LH in selected ruminant models.

The physiological mechanisms through which nutrition mediates its effects on reproduction are not well characterized. A variety of metabolic fuels, hormones, and neurotransmitters have been implicated in the communication of nutritional status to centers that control reproduction (Wade *et al.,* 1996). Evidence suggests that leptin, a protein hormone synthesized mainly by adipocytes, plays a major role in this communication network (Frieman and Halaas, 1998; Li *et al.,* 1998; Nagatani *et al.,* 1998). In genetically leptin-deficient mice (*ob/ob),* which are obese and infertile, leptin treatment induces weight loss and restores fertility (Barash *et al.,* 1996; Mounzih *et al.,*

1997). Moreover, it has been reported that leptin advances puberty in normal mice (Ahima *et al.,* 1997; Chehab *et al.,* 1997). In rats (Nagatani *et al.,* 1998), castrated sheep (Nagatani *et al.,* 2000), and peripubertal heifers (Maciel *et al.,* 2003a), leptin prevents fasting-mediated reductions in LH pulsatility. Similarly, intracerebroventricular (ICV) infusions of recombinant human leptin increase secretion of LH in chronically undernourished ewes (Henry *et al.,* 2001). Collectively, these observations indicate that leptin provides information to the central reproductive axis on the availability of energy stores to be utilized during periods of nutrient restriction.

The long-term aims of studies reported in this dissertation are to understand how information regarding nutritional and metabolic status is relayed to centers that control reproduction in cattle and apply this information to improve reproductive efficiency. Specific objectives were 1) to determine the effects of short-term nutrient restriction on leptin gene expression in adipose tissue, leptin receptor (LR) gene expression in the adenohypophysis, and circulating leptin in mature, ovariectomized cows in the presence of physiological levels of estradiol; and 2) to investigate *in vivo* and *in vitro* the responsiveness of the hypothalamic-adenohypophyseal axis of fasted and non-fasted cows to leptin.

CHAPTER II

LITERATURE REVIEW

The Hypothalamic-Gonadotropic Axis

The normal functions of gonads are dependent upon secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the adenohypophysis. Secretion of gonadotropins, in turn, is regulated by the hypothalamic peptide, gonadotropin-releasing hormone (GnRH) and by gonadal steroids and peptides (Knobil, 1980). Gonadotropin-releasing hormone released into the hypothalamic-hypophyseal portal circulation from neuron terminals is distributed in the adenohypophysis, binds to GnRH receptors on gonadotropes, and stimulates synthesis and secretion of gonadotropins.

Hypothalamic-adenohypophyseal complex

The hypothalamus is considered classically to be limited a) anteriorly by the optic chiasma and anterior commissure, including the immediately anterior area – the preoptic region; b) posteriorly by the mammillary bodies; c) laterally by the internal capsule; and d) dorsally by the intraventricular foramen (Dubois, 1993). Hypophysiotropic neurosecretory neurons, which have cell bodies located in several hypothatalamic nuclei, project towards the infundibulum, or median eminence, through

the tubero-infundibular tract and preoptico-infundibular tract (Page, 1994). Neuron terminals culminate next to a complex capillary network, the hypothalamic-hypophyseal portal system, at the level of the infundibulum. The hypothalamic-hypophyseal portal system, first described by Popa and Fielding (1930) and ascribed functional importance by Green and Harris (1947), is formed by vascular networks arising from the superior adenohypophyseal artery, which forms a capillary plexus of short and long capillary loops at the level of the infundibulum. Blood is collected into adenohypophyseal vessels at the level of the adenohypophyseal stalk and forms another network of capillaries in the adenohypophysis (Page, 1994). Therefore, hypophysiotropic hormones released from neuron terminals at the infundibulum are distributed in the adenohypophysis before reaching the general circulation.

Gonadotropin-releasing hormone neuron perikarya are localized in multiple areas in the hypothalamus and distribution patterns varies accordingly to the species. Perikarya of GnRH neurons are concentrated in the anterior diencephalon (preoptic region), but are also found distributed from the diagonal band of Broca (DBB) to the premamillary region of the hypothalamus. In rats, immunocytochemical studies have demonstrated GnRH cell bodies in the DBB, stria terminalis, septal nuclei, medial preoptic area, anterior hypothalamic area, and in the retrochiasmatic area of the medial basal hypothalamus (King *et al.,* 1974; Baker *et al.*, 1975; Naik, 1975; Setalo *et al.,* 1975; Bennet-Clark and Joseph, 1982; King *et al.,* 1982; Witkin *et al.,* 1982; Kelly *et al.,* 1982). In the rhesus monkey, large numbers of GnRH-containing perikarya have been observed in the medial and lateral preoptic nuclei, the bed nucleus of the stria terminalis,

and the organum vasculosum of the lamina terminalis (Silverman *et al.,* 1977; Silverman *et al.,* 1982). In addition, cell bodies are also located in the anterior hypothalamus, arcuate (infundibular) and premamillary nuclei (Silverman *et al.,* 1982). Despite the anatomical presence of GnRH cell bodies in the preoptic area (POA) and anterior portions of the hypothalamus of primates, physiological studies have demonstrated that GnRH neurons residing within the arcuate nucleus are the major population controlling reproductive cycles in primates, including humans (Knobil, 1980).

In pigs, in addition to localization of GnRH perikarya in the preoptic region, perikarya were observed in the lateral hypothalamic area, paraventricular, periventricular, and suprachiasmatic nuclei, and medial basal hypothalamus (MBH) (Kineman *et al.,* 1988). In sheep, similar to rats and pigs, the majority of GnRH perikarya are found in anterior portions of the diencephalon, mainly in the medial POA (Lehman *et al.,* 1986; Caldani *et al.,* 1988). There are also cell bodies located within the MBH, including the arcuate and ventromedial nuclei (Lehman *et al.,* 1986; Jansen *et al.,* 1997). In cattle, the immunolocalization of GnRH perikarya follows a pattern similar to sheep, except that medial basal hypothalamic localization of perikarya has not been confirmed. Although Dees and McArthur (1981) reported immunolocalization of GnRH-positive cell bodies within the arcuate nucleus, studies by Leshin and collaborators (1988, 1992) did not support this finding. Perikarya for GnRH neurons in cattle were observed in the DBB, medial and lateral preoptic areas, and ended in the anterior hypothalamic area (Leshin *et al.,* 1988, 1992).

The adenohypophysis consists of five major endocrine cell types: somatotropes, corticotropes, thyrotropes, lactotropes, and gonadotropes. Gonadotropes synthesize and secrete LH, FSH, or both (Herbert, 1976; Inoue and Kurosumi, 1984). However, Bastings *et al.* (1991) demonstrated that in bovine adenohypophysis, gonadotropes synthesize either LH or FSH, and no colocalization of gonadotropins was observed. Immunolocalization of LH and FSH in separate cells has also been observed in chicken pituitaries (Proudman *et al.,* 1999; Puebla-Osorio *et al.,* 2002). Therefore, it is still controversial as to whether one or two distinct populations of gonadotropes exist.

Regulation of hypothalamic secretion of GnRH

Determination of concentrations of GnRH in blood collected from the hypothalamic-hypophyseal portal circulation of sheep demonstrates that this hypophysiotropic hormone is released in rapid bursts and becomes undetectable in a few minutes (Caraty and Locatelli, 1988). Although it is generally accepted that there is an endogenous, synchronized rhythm in the release of GnRH, the origin of the pulsatile nature of release is not well understood. Observations that murine GnRH-secreting immortalized cells, GT1 cells, secrete GnRH in a pulsatile mode (Martinez de la Escalera *et al.,* 1992), suggest that GnRH neurons have an intrinsic ability to generate a pulse. However, synchronization and regulation of the release of GnRH from neurons involves not only intrinsic control, but also external inputs (Caldani *et al.,* 1993). Many neuronal and hormonal factors influence the frequency and amplitude of the release of GnRH, which defines the physiological reproductive state of the animal.

Gonadal steroids markedly influence the release of GnRH. Estradiol, progesterone, and testosterone decrease the frequency of pulses of GnRH (Karsch *et al.,* 1987; Jackson *et al.,* 1991; Kasa-Vubu *et al.,* 1992; Evans *et al.,* 1994), which is characterized as the steroid negative feedback mechanism. However, when concentrations of progesterone are decreased, estradiol induces an increase in frequency of pulses of GnRH and induces the preovulatory GnRH/LH surge (Evans *et al,* 1994), described as the positive feedback mechanism of estradiol. In the rat, however, the proestrus surge of GnRH/LH coincides with a surge of progesterone, which is believed to enhance sensitivity of the adenohypophysis to respond to GnRH during the estradiol-induced gonadotropin surge (Freeman, 1994). Although estrogen receptor (ER) and progesterone receptor (PR) have not been found in GnRH neurons in sheep or cattle, there is some evidence that ER-β (Hrabovszky *et al.,* 2001; Kallo *et al.,* 2001) and PR (King *et al.,* 1995) are present in some GnRH neurons in rodents. Thus, it is possible that progesterone and estradiol affects GnRH neurons directly. Nevertheless, indirect actions of gonadal steroid hormones through neural afferents on GnRH neurons have been demonstrated and seem to be a major mechanism through which the physiological effects of gonadal steroids regulate GnRH release.

Many neurotransmitters are involved on the regulation of hypophysiotropic hormone release into the portal circulation and potentially can control the function of GnRH neurons (Reichlin, 1998). Included are biogenic amines (dopamine, adrenaline, noradrenaline, and serotonine), excitatory (glutamate and aspartate) and inhibitory (γ-aminobutyric acid – GABA) amino acids, neuropeptides (neuropeptide Y and

proopiomelanocortin derivates), gaseous neurotransmitters (nitric oxide), cytokines (interleukin 1 and 6), and neurotropic growth factors (nerve growth factor). Contacts with afferent inputs may occur at the level of the GnRH cell body and/or on axon terminals.

Monoaminergic, gabanergic, and peptidergic neurons affect directly or indirectly GnRH neuronal functions (Smith and Jennes, 2001). Monoaminergic afferents include dopamine, noreadrenaline, and serotonin neurons. Dopamine neurons originate within the diencephalon, whereas noradrenaline and serotonin fibers arise from the brainstem (Reichlin, 1998; Smith and Jennes, 2001). The role of noradrenaline in the regulation of release of GnRH remains confusing. Inhibition of noradrenaline synthesis, blockade of α-adrenergic receptors by antagonists, and destruction of adrenergic bundles impairs noradrenalin inputs and diminishes pulsatility of LH, suggesting that noradrenalin stimulates the release of LH (Kordon *et al.,* 1994), supposedly as an effect on GnRH neuron activity. In contrast, infusion of noreadrenaline into the thrird ventricle inhibits frequency of pulses of LH (Gallo, 1984). In sheep, noradrenaline agonist, clonidine, suppresses the release of LH (Goodman, 1989), an effect that is blocked by the noradrenaline antagonist. Dopamine is involved in the increased negative feedback of estradiol during seasonal anestrous in ewes. Dopamine antagonists increase the release of LH in intact ewes during anestrus (Meyer and Goodman, 1986). Moreover, Goodman (1989) suggested that noradrenaline and dopamine may act in series because the ability of clonidine to suppress LH release during anestrus is blocked by the dopamine antagonist, pimozide (Goodman, 1989).

The role of serotonin in regulating GnRH neuronal activity has been demonstrated by the diminished expression of the GnRH gene in rats treated with a serotonin agonist, an effect blocked by serotonin receptor antagonists (Li and Pelletier, 1995). However, the definitive role of serotonin in controlling GnRH secretion is not clear because both positive and negative effects on the release of LH have been observed depending upon the physiological model (Vitale and Chiocchio, 1993). Nevertheless, the serotonin antagonist cyproheptadine increased LH pulse frequency during the anestrous season in ovariectomized ewes (Meyer and Goodman, 1986).

Gamma amino-butyric fibers synapse with GnRH neurons (Leranth *et al.,* 1985), indicating that GABA neurons can directly modulate GnRH neuronal function. Gamma-aminobutyric acid is one of the major inhibitory neurotransmitters of the central nervous system (Reichlin, 1998), and evidence indicates that it is inhibitory to secretion of GnRH in rodents (Smith and Jennes, 2001). Stimulation of $GABA_A$ receptor by an agonist suppressed GnRH release in follicular-phase ewes (Tomaszewska-Zaremba *et al.,* 2003). However, in male sheep, baclofen, a $GABA_B$ receptor agonist, infused by microdialysis into the MBH of intact rams, caused an acute increase in the release of GnRH into the portal blood (Jackson *et al.,* 2000). In contrast to $GABA_A$ receptor, which is considered to be mainly post-synaptic, $GABA_B$ is considered to be a pre-synaptic receptor and functions as an autoreceptor to suppress GABA neurons (Jackson *et al.,* 2000). Therefore, based on observation in rams, stimulation of $GABA_B$ receptors can inhibit the release of GABA and consequently attenuate the inhibitory effects of GABA on the release of GnRH.

Other amino-acid neurotransmitters are also able to modulate secretion of GnRH. Glutamate and aspartate, for example, are endogenous excitatory amino acids that have been demonstrated to increase GnRH and LH release (Downing *et al.,* 1996; Brann and Mahesh, 1997). Excitatory amino acid receptor antagonists inhibit the proestrus surge of LH in rats (Brann and Mahesh, 1991) and decrease concentrations of LH in the circulation in rats (Ping *et al.,* 1994) and sheep (Hileman *et al.,* 1992).

Gonadotropin-releasing hormone neurons also receive inputs from neurons containing neuropeptides as neurotransmitters. Examples of peptides that mediate neuronal communications are neuropeptide Y (NPY), neurotensin, and endogenous opioids. Some show stimulatory effects and others suppressive effects on the release of GnRH. Neuropeptide Y has dual effects in rats. In ovariectomized rats without estradiol treatment, NPY decreases the release of LH (Sahu *et al.,* 1987). In contrast, in ovariectomized rats treated with estradiol, NPY stimulates the secretion of LH (Sahu *et al.,* 1987). In monkeys, similar to rats, NPY has positive and negative effects on the release of gonadotropin. Intracerebroventricular infusion of NPY inhibits LH in ovariectomized monkeys with or without estradiol replacement (Kaynard *et al.,* 1990). In contrast, hypothalamic explants perifused with NPY released more GnRH than control-treated explants (Pau *et al.,* 1991) and infusion of NPY antiserum into the infundibulum with a push-pull pump suppressed the release of GnRH (Woller *et al.,* 1992). In sheep and cattle, NPY has negative effects on the release of GnRH/LH (McShane *et al.,* 1992; Barker-Gibb *et al.,* 1995; Gazal *et al.,* 1998).

Neurotensin is proposed to be involved in mediating the effects of estradiol on the generation of the GnRH surge in rats (Smith and Jennes, 2001). Neurotensin is expressed in neurons that are in close apposition to GnRH neurons in the POA (Hoffman, 1985) and GnRH neurons express the neurotensin receptor (Smith and Wise, 2001). Moreover, infusion of neurotensin in the POA amplifies the preovulatory surge of LH in rats (Ferris *et al.,* 1984). Information on effects of neurotensin in species other than rodents is limited.

Endogenous opioid peptides (enkephalins, dynorphins, and endorphis) are present in the hypothalamus and originate from three precursors: proenkephalin, prodynorphin, and proopiomelanocortin (POMC). Opioids have a profound effect on GnRH neuronal function. Opiates are generally involved in suppression of release of LH (Smith and Jennes, 2001) and have been implicated in mediating the inhibitory effects of progesterone in rats (Kalra, 1993) and in sheep (Whisnant and Goodman, 1988). Basal release of GnRH from the bovine infundibulum is increased by treatment with the opioid receptor antagonist naloxone (Leshin *et al.,* 1991). Moreover, naloxone given to postpartum cows induces an increase in concentrations of LH in the circulation (Whisnant *et al.,* 1986).

Regulation of adenohypophyseal release of gonadotropins

The release of LH and FSH, particularly when concentrations are determined in portal blood, is characterized as pulsatile, which reflects a rapid and intense increase in their concentrations in circulation followed by an exponential decrease (Caldani *et al.,*

1993; Padmanabhan *et al.,* 2002a). However, this pulsatile release is not spontaneous, but induced by GnRH. The gonadotropins, LH and FSH, consists of association of two protein subunits, α and β. The α subunit is common to both gonadotropins and the thyrotropin, thyroid stimulating hormone (TSH). The β subunit confers specificity to each type of hormone (Bousfield *et al.,* 1994). Secretion of gonadotropins is regulated by the interaction of hypothalamic GnRH with gonadotropes and feedback of gonadal steroids and peptides (Caldani *et al.,* 1993). In addition, intra-adenohypophyseal factors may also regulate the release of LH and FSH (Padmanabhan *et al.,* 2002a).

Radio-frequency lesions of the arcuate nucleus in monkeys, which cause the destruction of GnRH neurons, elicit a fall in circulating concentrations of gonadotropins (Knobil, 1980). Similarly, after surgical hypothalamic-hypophyseal disconnection in sheep, concentrations of gonadotropins in the circulation and mRNA for the α and β subunits of LH and FSH diminish drastically (Clarke *et al.,* 1983; Hamernik *et al.,* 1986). In these models, pulsatile injections of GnRH restore concentrations of gonadotropins in the circulation (Belchetz *et al.,* 1978; Hamenik and Nett, 1988). Moreover, treatment with pulsatile injections of GnRH increases mean concentrations of LH, FSH, and estradiol in individuals with hypothalamic hypogonadism (Woodhouse *et al.,* 1984).

Multi-unit electrical activity can be detected in the MBH before each pulse of LH (Knobil, 1989) coincident with an activation of GnRH neurons secretory preceding the release of LH from the adenohypophysis. Simultaneous measurements of release of GnRH into the hypothalamic-hypophyseal portal blood and gonadotropins from

adenohypophysis have been possible by collection of hypophyseal portal blood using "portal cannulation" (Clarke and Cummins, 1982; Caraty and Locateli, 1988) or by perifusion with "push-pull cannula" (Levine *et al.,* 1982). Both methods have demonstrated a high correlation between pulses of GnRH and gonadotropins, particularly LH. Moreover, in a physiological state in which increases in mean concentrations of LH in the peripheral circulation are observed, such as during the preovulatory surge of LH, increases in the release of GnRH into the portal blood immediately precede or occur simultaneously to the release of LH (Clarke and Cummins, 1985).

Gonadotropin-releasing hormone acts on the adenohypophysis by binding to a specific GnRH receptor (GnRH-R). The GnRH-R belongs to the G-protein coupled receptor family of transmembrane receptors (Tsutsumi *et al.,* 1992). G-proteins are heterotrimeric proteins composed of α, β, and γ subunits. Upon stimulation, Gα dissociates from a Gβγ dimer and becomes active. There are several subtypes of Gα (Kraus *et al.,* 2001) and examples are: Gαs, which is stimulatory to adenyl cyclase (AC) and induces production of cAMP; Gαi, which is inhibitory to the AC; and Gαq, which activates membrane-associated phospholipase C (PLC). Binding of GnRH to its receptor activates Gαq protein and PLC, and ultimately causes hydrolysis of membrane-bound phosphoinositides that generates diacylglycerol (DAG) and Inositol-3-phosphate (IP3). Diacylglycerol activates protein kinase C (PKC), and IP3 leads to Ca^{++} mobilization (Stojilkovic *et al.,* 1994). Both pathways are involved in GnRH stimulation of gonadotropin synthesis. In addition, mobilization of intracellular Ca^{++} and an influx

of extracellular Ca^{++} are important mechanism triggering gonadotropin exocytosis (Zorec, 1996). In addition to the cellular mechanisms described above, GnRH-R has been shown to activate other signaling mechanisms such as mitogen-activated protein kinase (MAPK) (Haisenleder *et al.,* 1998). Activation of MAPK pathway by GnRH involves Ca^{++} dependent and independent mechanisms (Mulvaney and Roberson, 2000). The role of GnRH-stimulated MAPK signaling seems to be relevant primarily in regulating α and β subunit gene expression and is not involved in the secretion of gonadotropins (Kraus *et al.,* 2001).

Activation of intra- and extracellular Ca^{++} is essential for the release of gonadotropins from the adenohypophysis (Naor *et al.,* 1995; Stojilkovic and Catt, 1995). Blocking Ca^{++} mobilization impairs exocytosis of LH and FSH (Bates and Conn, 1984). Following an increase in intracellular Ca^{++} concentrations, the exocytosis machinery becomes activated. Molecules such as N-ethylylmaleimide-sensitive factor (NSF), soluble NSF attachment protein receptor (SNARE), synaptosome-associated protein (SNAP), and vesicle-associated membrane protein (VAMP) are essential for fusion of secretory vesicles to the cell membrane (Brunger, 2000). Although mechanisms of vesicle fusion during exocytosis have not been fully characterized for the release of gonadotropins, these proteins are localized in gonadotropes (Thomas *et al.,* 1998).

In addition to the regulatory effects of gonadal steroids on the secretion of gonadotropins at the level of hypothalamic neurons, gonadal steroids also have a direct effect at the level of the adenohypophysis. Progesterone and estrogen receptors are present in rat gonadotropes and estradiol stimulates PR gene expression (Sprangers *et*

al., 1990). Basal and GnRH-stimulated release of LH from rat (Drouin *et al.,* 1976) and bovine (Baratta *et al.,* 1994) adenohypophyseal cells are increased by estradiol. However, progesterone diminishes the estradiol-stimulated increase in responsiveness to GnRH in adenohypophyseal cells (Drouin and Labrie, 1981; Baratta *et al.,* 1994). In the ovariectomized, hypothalamic-hypophyseal disconnected ewe treated with hourly pulses of GnRH, exogenous estradiol induces a biphasic response in the release of LH with an initial suppression followed by stimulation (Mercer *et al.,* 1993). However, in these experiments, the estradiol-induced surge of LH was greater in ovariectomized, hypothalamic-hypophyseal intact ewe. In contrast to the biphasic effect on LH, mean circulating concentrations of FSH and FSHβ mRNA are diminished by estradiol treatment (Mercer *et al.,* 1993).

Gonadal peptides, such as inhibins, follistatins, and activins affect the release of FSH from the adenohypophysis without apparent effects on secretion of LH (Rivier *et al.,* 1986; Padmanabhan *et al.,* 2002a). However, an inhibin-induced decrease in the number of binding sites for GnRH has been reported (Wang *et al.,* 1988). Inhibins are heterodimeric glycoproteins with α and β subunits (Padmanabhan and Sharma, 2001). The α subunit is common to all inhibins, but there are 2 forms of β subunits: β_A and β_B, and synthesis and secretion of inhibins are stimulated by FSH. Release of FSH is reduced by inhibin treatment in rats (Hermans *et al.,* 1980), sheep, (Martin *et al.,* 1986) and cows (Beard *et al.,* 1989) and prevents the post-castration increase in circulating FSH (Sairam *et al.,* 1981). Immunization against inhibin increases ovulation rate in several species (Henderson *et al.,* 1984; Rivier and Vale, 1989; Scanlon *et al.,* 1993;

Medan *et al.,* 2003). Inhibin decreases the expression of FSHβ gene (Beard *et al.,* 1989) in gonadotropes by binding to inhibin-binding proteins (or receptor) or antagonizing the stimulatory action of activins by competing for the activin receptor (Gray *et al.,* 2000). No action of inhibins has been reported at the central nervous system level relative to the control of gonadotropin secretion (de Greef *et al.,* 1987).

Activins are peptide dimers of the inhibin β subunit ($\beta_A\beta_A$, $\beta_A\beta_B$, and $\beta_B\beta_B$) that cause an increase in the secretion of FSH (Padmanabhan and Sharma, 2001). In rats, activin A has been shown to stimulate the secretion of FSH and expression of FSHβ mRNA independent of and in synergy with the GnRH agonist buserelin (Gajewska *et al.,* 2002). In ovine adenohypophyseal cells, activin also increases the release of FSH without affecting the release of LH (Gregg *et al.,* 1991). Follistatin, another non-steroidal gonadal regulator of secretion of FSH, functions as a high affinity binding protein for activins and exerts its actions by impairing binding of activins to their receptor (de Winter *et al.,* 1996; Padmanabhan *et al.,* 2002b).

Initially, it was considered that inhibins, activins, and follistatins produced only in gonads acted as endocrine factors at the level of the adenohypophysis to regulate secretion of FSH. However, recent findings have demonstrated that these proteins are expressed within the adenohypophysis and paracrine/autocrine actions may represent an important aspect of the regulation of FSH synthesis and secretion (Padmanabhan and Sharma, 2001).

Nutritional Effects on Hypothalamic-Gonadotropic Function

Severe nutrient restriction suppresses secretion of LH in both sexually-mature and prepubertal mammals, including cattle (Day *et al.,* 1986a; McCann and Hansel, 1986) and sheep (Foster *et al.,* 1989; Keisler and Lucy, 1996). Feed-restricted, ovariectomized lambs exhibiting a low frequency of LH pulses respond to exogenous stimulation by GnRH which produces corresponding pulses of LH (Foster *et al.,* 1989). Although restricted and control-fed heifers respond to exogenous administration of physiological doses of GnRH by releasing LH, the release of LH was lower in restricted than in control heifers (Day *et al.,* 1986a). Restriction of food intake does not change GnRH mRNA in the POA of rats (Leonhardt *et al.,* 1999) or sheep (McShane *et al.,* 1993), although diminished LHβ and α subunits, and GnRH receptor mRNA in the adenohypophysis is observed (Leonhardt *et al.,* 1999). However, pulsatile administration of GnRH in feed-restricted ewes restores circulating concentrations of LH to normal and increases pituitary LH mRNA content (Kile *et al.,* 1991). These observations suggest that undernutrition impairs pulsatile LH secretion by diminishing hypothalamic GnRH release. Therefore, it has been generally accepted that the diminished pulsatile release of LH is the result of a reduced frequency of GnRH pulses (Williams, 1999). However, diminished amplitude of GnRH pulses may also play a role in the reduced concentrations of LH observed during undernutrition. Simultaneous blood sampling from the adenohypophyseal portal system and the jugular vein has demonstrated that the reduction in frequency of LH pulses in feed-restricted female lambs is greater than the reduction in frequency of GnRH pulses (I'Anson *et al.,* 2000).

Specifically, pulses of GnRH with low amplitudes did not always result in concomitant LH pulses. This suggests that gonadotropes of long-term, feed-restricted lambs may be less sensitive to low amplitude pulses of GnRH. Although decreased GnRH receptor numbers in gonadotropes, as suggested in the undernourished rat (Leonhardt *et al.,* 1999), may account for decreased responsiveness to GnRH, mechanisms for a putative decrease in sensitivity to GnRH have not been fully determined. Another possibility is that small pulses of GnRH, that are not adequate to stimulate release of LH, may prime the adenohypophysis to respond to larger pulses (Clarke and Cummins, 1987).

Short-term restriction of feed intake also impacts the hypothalamic-gonadotropic axis of mice (Ahima *et al.,* 1996), rats (Cagampang *et al.,* 1990), monkeys (Helmreich and Cameron, 1992), and humans (Samuels and Kramer, 1996). Gonadal steroids appear to have a marked effect on the response of the hypothalamic-gonadotropic axis to acute food deprivation. Circulating luteinizing hormone is reduced by 48 h of feed restriction in ovariectomized rats implanted with estradiol compared to those implanted with vehicle alone (Cagampang *et al.,* 1991). The site for this action of estradiol on fasted rats seems to be the paraventricular nucleus (Nagatani *et al.,* 1996) and may involve interactions with activation of sympathomedullary axis (Cagampang *et al.,* 1999) and endogenous opioids (Cagampang *et al.,* 1991).

In contrast to marked effects of severe undernutrition, short-term feed-restriction does not seem to clearly impact reproduction in sexually-mature ruminants. Short-term fasting did not affect circulating concentrations of LH in mature cows (McCann and Hansel, 1986; Kadokawa and Yamada, 1999) or ewes (Nagatani *et al.,* 2000), despite

clear effects on indicators of metabolic status, such as insulin, insulin-like growth factor I (IGF-I), and non-sterified fatty acids (NEFA). However, recent reports have shown that 48 to 72 h of total feed restriction reduces LH pulsatility in prepubertal heifers (Amstalden *et al.,* 2000; Maciel *et al.,* 2003a) and castrated male rams implanted with estradiol (Nagatani *et al.,* 2000). Therefore, body energy reserves, gender, maturity of the central reproductive axis, and interactions with estradiol negative feedback may be important determinants of the response to acute diet restriction in ruminants.

Metabolic and Neuroendocrine Mediators of Nutritional Effects on the Hypothalamic-Gonadotropic Axis

Glucose as a metabolic fuel

Glucose serves as the main energy source for the central nervous system. Restriction of nutrient intake reduces concentrations of glucose in the circulation of humans (Kolaczynski *et al.,* 1996), rodents (Schneider and Wade, 1989; Cagampang *et al.,* 1990), monkeys (Schreihofer *et al.,* 1996), and less rapidly in ruminants (Pell and Bergman, 1983; McCann and Hansel, 1986). Hypoglycemia induced by insulin reduces mean plasma concentrations of LH and mean amplitude and frequency of pulses of LH in ewes (Clarke *et al.,* 1990; Adam and Findlay, 1998). This effect is prevented by infusion of glucose. Moreover, intravenous or central infusion of 2-deoxyglucose (2DG), a competitive antagonist of glucose metabolism, reduces secretion of LH (Bucholtz *et al.,* 1996). However, simultaneous administration of either GnRH or N-

methyl-D-aspartate (NMDA), a GnRH secretagogue, prevents the 2DG-induced reduction in LH. Therefore, these observations suggest that glucose-sensitive mechanisms within the brain regulate central reproductive hormone release. In addition, effects of insulin-induced hypoglycemia on the reduction of release of LH have been linked to activation of neurons in the paraventricular nucleus of the hypothalamus and may involve activation of corticotrophin-releasing hormone neurons (Adam and Findlay, 1998). However, the estrous cycle in hamsters is disrupted only when both glucose and fatty acid metabolism are impaired (Schneider and Wade, 1989). In addition, restoration of LH pulsatility after refeeding in fasted monkeys occurs in monkeys fed carbohydrates, which increased blood glucose, and in monkeys fed proteins and fat, which did not increase blood glucose (Schreihofer *et al.,* 1996). Therefore, although it is generally accepted that glucose is essential for normal brain function, the role that glucose plays on the regulation of the hypothalamic-gonadotropic axis is still not clear.

Insulin as a metabolic hormone

The positive association of insulin to nutritional status leads to the hypothesis that insulin serves as a metabolic hormone signaling energy reserves to the brain. Insulin receptors are located in the hypothalamus (van Houten *et al.,* 1980) and acute (Kolaczynski *et al.,* 1996; Ahima *et al.,* 1996; Williams *et al.,* 1996; Nagatani *et al.,* 1998) or chronic (McCann and Hansel, 1986; Hileman *et al.,* 1993) undernutrition diminishes concentrations of insulin in the circulation. However, the effects of intracerebroventricular infusions of insulin have been contradictory. Chronic central

infusions of insulin or insulin plus glucose increase mean concentrations of LH in feed-restricted ewes (Adam and Findlay, 1998; Daniel *et al.,* 2000). However, acute infusions of insulin alone have no effects (Hileman *et al.,* 1993). Moreover, increases in mean concentrations of insulin as a result of chronic administration of glucose are not followed by changes in secretion of LH during the estrous cycle of ewes (Rubio *et al.,* 1997). Nevertheless, insulin increases the GnRH-stimulated release of LH from adenohypophyseal cells in culture (Adashi *et al.,* 1981; Soldani *et al.,* 1994).

The GH, IGF-I, and IGF-binding protein axis

Under adequate nutritional conditions, increases in GH usually result in an increase in circulating concentrations of IGF-I (Thissen *et al.,* 1994; Keisler and Lucy, 1996; Armstrong and Benoit, 1996; Williams, 1999). Growth hormone stimulates synthesis of IGF-I by direct effects on hepatocytes, which are the main source of circulating IGF-I. Most of the IGF-I circulates bound to specific binding proteins (IGFBP), which modulates bioavailability of IGF-I (Thissen *et al.,* 1994). Binding of radiolabeled IGF-I has been observed in the hypothalamus and adenohypophysis of sheep, and IGF-I receptor mRNA is localized in the adenohypophysis (Adam *et al.,* 2000). Circulating concentrations of IGF-I increase before puberty in heifers (Yelich *et al.,* 1996; Garcia *et al.,* 2002a), and limiting weight gain before puberty decreases mean circulating concentrations of IGF-I and delays puberty compared to heifers on a higher rate of weight gain (Yelich *et al.,* 1996). Moreover, intracerebroventricular (ICV) infusion of IGF-I advances puberty in rats (Hiney *et al.,* 1996), suggesting a possible

role for IGF-I on the onset of puberty. Insulin-like growth factor I increases basal (Adam *et al.,* 2000) and GnRH-stimulated (Soldani *et al.,* 1994) release of LH from ovine and rat adenohypophyseal cells, respectively.

Estradiol affects the GH:IGF-I:IGFBP axis and its ability to regulate the release of gonadotropin. Ovariectomized cows with estradiol implants have higher concentrations of GH and IGF-I than cows without implants (Simpson *et al.,* 1997), but this effect seems to be impaired in feed-restricted cows (Richards *et al.,* 1991). In bovine adenohypophyseal cells, IGF-I increases GnRH-stimulated release of LH, effect that is enhanced by simultaneous treatment with estradiol (Hashizume *et al.,* 2002).

Neuropeptide Y as neurotransmitter regulating GnRH neurons

Neuropeptide Y is a potent stimulator of feeding behavior in several species (Stanley and Leibowitz, 1984; Parrott *et al.,* 1986; Miner *et al.,* 1989; Larsen *et al.,* 1999) and has been shown to be involved in regulation of energy metabolism and reproduction. Chronic (McShane *et al.,* 1993; Archer *et al.,* 2002) or acute (Li *et al.,* 1998; Grove *et al.,* 2003) restriction of feed intake increases hypothalamic NPY mRNA. Neuropeptide Y receptors are abundant in the hypothalamus (Parker and Herzog, 1999). Actions of NPY on reproduction involve effects on GnRH neurons. Although it is not clear whether NPY neurons have direct contact on GnRH neurons, GnRH perikarya in the preoptic region and terminal fibers in the infundibulum are found in close proximity to NPY-containing neurons (Li *et al.,* 1999). Modulation of LH release by direct effects of NPY at the adenohypophyseal level is also possible (Pau *et al.,* 1991; O'Conner *et al.,* 1993).

Neuropeptide Y has a bimodal effect on gonadotropin release from rodents. In ovariectomized rats treated with estradiol, NPY stimulates the release of LH. In contrast, in ovariectomized rats without estradiol treatment, NPY inhibits LH release, demonstrating an important interaction with gonadal steroids (Sahu *et al.,* 1987). In the rhesus monkey, the effect of NPY in regulating the release of LH depends on the site of action. In ovariectomized monkeys in presence or absence of estradiol, NPY infused into the ventricular system has an inhibitory effect, whereas, NPY infused into the infundibulum has a stimulatory effect on GnRH release (Pau *et al.,* 1995). In ruminants, NPY has inhibitory effects on gonadotropin release, independent of the estradiol milieu (McShane *et al.,* 1992; Gazal *et al.,* 1998; Thomas *et al.,* 1999; Morrison *et al.,* 2003). Intracerebroventricular infusions of NPY reduce the release of both GnRH into the cerebrospinal fluid (CSF) and LH in blood of ovariectomized, estradiol-implanted cows (Gazal *et al.,* 1998). Therefore, NPY may be involved in mediating suppression of gonadotropin secretion observed during undernutrition.

Melanocyte-stimulating hormone

A peptide product of post-translation modification of POMC, melanocyte-stimulating hormone (MSH) has been implicated in stimulating gonadotropin release in rodents (Alde and Celis, 1980), humans (Reid *et al.,* 1984), and monkeys (Wardlaw and Ferin, 1990). Ovulation rate and mean concentrations of LH are increased in rats treated with α-MSH, but not β-MSH, suggesting distinct functions between MSH types (Alde and Celis, 1980). Administration of antiserum to α-MSH blocks the proestrus increase in LH

and ovulation (Caballero and Celis, 1993). Moreover, α-MSH advances by two days the vaginal opening in prepubertal rats when given to 28 day-old rats, indicating a triggering of the onset of puberty (Durando *et al.,* 1989). This effect is associated with subsequent increases in mean concentrations of LH and progesterone in the circulation. However, inhibitory effects of α-MSH on LH release in vivo are seen when given into the third ventricle of ovariectomized rats without estradiol treatment (Khorram *et al.,* 1984). In women, α-MSH induces the release of LH during the luteal phase, but not during the follicular phase (Reid *et al.,* 1984). Therefore, these observations suggest that gonadal steroid milieu modulates responses to α-MSH. Moreover, α-MSH attenuates the inhibitory effects of endogenous opioids on the release of LH in rhesus monkeys (Wardlaw and Ferin, 1990).

Melanocortin-stimulating hormone binds to melanocotin receptors (MCR). Melanocortin receptor 3 and 4 are the most abundant in the brain and are considered to be the neuroendocrine and behavioral mediators of MSH effects (Schioth and Watanobe, 2002). Food restriction increases, whereas obesity decreases numbers of MCR-4 receptors in the hypothalamus (Harrold *et al.,* 1999). Therefore, MCR-3 and -4 seem to be the central mediators of α-MSH and its effects on the hypothalamic-gonadotropic axis.

Agouti-related peptide

Agouti-related peptide (AgRP) is an endogenous antagonist of the melanocortin 3 and 4 receptors and is associated with stimulation of feeding behavior (Small *et al.,* 2001). The majority of neurons expressing AgRP in the rat also express NPY (Stanley and

Leibowitz, 1984). A peptide fragment of AgRP blocks the feed-reducing effects of α-MSH, demonstrating antagonist effects of the MSH system (Rossi *et al.,* 1998). The effects of AgRP on reproduction can be seen by its effects on estradiol/progesterone-induced LH and prolactin surges in ovariectomized rats. In this model, AgRP prevents the surge of LH and prolactin (Schioth *et al.,* 2001).

Ghrelin

An endogenous growth hormone secretagogue synthesized and secreted mainly in the stomach, Ghrelin has also found to be expressed in the pituitary, kidney, and hypothalamus (Horvath *et al.,* 2001). Central administration of ghrelin to rats induces food intake and decreases lipolysis, promoting adiposity (Tschop *et al.,* 2000). Circulating concentrations of ghrelin are increased by fasting and hypoglycemia (Horvath *et al.,* 2001). Receptors for ghrelin are present in the hypothalamus, particularly in areas where NPY neurons are abundant, such as the arcuate nucleus (Horvath *et al.,* 2001). Neuropeptide Y and AgRP seem to be central mediators of ghrelin action. Antisera and antagonists of NPY and AgRP interfere with the effects of ghrelin on induction of appetite (Nakazato *et al.,* 2001). Intracerebroventricular infusions of ghrelin suppress the release of LH in rats (Furuta *et al.,* 2001). This suggests that ghrelin may regulate the hypothalamic-gonadotropic axis in addition to its effects on feed intake and GH release.

Galanin

Originally found to be synthesized in the intestine, galanin is widely expressed in the hypothalamus and infundibulum, where it is highly concentrated (Kaplan *et al.,* 1988). Galanin has been found to be colocalized within some GnRH neurons and to be secreted in synergy with GnRH into the hypothalamic-hypophyseal portal circulation (Lopez *et al.,* 1991). Secretion of LH in male (Scheffen *et al.,* 2003) and female (Splett *et al.,* 2003) rats is enhanced by galanin. However, this effect seems to be dependent on gonadal steroids. Enhancement of GnRH-stimulated release of LH by galanin was observed only when male (Scheffen *et al.,* 2003) and female (Splett *et al.,* 2003) gonadectomized rats were treated with testosterone and estradiol or estradiol plus progesterone, respectively. Without gonadal steroid treatment, effects of galanin on LH release are inhibitory. The galanin receptor is observed in close proximity to and on GnRH neurons, suggesting the galanin can indirectly and directly modulate GnRH neuronal function (Mitchell *et al.,* 1999). However, its effects vary depending on sex and stages of the estrous cycle. In female rats, GnRH neurons expressing galanin receptors are observed during all stages of the estrous cycle, except during diestrus. In male rats, no coexpression of GnRH and galanin receptor is observed (Mitchell *et al.,* 1999). It is not clear whether galanin has similar effects on gonadotropin release in species other than the rat. In monkeys, intravenous injected galanin stimulated release of LH (Finn *et al.,* 2000). However, galanin perfused using push-pull perfusion into the arcuate nucleus and infundibulum did not affect secretion of LH. Receptors for galanin are present on gonadotropes (Depczynski *et al.,* 1998) and

direct stimulatory effects of galanin on LH release have also been reported (Scheffen *et al.,* 2003).

Orexin

Orexins A and B are orexigenic hypothalamic peptides that have been demonstrated to influence energy balance and release of GnRH (Pu *et al.,* 1998; Sweet *et al.,* 1999). Similar to NPY, orexin stimulates LH release in ovariectomized, estradiol/progesterone-treated rats, but inhibits release of LH in ovariectomized rats without gonadal steroid treatment (Pu *et al.,* 1998). However, in ovariectomized rats treated with low doses of estradiol, orexin A suppressed the release of LH (Furuta *et al.,* 2002), suggesting that an important interaction between gonadal steroids and orexin effects exist. Moreover, the dual effect of orexin on secretion of GnRH seems to be also dependent on site of delivery. Orexin infused into more anterior regions of the preoptic area in rats has stimulatory effects (Small *et al.,* 2003), whereas orexin infused into the medial POA and arcuate and infundibular regions has inhibitory effects on the release of LH. Campbell *et al.* (2003) have demonstrated that in the rat, the orexin receptor is localized in GnRH neurons and that orexin neurons synapse with GnRH perikarya, but not with GnRH-neuronal terminals at the infundibulum. In sheep, GnRH neurons are in close contact with orexin neurons (Iqbal *et al.,* 2001). However, ICV infusion of orexin B in sheep did not effect the release of LH, despite transient increases in food intake (Sartin *et al.,* 2001).

Leptin as a Putative Signal of Nutritional Status to the Hypothalamic-Gonadotropic Axis

Leptin, a 167-amino acid hormone synthesized and secreted primarily by adipocytes, has been implicated in communicating nutritional status to the brain and is involved in regulation of food intake, metabolism, and reproduction (Houseknecht *et al.,* 1998). Leptin is expressed in placenta (Hoggard *et al.,* 1997a), stomach (Bado *et al.,* 1998), skeletal muscle (Wang *et al.,* 1998), brain (Morash *et al.,*1999; Wiesner *et al.,* 1999), and adenohypophysis (Morash *et al.,* 1999). Brain regions in which leptin mRNA is detected are the cerebral cortex, cerebellum, hypothalamus, and pineal gland. However, it is not clear whether neurons are the source of leptin in the central nervous system. Leptin mRNA and protein were detected in a glial cell line (rat glioblastoma cells) (Morash *et al.,* 1999), suggesting that leptin synthesis within the brain may come from cells other than neurons.

Leptin was initially studied in genetically obese (*ob/ob*) mice, which have a mutation in the gene encoding leptin that leads to a truncated, nonfunctional protein (Zhang *et al.,* 1994). Obese (*ob/ob*) mice are infertile and have atrophic reproductive organs (Barash *et al.,* 1996). Leptin treatment induced weight loss and development of reproductive organs, and restored reproduction in male (Mounzih *et al.* 1997) and female (Barash *et al.,* 1996) *ob/ob* mice. In normal prepubertal mice, leptin treatment induced earlier onset of estrous cycles (Ahima *et al.,* 1997; Chehab *et al.,* 1997). In *db/db* mice, a mutation of the leptin receptor (LR) gene results in synthesis of a short isoform of the receptor without intracellular signaling transduction capacity (Chua *et al.,* 1996).

Similar to *ob/ob* mice, *db/db* mice are hyperphagic, obese, and incapable of undergoing normal pubertal development; however, in contrast to *ob/ob* mice, *db/db* mice are insensitive to leptin (Chua *et al.,* 1996).

In normal mice (Frederich *et al.,* 1995), humans (Ahren *et al.,* 1997), sheep (Delavaud *et al.,* 2000), and cattle (Ehrhardt *et al.,* 2000), circulating concentrations of leptin are correlated positively with adiposity and increases in body weight in developing heifers (Garcia *et al.,* 2002a). Exogenous treatment with leptin reduces feed intake in several species (van Heek *et al.,* 1997; Barb *et al.,* 1998; Ahima *et al.,* 1999; Henry *et al.* 1999).

Cellular effects of leptin seem to be mediated via activation of the LR, a member of the class I cytokine receptor family (Tartaglia, 1997). Six isoforms of the LR have been identified (Tartaglia, 1997), but only the long form seems to be fully capable of signal transduction (Bjorbaek *et al.,* 1997). Upon ligand binding, the LR undergoes homodimerization, which seems to be required for signaling activity, and activates receptor-associated Janus kinases (JAK) (White *et al.,* 1997). Phospho-tyrosine residues of JAK interact with the SH2 domain of signal transducers and activators of transcription (STAT) (Bjorbaek *et al.,* 1997), a family of transcription factors that are targets of JAK proteins. Upon interaction with JAK, STAT becomes phosporylated, dimerizes, translocates to the nucleus, and modulates transcription of target genes (White *et al.,* 1997; Houseknecht and Portocarrero, 1998).

It has been shown that the long form of the LR has binding sites for JAK and STAT (Tartaglia *et al.,* 1995) and is capable of activation of JAK2 and STAT-3, -5, and

-6 (Bjorbaek *et al.,* 1997). The *db/db* mutation in mice causes incorrect splicing of LR mRNA, producing a receptor that lacks binding sites for JAK proteins and is unable to activate the JAK-STAT pathway (Ghilardi *et al.,* 1996). Activation of the long form of the LR also leads to activation of other signaling pathways, such as MAPK, insulin-receptor substrate (IRS) -1 (Bjorbaek *et al.,* 1997), and phosphatidylinositol-3 kinase (Niswender *et al.,* 2001).

The long form of LR has been reported to occur in several tissues, including brain, adenohypophysis, lung, kidney, liver, adipose tissue, pancreas, and muscle (Hoggard *et al.,* 1997b; Lollmann *et al.,* 1997; Zamorano *et al.,* 1997). It is abundant in the hypothalamus, especially in the ventromedial hypothalamus and arcuate nucleus (Dyer *et al.,* 1997a; Houseknecht and Portocarrero, 1998; Williams *et al.,* 1999). In addition, expression of the long form of the LR in the hypothalamus is increased in feed-restricted ewes (Dyer *et al.,* 1997a). Short isoforms of the LR have a truncated intracellular domain and are unable to activate STAT proteins, but seem to activate MAPK pathways (Bjorbaek *et al.,* 1997). Although the physiological significance of signaling by LR short isoforms has not yet been determined, it is suggested that they might mediate leptin transport across the blood-brain barrier (Bjorbaek *et al.,* 1997). The shortest isoform of the LR lacks the transmembrane and intracellular domains and may function as a soluble receptor for leptin in the circulation (Houseknecht and Prtocarrero, 1998).

Leptin receptor has been detected in GT1-7 cells, an immortalized GnRH-secreting cell line (Magni *et al.,* 1999), and treatment with leptin stimulated GnRH

secretion in these cells. However, GnRH neurons seem to not express LR (Finn *et al.,* 1998). Therefore, it is likely that the effects of leptin on the regulation of hypothalamic release of GnRH are indirect, via interaction with other neuronal systems. Nevertheless, leptin increased secretion of GnRH by rat (Yu *et al.,* 1997; Woller *et al.,* 2001) and pig (Barb *et al.,* 1999) infundibular-hypothalamic explants. Leptin also affects the hypothalamic-gonadotropic axis by direct effects at the level of the adenohypophysis. Leptin increases the secretion of LH from rat adenohypophyseal explants (Yu *et al.,* 1997). However at higher doses ($10^{-5}$and 10^{-6}M), leptin loses its ability to stimulate the release of LH and GnRH from adenohypophyseal and hypothalamic explants, respectively (Yu *et al.,* 1997). Moreover, LR has been colocalized in gonadotropes of ewes (Iqbal *et al.,* 2000), demonstrating that leptin can act at both hypothalamic and adenohypophyseal levels to influence secretion of gonadotropins.

There is significant evidence that the effects of leptin on the central nervous system are mediated, in part, via NPY neurons. Messenger RNA for the LR has been detected in NPY neurons (Mercer *et al.,* 1996; Finn *et al.,* 1998; Williams *et al.,* 1999). Expression of NPY in the hypothalamus is increased in *ob/ob* mice and treatment with leptin diminishes hypothalamic NPY in this mutant mouse (Stephens *et al.,* 1995). An interesting correlate is that feed restriction increases NPY mRNA in sheep (McShane *et al.,* 1993), and ICV infusion of NPY decreases circulating concentrations of LH in sheep (McShane *et al.,* 1992) and cows (Gazal *et al.,* 1998). In addition, ICV infusion of leptin prevented NPY-induced feed intake in rats (Sahu, 1998). However, in the ovariectomized, estradiol implanted cow, pretreatment with leptin did not prevent the

NPY-mediated decrease in plasma concentrations of LH (Garcia *et al.,* 2002b). Leptin prevents the increase in NPY mRNA in fasted rats (Schwartz *et al.,* 1996; Ahima *et al.,* 1999) and modest increases in concentrations of leptin by subcutaneous administration decreased NPY mRNA in the arcuate nucleus (Schwartz *et al.,* 1996).

Another possible target for the effects of leptin in the central nervous system is POMC-expressing neurons. Leptin receptor mRNA is localized in POMC neurons of the arcuate nucleus (Cheung *et al.,* 1997; Finn *et al.,* 1998). Moreover, *ob/ob* and normal mice fasted for 48 h have POMC mRNA reduced in the arcuate nucleus. Exogenous leptin treatment raises POMC mRNA levels in both subjects (Schwartz *et al.,* 1997; Thornton *et al.,* 1997; Ahima *et al.,* 1999). However, constant infusion of leptin in *ad libitum* fed rats reduces food intake and NPY mRNA, but no effect on POMC mRNA is observed (Ahima *et al.,* 1999).

Additional neuronal systems may be involved on mediating the central effects of leptin. Neurons expressing GABA (Ovesjo *et al.,* 2001), AgRP (Wilson *et al.,* 1999), galanin (Iqbal *et al.,* 2001a), and orexin (Haknasson *et al.,* 1999; Iqbal *et al.,* 2001a,b) have been reported to contain LR, suggesting that those neurons are direct targets for leptin. Leptin reduces expression of AgRP in normal and leptin-deficient mice (Wilson *et al.,*1999) and prevents fasting-induced increases in AgRP (Korner *et al.,* 2001) and galanin (Sahu, 1998) mRNA in rats. In the mouse, although leptin reduces mRNA for galanin, LR was not observed in galanin-containing neurons (Cheung *et al.,* 2001).

Short-term fasting reduces circulating concentrations of leptin in humans (Kolaczynski *et al.,* 1996), rodents (Mizuno *et al.,* 1996; Li *et al.,* 1998; Mercer *et al.,*

1998), ruminants (Amstalden *et al.,* 2000; Nagatani *et al.,* 2000), and horses (McManus and Fitzgerald, 2000). In rats (Nagatani *et al.,* 1998), prepubertal heifers (Amstalden *et al.,* 2000), castrated lambs (Nagatani *et al.,* 2000), and ovariectomized gilts (Whisnant and Harrel, 2001) the fasting-mediated reduction in mean concentrations of leptin is associated with decreased pulsatility of LH. Moreover, administration of leptin to ovariectomized, estradiol-treated rats (Nagatani *et al.,* 1998), castrated, estradiol - implanted lambs (Nagatani *et al.,* 2000), and peripubertal heifers (Maciel *et al.,* 2003a) during fasting prevents the reduction in frequency of LH pulses. In ovariectomized, estradiol-implanted cows under acute nutrient restriction, leptin increases mean concentrations of LH (Zieba *et al.,* 2002). However, when higher doses (10 times greater) are used, leptin loses its ability to stimulate LH. Similarly, long-term undernourished, ovariectomized ewes, but not normal-fed ewes, exhibited increased secretion of LH after ICV infusions of recombinant hleptin (Henry *et al.,* 1999; Henry *et al.,* 2001).

Sex steroids may influence expression and secretion of leptin, expression of leptin receptor, and biological actions of leptin. Leptin mRNA decreases in adipose tissue after ovariectomy in rats and estradiol stimulates mRNA expression and secretion of leptin in adipocytes in culture (Machinal *et al.,* 1999). In contrast, leptin mRNA increases after castration of male rats and adipocytes in culture treated with dihydrotestosterone decrease expression of leptin without effects on the release of this hormone (Machinal *et al.,* 1999). Moreover, estradiol influences the effects of leptin on the release of gonadotropins in rats. Central infusion of leptin increases mean

concentrations of LH and FSH in ovariectomized rats treated with estradiol (Walczewska *et al.,* 1999). In contrast, ovariectomized rats without estradiol treatment did not respond to central infusions of leptin and high doses of estradiol attenuated the leptin-induced increase in the release of LH (Walczewska *et al.,* 1999). Moreover, effects of estradiol may also occur at the level of leptin receptor. The expression of the long form of leptin receptor is reduced in the hypothalamus after estradiol treatment in rats (Bennett *et al.,* 1998).

CHAPTER III

CENTRAL INFUSION OF RECOMBINANT OVINE LEPTIN NORMALIZES PLASMA INSULIN AND STIMULATES A NOVEL HYPERSECRETION OF LUTEINIZING HORMONE AFTER SHORT-TERM FASTING IN MATURE BEEF COWS*

Introduction

Nutrition has a marked impact on reproduction in mammals. Undernutrition reduces the secretion of LH, probably as a consequence of its effects on GnRH release from hypothalamic neurons (Day *et al.,* 1986a; Cameron, 1996; Kile *et al.,* 1991; I'Anson *et al.,* 2000). A variety of metabolic fuels, hormones, and neurotransmitters have been implicated in the communication of nutritional status to centers that control reproduction (Wade *et al.,* 1996; Keisler and Lucy, 1996), and evidence now supports the contention that leptin, a protein hormone synthesized mainly by adipocytes, plays a major role in this communication network (Friedman and Halaas, 1998; Li *et al.,* 1998; Nagatani *et al.,* 1998).

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In ruminants, it is generally recognized that the link between the central reproductive axis and acute changes in nutrient intake is less distinct than in monogastrics, and short-term feed restriction, as a general rule, does not measurably reduces pulsatile LH release (McCann and Hansel, 1986; Kadokawa and Yamada, 1999). However, this belief has been tempered by recent observations in our laboratory (Amstalden *et al.,* 2000) and the laboratory of others (Nagatani *et al.,* 2000) involving heifers and castrate rams. In prepubertal heifers, circulating leptin, leptin mRNA in adipose tissue, and LH pulsatility were reduced by short-term fasting (Amstalden *et al.,* 2000). Moreover, 72-h feed restriction reduced pulsatile LH release in estradiol-implanted, castrated ram lambs (Nagatani *et al.,* 2000), and subcutaneous administration of human leptin (hleptin) prevented these changes. Collectively, these and other observations indicate that the interactions between estradiol negative feedback, body energy reserves, and perhaps maturity of the central reproductive axis, are important determinants of the response to acute diet restriction in ruminants. Importantly, these responses may be linked to circulating leptin, to changes in responsiveness to leptin, or to interactions between leptin and other metabolic hormones. Thus, leptin could potentially provide information on the availability of energy stores that can be utilized during periods of nutrient restriction. However, no investigations have examined these relationships in mature cattle.

The current experiments were designed to 1) examine the effects of short-term fasting on leptin gene activity and circulating leptin in mature, ovariectomized beef cows in the presence of physiological levels of estradiol, and 2) determine responsiveness of

the hypothalamic-adenohypophyseal axis of fasted and non-fasted cows to recombinant ovine leptin (oleptin). Because nutritionally-mediated changes in circulating leptin and LH are also tightly linked to insulin secretion (McCann and Hansel, 1986; Ahima *et al.,* 1996; Amstalden *et al.,* 2000), and because leptin has been shown to influence pancreatic endocrine function via changes in sympathetic tone (Mizuno *et al.,* 1998), a secondary interest was to examine insulin secretion after centrally-administered leptin in fasted and non-fasted cows.

Materials and Methods

All animal-related procedures employed in these studies were approved by the Institutional Agricultural Animal Care and Use Committee (IAACUC) of The Texas A&M University System.

Animal model and intracerebroventricular delivery of recombinant oleptin

The animal model employed in these studies was the ovariectomized, mature beef cow bearing an estradiol implant. These implants are designed to produce basal circulating concentrations of estradiol-17β of 2-5 pg ml^{-1} as reported previously (Gazal *et al.,* 1998). Ovariectomized, steroid-treated females and castrated, steroid-treated males provide excellent models for studying the effects of nutrition on neuroendocrine control of gonadotrophin secretion in ruminants (Imakawa *et al.,* 1986; Day *et al.,* 1986b; Kile *et al.,* 1991; Nagatani *et al.,* 2000). Using this approach, hormonal implants can provide a constant level of steroid (estradiol) negative feedback without the

complications associated with ovarian cyclicity. Cows used in the current experiments had mean concentrations of estradiol of 3.3 ± 0.4 pg ml^{-1}.

Availability of oleptin preparations for use in biological studies is limited. In the current experiment, the recombinant oleptin (Gertler *et al.,* 1998) used previously (Amstalden *et al.,* 2000) in a ruminant-specific radioimmunoassay (RIA) (Delavaud *et al.,* 2000) was utilized for leptin infusion. To minimize the amounts necessary to conduct the experiments, and because leptin effects are to a large degree effected at the hypothalamic level (Frieman and Halaas, 1998; Li *et al.,* 1998; Nagatani *et al.,* 1998), we chose to infuse leptin directly into the cerebroventricles using an ICV cannulation model similar to that established and reported previously from this laboratory (Gazal *et al.,* 1998).

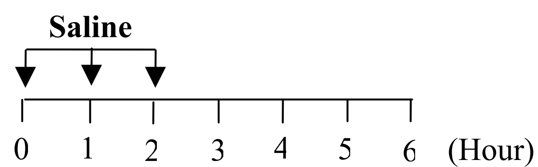
Procedures

Experiment 1A tested the hypotheses that 1) short-term fasting would reduce circulating concentrations of leptin and insulin in mature, ovariectomized, estradiol-implanted cows; 2) ICV infusions of recombinant oleptin would attenuate fasting-mediated reductions in insulin; and 3) central infusion of recombinant oleptin would stimulate LH secretion, particularly in fasted cows. Thirteen mature, ovariectomized cross-bred beef cows (Texas Agricultural Experiment Station herd, Beeville, TX), each bearing subcutaneous estradiol implant, were used in this study. Twelve of 13 cows were surgically fitted with ICV cannulas (Control, n = 6; Fasted, n = 6) as described previously (Gazal *et al.,* 1998), except that cannulas were inserted into the lateral rather

than the third ventricle. The location and function of cannulas were verified by radiography and continuous flow of cerebrospinal fluid. A period of at least 3 weeks was allowed for cows to recover from ICV surgery. Cows were fed once daily at 0700 h a diet formulated to provide 100% of the National Research Council (NRC) (1996) requirements for maintenance before the start of the experiment, and their average body condition score was 6 ± 0.12 on a scale of 1 to 9 (1 = emaciated; 9 = obese).

Each cow was assigned to one of two dietary groups: 1) Control; cows were fed 100% of the NRC requirements (NRC, 1996) for maintenance and had free access to water (*n* = 6) and 2) Fasted; cows were fasted for 60 h with free access to water (*n* = 7). On the day before the start of dietary treatments (Day –1), cows were fitted with jugular catheters (polyethylene tubing, 1.4 mm i.d., 1.9 mm o.d.; Becton Dickinson, Parsippany, NJ) for intensive blood sampling. At the same time, patency of ICV cannulas was verified, and ICV cows were treated prophylactically with antibiotics (oxytetracycline HCl, 9 mg kg^{-1} daily and oral sulfadimethoxine, 125 mg kg^{-1} once). Cows were placed in stanchions after control cows had been fed and allowed to stand without further restraint during periods of intensive blood sampling, which started at 0900 h on Days 0 and 2. Blood was collected semiremotely at 10-min intervals for 6 h on Day 0 and for 12 h on Day 2 of the experiment via an extension connected to the jugular catheter. Blood samples were dispensed into tubes containing 150 µl of a solution containing heparin (1000 IU ml^{-1}) and 5% EDTA and placed immediately on ice. Plasma was harvested by centrifugation and stored at -20°C until hormone analysis. At the beginning of the intensive sampling periods on Days 0 (6 h) and 2 (12 h), sterile saline

Day 0 – beginning of dietary treatment



Day 2

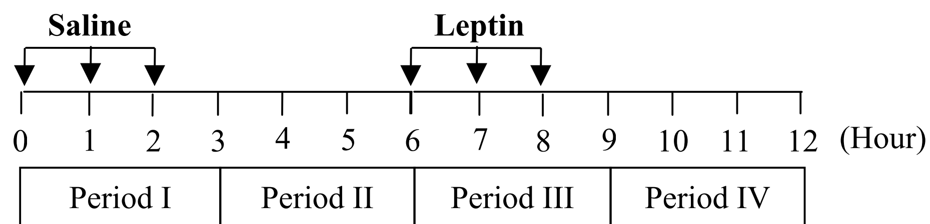


Fig. 1. Timeline for experimental procedures in Experiment 1A. Blood was collected at 10-min intervals for 6 h on Day 0 and for 12 h on Day 2. The ICV infusions of saline or recombinant oleptin are indicated by arrows.

(200 μl) was infused as a control into the ICV cannula at 0, 1, and 2 h. Similarly, on Day 2, cows in each group received ICV infusions of 200 μg recombinant oleptin (Gertler *et al.,* 1998) in 100-μl saline, followed by a 100-μl injection of saline to flush the cannula at 6, 7, and 8 h (Fig. 1). The dose of leptin used in this experiment was determined on the basis of extrapolation from previously reported experiments in pigs (Barb *et al.,* 1998) and sheep (Henry *et al.,* 1999) in combination with preliminary experiments using hypothalamic explants and ICV-cannulated cows in this laboratory (unpublished results). The heads of the cows were briefly restrained during each ICV infusion. The fasted cow without an ICV cannula was not treated with saline or leptin and data from this animal contributed only to the study of fasting effects.

Because of confounding effects of leptin infusion, leptin gene activity could not be determined in the initial group of cows. Therefore, to test whether the leptin gene was responsive to short-term fasting in mature cows, an additional 12 ovariectomized, estradiol-implanted cows were used in a complementary experiment (Experiment 1B). This experiment also allowed us to determine whether patterns of leptin in the circulation are affected by period of the day, which if observed, could complicate our interpretation of the effects of centrally infused leptin in Experiment 1A. Before the onset of the experiment, cattle were fed for maintenance according to NRC recommendations (1996). The average body condition score was 6.1 ± 0.2, as described previously. Each cow was assigned to one of two dietary groups: 1) Control: cows were fed Coastal bermudagrass hay *ad libitum* and had free access to water (n = 6) and 2) Fasted: cows were fasted for 60 h with free access to water (n = 6). Subcutaneous fat

samples were collected lateral to the tail head by aseptic biopsy using epidural anesthesia (2% lidocaine HCl) at the beginning (Day 0) and end (Day 2) of the dietary treatment. Fat samples were snap frozen in liquid nitrogen and stored at -80°C until Northern blot analysis for leptin mRNA. Blood samples were collected by caudal venipuncture every h on Day 2 of the experiment and processed as described previously.

Radioimmunoassay and colorimetric assays

Circulating concentrations of leptin were determined using a highly specific ovine leptin radioimmunoassay (RIA) validated for use in bovine serum (Delavaud *et al.,* 2000). Use of this assay for determining plasma concentrations of leptin in bovine has been validated and reported previously by our laboratory (Amstalden *et al.,* 2000). Determinations for circulating leptin were performed in samples collected every 3 h for 6 h on Days 0 (Experiment 1A) and 2 (Experiments 1A and 1B), and every h for samples collected on the final 6 h on Day 2 (Experiments 1A and 1B). Plasma concentrations of insulin were determined as validated previously 24(Ryan *et al.,* 1995) in samples collected for leptin determinations. Circulating concentrations of LH were determined with a validated assay (McVey *et al.,* 1991) for samples collected at 10-min intervals for 6 h on Day 0 and for 12 h on Day 2 (Experiment 1A). Serum estradiol was assayed in extracted samples as reported previously (Talavera *et al.,* 1985). Intraassay and interassay coefficients of variation for the preceding assays averaged 7 and 15%, respectively. Plasma glucose was determined in samples collected at 0 and 6 h on Day 0

and at 0, 6, and 12 h on Day 2 using the Sigma colorimetric assay (510A; Sigma, St. Louis, MO), according the to manufacturer's instructions.

Northern blot analysis

Total cellular RNA was isolated from 0.7 g of subcutaneous adipose tissue as previously described (Amstalden *et al.,* 2000). Fifteen micrograms of RNA were loaded on 1% agarose gels, separated by electrophoresis, and transferred onto nylon membranes. Ultraviolet transillumination of ethidium bromide-stained RNA was used to quantify 18S rRNA bands using a Fluor-S MultiImager System (Bio-Rad Laboratories, Hercules, CA). Blots were hybridized with ^{32}P-labeled RNA probe generated from a 350-bp ovine leptin cDNA (Genebank accession U62123) (Dyer *et al.,* 1997b). Hybridization signals were quantitated with an Instant Imager (Packard Instrument Co., Downers Grove, IL) and normalized with 18S rRNA.

Statistical analysis

Hormone data were analyzed by analysis of variance (ANOVA) for repeated measures using the general linear models procedure (PROC GLM) of Statistical Analysis System (SAS 8.1) (SAS Institute Inc., Cary, NC). Frequency and amplitude of LH pulses were determined using a pulse detection algorithm, Pulsefit 1.2 (Kushler and Brown, 1991). Sources of variation were diet, day, cow(diet), and appropriate interactions. The least significant means procedure was used to compare means when significant differences were detected. Because of random differences in LH concentrations between groups on Day 0, analysis of covariance (ANACOVA) was used

to compare treatment means on Day 2. Mean concentrations of LH for each cow on Day 0 were used as the covariate to test effects of dietary treatment on LH secretion. Similarly, mean amplitude of LH pulses on Day 0 was used as a covariate in an ANACOVA procedure to test effects of dietary treatment on mean amplitude of LH pulses. To test the temporal effects of leptin administration on plasma LH and insulin, the 12-h intensive sampling period on Day 2 was subdivided in 4 periods (I - IV). Hormone data were analyzed using ANOVA for repeated measures (PROC GLM procedure of SAS). Sources of variation were diet, period, cow(diet), and appropriate interactions. When a significant difference was detected, the least significant means procedure was used to compare means. Leptin mRNA data were transformed to percentage values relative to time 0 (Day 0) and analyzed by the *t*-test procedure of SAS.

Results

One control and one fasted cow exhibited alterations in the pattern of LH secretion on Day 0 or 2 after ICV infusions of saline. Therefore, LH data from these cows were not considered further. However, secretion of insulin, leptin, and glucose were considered normal and were included in all analyses.

Effects of short-term fasting on leptin gene expression in adipose tissue; on circulating insulin, leptin, and glucose; and on pulsatile LH release

In Experiment 1A, postprandial increases of circulating insulin in the Control group resulted in overall mean concentrations of insulin that were greater ($P < 0.01$) in

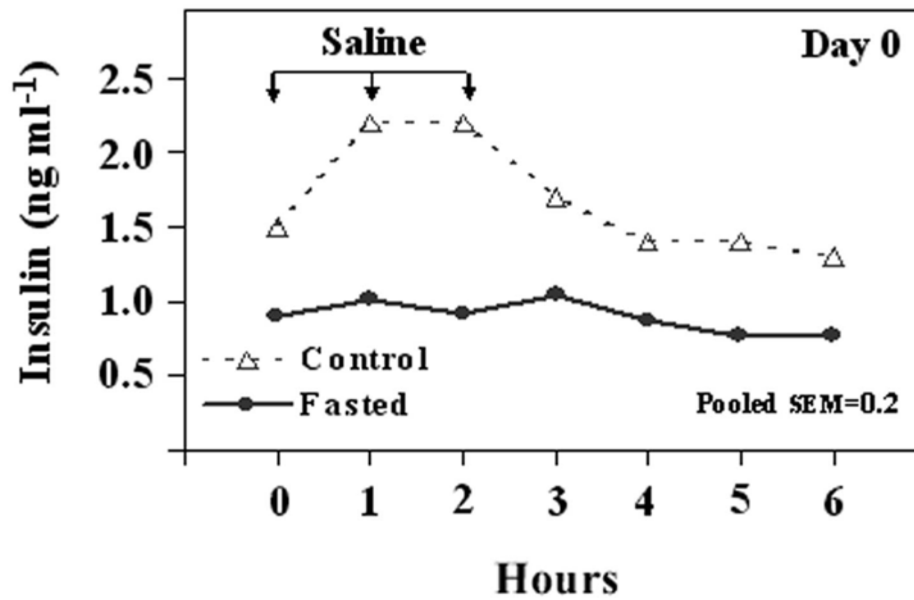


Fig. 2. Mean plasma concentrations of insulin in control and fasted cows on Day 0 in Experiment 1A. A postprandial increase in circulating insulin was observed in control cows after they received their morning feed. Therefore, the overall mean concentration of insulin was greater ($P < 0.01$) in control than in fasted cows.

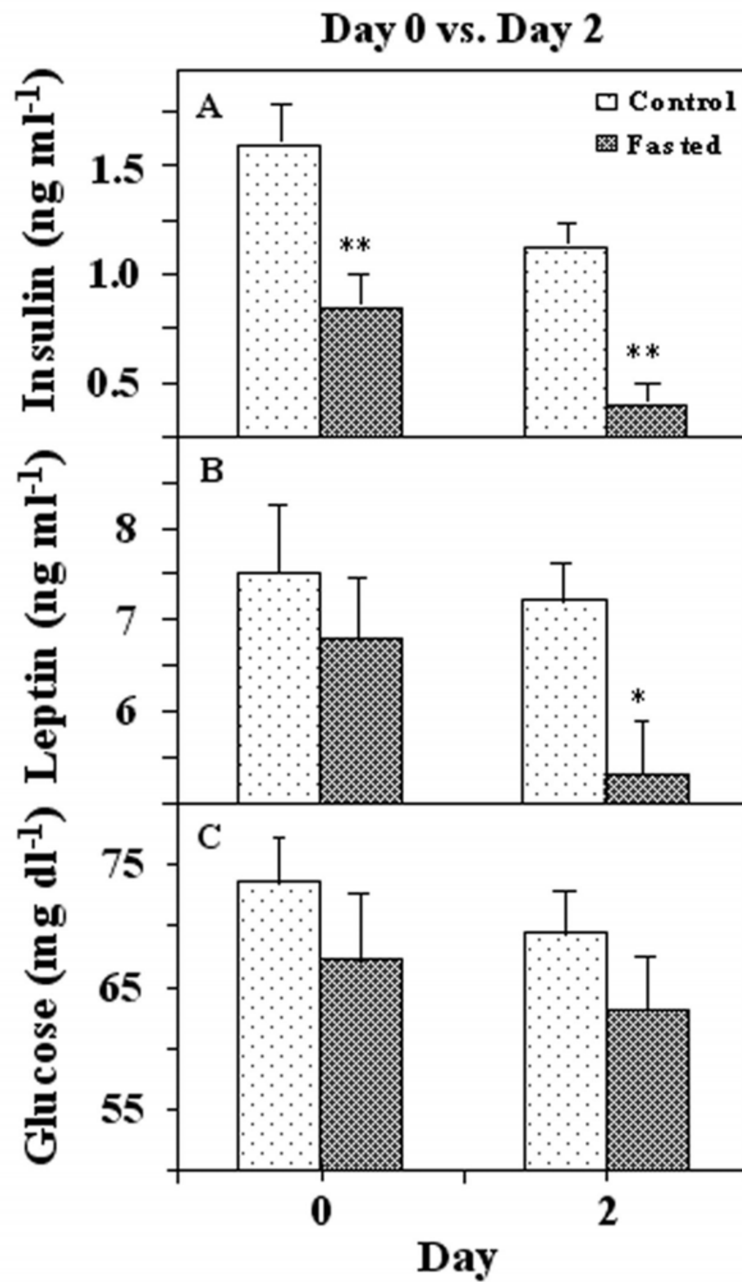


Fig. 3. Mean plasma concentrations of insulin (A), leptin (B), and glucose (C) in control and fasted cows on Days 0 and 2 in Experiment 1A. Mean concentrations of insulin were greater (**, $P < 0.01$) in control than in fasted cows on Day 0. Fasting reduced plasma insulin (**, $P < 0.01$) and leptin (*, $P < 0.04$) concentrations on Day 2. Mean concentrations of glucose did not differ ($P > 0.1$) between or within group on any day.

the Control than in the Fasted group on Day 0 (Figs. 2 and 3). In addition, fasting caused a 49% decrease ($P < 0.001$) in circulating insulin on Day 2 compared to Day 0 and concentrations were lower ($P < 0.01$) than in controls (Fig. 3). Circulating concentrations of leptin were lower in the Fasted than in the Control group ($P < 0.04$). Plasma concentrations of glucose did not differ between and within groups on any day (Fig. 3). Fasting caused a 30% reduction ($P < 0.001$) in leptin mRNA expression in adipose tissue on Day 2 (Experiment 1B; Fig. 4).

The mean concentration of LH and mean amplitude of LH pulses were greater (dietXday; $P < 0.01$) in the Fasted compared to the Control group at the start of the experiment on Day 0 (6-h sampling window). Because differences in feed intake involving only a few hours do not affect LH secretion in ruminants (Day *et al.,* 1986a), differences in LH secretion patterns on Day 0 were considered to be innate to the individual animals and not a consequence of diet. Using ANCOVA to adjust for the difference on Day 0, we observed no effect of fasting on any LH variable in this experiment.

Effects of ICV infusions of oleptin on plasma insulin, leptin, glucose, and LH secretion

On Day 2 of the experiment (Fig. 5), mean concentrations of insulin were lower ($P < 0.01$) in fasted compared to control cows during Periods I and II. However, after the start of leptin infusions, plasma insulin began to increase steadily ($P < 0.01$) in the Fasted group and reached concentrations similar to those observed before fasting and not different from the Control group at Hours 9, 10, and 12 ($P > 0.10$; Fig. 5). Circulating

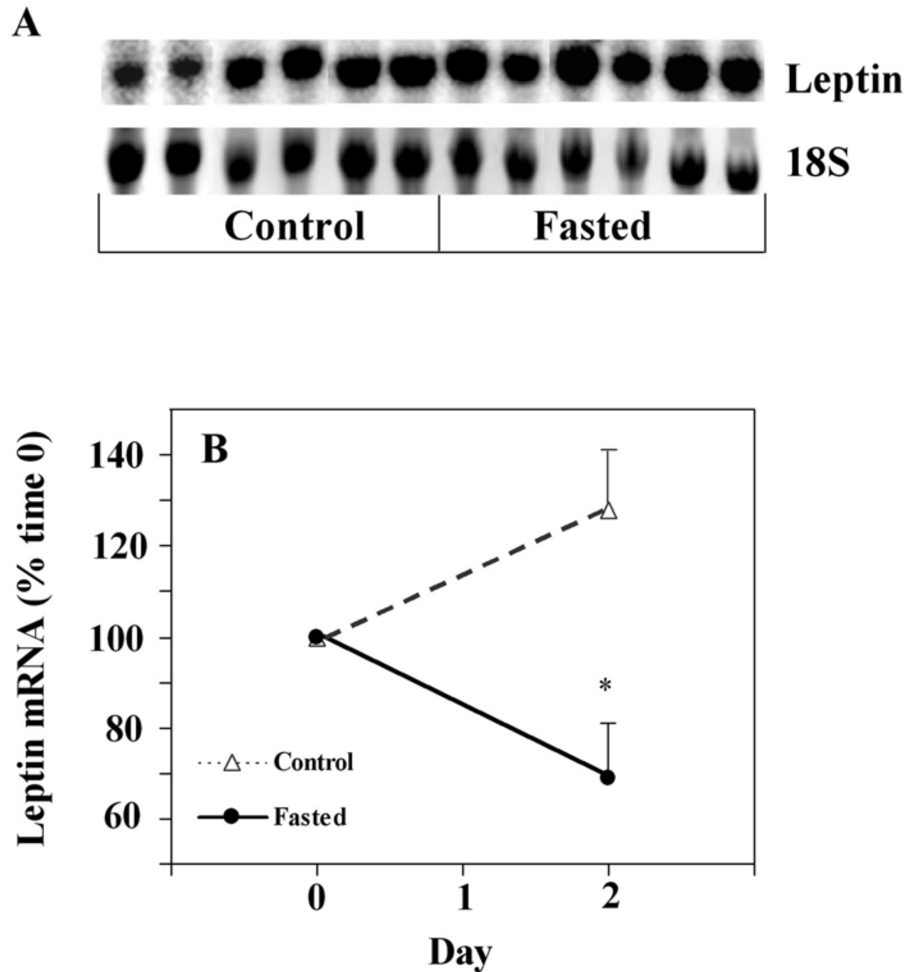


Fig. 4. Leptin gene expression in adipose tissue of control and fasted cows in Experiment 1B. A) Hybridization signals for leptin mRNA (top) and UV transillumination of ethidium bromide-stained 18S rRNA (bottom) in 3 representative control and 3 representative fasted cows. Lanes represent consecutive prefasting and postfasting periods for each control and fasted cow. B) Leptin mRNA expression is presented as the mean ± SEM percentage of Time 0 (Day 0) in all control (n = 6) and fasted (n = 6) cows. Leptin mRNA expression was lower (*, $P < 0.001$) in the Fasted group compared with the Control group on Day 2.

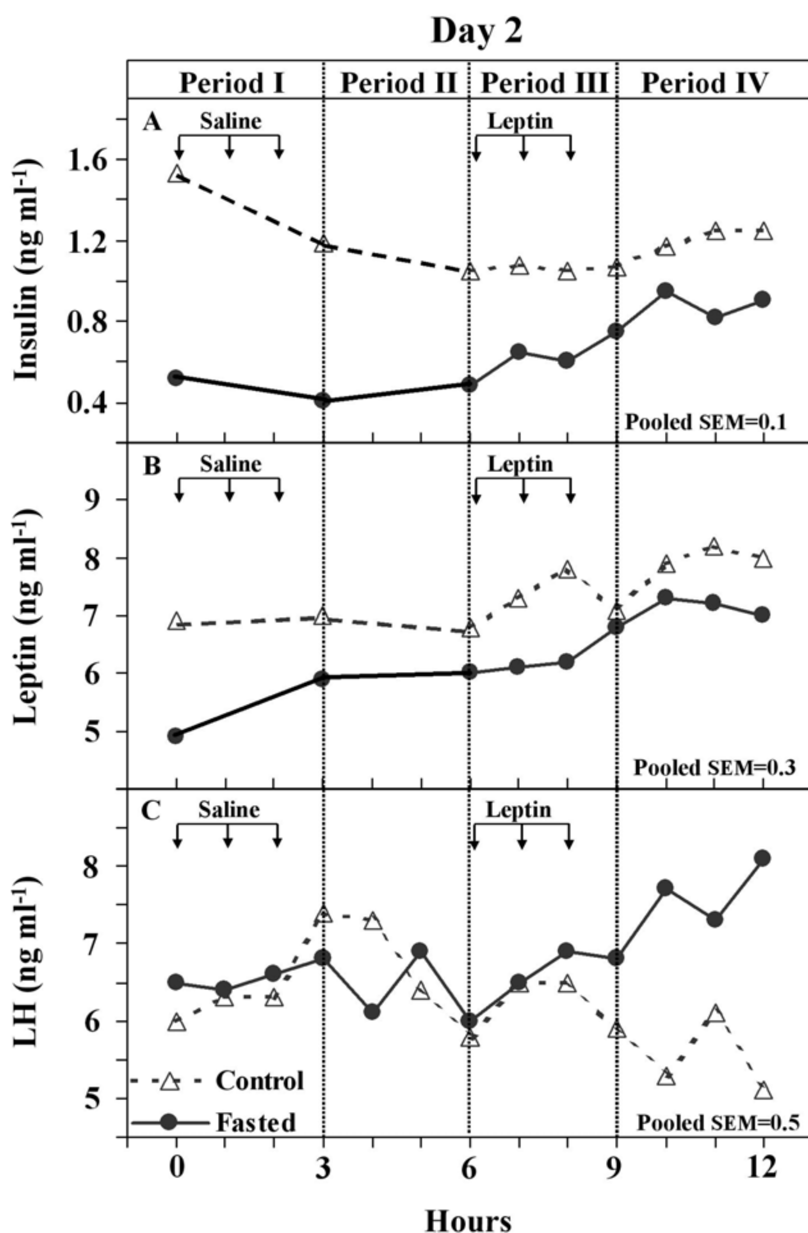


Fig. 5. Mean concentrations of insulin (A), leptin (B), and LH (C) on Day 2 in Experiment 1A. The ICV infusions of recombinant oleptin increased (*P* < 0.01) mean concentrations of insulin in the Fasted group (Period III and IV) to levels observed before fasting and not different from those in controls at Hours 9, 10, and 12 (*P* > 0.1). Mean concentrations of leptin were greater (*P* < 0.01) during Period IV in both Control and Fasted groups than during previous periods. A marked increase (*P* < 0.03) in mean concentrations of LH in fasted cows was observed during Period IV after ICV infusions of leptin.

concentrations of leptin were greater (*P* < 0.01) during Period IV (after leptin infusion) than during previous periods in both Control and Fasted groups. This increase in circulating leptin was not seen in control-fed and fasted cows that were not infused centrally with leptin (Experiment 1B, data not shown). Mean concentrations of glucose were not affected by leptin infusions (*P* > 0.1; data not shown).

A remarkable increase (*P* < 0.03) in mean concentrations of LH was observed in fasted cows during Period IV after ICV infusions of leptin compared with concentrations in the previous three periods and with concentrations in control cows during Period IV. Although mean concentrations of LH in the Control group during Period IV were lower (*P* < 0.05) than those for Periods I through III, this observation did not account for the positive effect of leptin in the Fasted group. However, mean size of LH pulses, determined by the area under each pulse, was greater (*P* < 0.04) in the Fasted than in the Control group. Increased pulse size appeared to occur as a result of an extended duration of individual pulses in four of five cows (*P* < 0.01) after ICV infusion of leptin, as neither amplitude nor frequency of pulses per se differed between groups. Figure 6 shows individual patterns of LH secretion in two representative fasted cows on Day 2 of the experiment. An increase in baseline concentrations of LH and area under each pulse after leptin treatment in the two representative fasted cows can be readily observed. For the two representative control-fed cows, concentrations of LH and amplitude of pulses appear to decline after leptin, which is consistent with the reduction in mean concentrations observed for this group during Period IV.

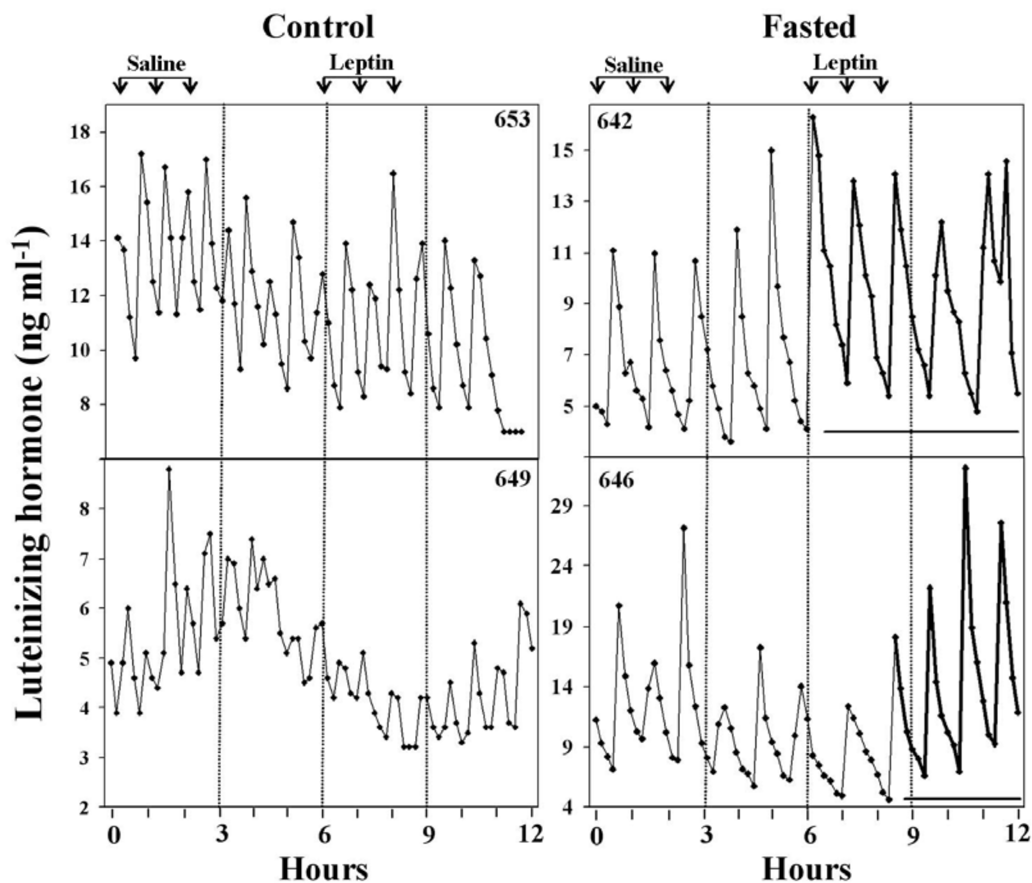


Fig. 6. Individual patterns of LH secretion in 2 representative control and 2 representative fasted cows on Day 2 of Experiment 1A. For fasted cows, note the increase in the baseline secretion (area denoted by black bars) after initiation of leptin infusions and augmentation of pulses of LH after ICV infusion of recombinant oleptin. In control cows, LH tended to decline after leptin infusions.

Discussion

Two novel observations were made in this study regarding the interaction of nutritional status and exogenous leptin treatment in cattle, including normalization of fasting-mediated declines in circulating insulin and heightened secretion of LH in mature cows treated with leptin. These effects were observed in the presence of fasting-mediated reductions in plasma leptin, which in cows not treated with leptin (Experiment 1B) were associated with diminished leptin gene expression in adipose tissue.

Declines in leptin mRNA and circulating leptin were similar to that reported previously in prepubertal heifers (Amstalden *et al.,* 2000) and in several other species (Kolaczynski *et al.,* 1996; Li *et al.,* 1998; Mercer *et al.,* 1998; Nagatani *et al.,* 1998; McManus and Fitzgerald, 2000; Nagatani *et al.,* 2000), confirming that leptin synthesis and secretion are acutely responsive to changes in nutritional status. Circulating concentrations of leptin increased in both Control and Fasted groups after ICV infusions of recombinant oleptin. The outcome of central infusions of leptin has been reported in the mouse. Radiolabeled leptin (^{125}I-leptin) injected into the lateral ventricle appeared in the circulation in less than 5 min, reaching peak concentrations within 20 min that were similar to those observed 20 min after i.v. infusion of a similar quantity (Maness *et al.,* 1998). In addition, periventricular diffusion of ^{125}I-leptin was detected up to 600 μm from the midline into the hypothalamus within 30 min, demonstrating the potential for infusion of leptin into the ventricular system to act within hypothalamic neurons. In humans, leptin secretion has been reported to follow a circadian rhythm (Licinio *et al.,* 1998), with a nadir early in the morning (0800 – 0900h), an increase during the day, and

a peak between 2400 and 0200 h. A circadian rhythm for leptin secretion has not been reported in ruminants. However, in Experiment 1B, we collected blood samples for 12 h to determine if circulating concentrations of leptin would change between 900 h and 2100 h. Had such changes been observed, their occurrence would have complicated our interpretation of the increased concentrations of leptin observed after ICV infusions in Experiment 1A. No evidence of increased circulating leptin during the evening hours was observed in cows not infused with leptin (Experiment 1B). Therefore, the increases observed in circulating leptin for both groups after central infusion of oleptin (Experiment 1A) probably were not confounded by a potential diurnal variability.

Lower mean concentrations of insulin on Day 0 in fasted cows were due mainly to postprandial increases in insulin in control cows fed in the morning before the beginning of blood sampling and have been reported previously in cattle (Blum *et al.,* 2000). Such increases in insulin can occur either as a physiological response to increasing plasma glucose or as parasympathetic responses to feeding (Herath *et al.,* 1999; D'Alessio *et al.,* 2001). In our study, circulating glucose was not affected by feeding or short-term fasting or by central administration of leptin.

Importantly, ICV infusions of leptin, which resulted in an elevation of serum leptin, increased circulating concentrations of insulin in fasted cows to those observed before fasting and similar to those in controls. Because ICV leptin enters the peripheral circulation, central and peripheral effects of leptin could not be differentiated in the present experiments. However, the stimulatory effects of leptin on serum insulin in fasted cows supports previous observations in rat pancreatic β-cells (Tanizawa *et al.,*

1997; Shimizu *et al.,* 1997). In those studies, leptin stimulated basal insulin secretion from isolated rat pancreatic islets cultured in 5 – 7 mM glucose, but did not affect glucose-stimulated (25mM) insulin secretion. Paradoxically, others have reported either negative (Fehmann *et al.,* 1997; Kieffer *et al.,* 1997; Poitout *et al.,* 1998; Sivitz *et al.,* 1997) or neutral (Ahima *et al.,* 1996; Nagatani *et al.,* 2000) effects of leptin on insulin release. The basis for these conflicting observations is not clear, but has arisen from experiments involving a variety of experimental conditions and animal models. For example, hleptin injected subcutaneously over a period of 76 h to produce concentrations of leptin above 30 ng ml^{-1} does not affect insulin secretion during fasting in the castrated, estradiol-treated male sheep (Nagatani *et al.,* 2000). This is in contrast to the present study in fasted cows in which centrally infused leptin elevated plasma concentrations of leptin to prefasting concentrations (7 ng ml^{-1}), and increased circulating concentrations of insulin. Pancreatic β-cells express leptin receptors (Fehmann *et al.,* 1997; Kieffer *et al.,* 1997), and both Janus kinase (JAK)/signal transducer and activator of transcription (STAT), and mitogen activated protein kinase (MAPK) (Ghilardi *et al.,* 1997; Morton *et al.,* 1999) have been reported as intracellular mediators of leptin-receptor interaction. In addition, leptin has been shown to influence ATP-sensitive potassium channels in pancreatic β-cells (Poitout *et al.,* 1998). Although leptin has been reported to directly influence pancreatic insulin release, an indirect effect via the central nervous system cannot be discounted. Chemical sympathectomy prevented the suppressive effects of leptin on a glucose-induced increase in secretion of insulin (Mizuno *et al.,* 1998), suggesting an indirect effect of leptin on the pancreas via

the autonomic nervous system. In contrast, ICV infusions of leptin resulted in normalization of circulating concentrations of insulin during fasting-induced hypoinsulinemia in cows. If this effect is mediated via the central nervous system, it is in addition to the paradoxical variety of effects seen for leptin through direct action on pancreatic β-cells. Taken together, these reports suggest the potential for a series of complex actions of leptin on the pancreas that include effects on gene expression, plasma membrane polarization potential, and sympathetic tone. However, the relevance of these pathways to ruminant physiology and metabolism remains to be determined.

As expected, short-term fasting did not suppress LH secretion in these mature, ovariectomized, estradiol-implanted cows. Yet, fasted cows respond to leptin with a remarkable increase in the mean concentrations of LH. This occurred as a result of a 46.6% increase in the size of LH pulses in four of five fasted cows after central infusion of leptin. Two of the four fasted cows that responded to leptin showed an increase in size of LH pulses almost immediately after ICV infusions, whereas the other two cows that responded to leptin showed a delayed response (note the 2 representative fasted cows in Fig. 6). This variability in temporal response to centrally infused leptin may be associated with variability of diffusion of the hormone into the tissue among animals, either directly or via the bloodstream. Nevertheless, the increased size of LH pulses was extended throughout the end of the experiment, which can be seen by the significant increase in mean concentrations of LH in the Fasted group during Period IV. The increase did not involve heightened frequency of LH pulses, but this would not have been expected since pulse frequency was not suppressed in this model. Acute feed

restriction causes a rapid reduction in mean concentrations of LH in rodents, humans, and monkeys (Cagampang *et al.,* 1990; Cameron and Nosbish, 1991; Samuels and Kramer, 1996), but only chronic undernutrition has been shown to suppress LH release in mature cows (Richards *et al.,* 1991). Nonetheless, we have reported that 48 h of total feed restriction decreases LH pulse frequency in growing prepubertal heifers (Amstalden *et al.,* 2000), suggesting that the hypothalamic-pituitary axis of immature female cattle is more sensitive to acute perturbations in energy status than mature cows having larger adipose stores. It has been reported that undernutrition increases leptin receptor numbers in the hypothalamus of ewes (Dyer *et al.,* 1997a). Thus, it is possible that fasted cows had increased numbers of leptin receptors in the hypothalamus and became more sensitive to leptin.

Control-fed cows exhibited decreased mean concentrations of LH during Period IV in the present study, which can be seen in the individual control cows shown (Fig. 6). Whether this was also an effect of leptin is not clear. Hypothalamic explants of rats treated with low concentrations of leptin (10^{-12} and 10^{-10} M) show increased GnRH secretion compared to controls (Yu *et al.,* 1997). However, higher doses of leptin decreased secretion of GnRH into the media. Henry et al. (1999) did not observe an effect of centrally infused recombinant hleptin on LH secretion in normal-fed, ovariectomized ewes without estrogen replacement; however, subcutaneous administration of hleptin prevented the decrease in mean concentrations of LH in gonadectomized, estradiol-implanted male sheep fasted for 72 h (Nagatani *et al.,* 2000),

indicating that not only metabolic status, but also gonadal steroids may impact the effects of leptin on the reproductive axis.

In summary, the results of the present experiments demonstrate that short-term (60 h) feed restriction diminishes synthesis of leptin in adipose tissue and circulating leptin in mature cows, similar to results observed in prepubertal heifers. In contrast to immature cattle, secretion of LH was not affected by short-term fasting. However, fasted cows were clearly more sensitive than normal-fed cows to the stimulatory effects of leptin, resulting in an augmentation of size of LH pulses and an increase in the mean circulating concentration of LH. In addition to marked effects on LH secretion, leptin also stimulated an increase in circulating insulin in fasted cows. This effect, similar to that observed for LH, was clearly dependent upon the metabolic status of the animal. However, because of conflicting reports in the literature, the role of leptin in regulating pancreatic insulin secretion in ruminants remains unclear and requires additional study.

CHAPTER IV

EFFECTS OF LEPTIN ON GONADOTROPIN-RELEASING HORMONE RELEASE FROM HYPOTHALAMIC-INFUNDIBULAR EXPLANTS AND GONADOTROPIN RELEASE FROM ADENOHYPOPHYSEAL PRIMARY CELL CULTURES: FURTHER EVIDENCE THAT FULLY-NOURISHED CATTLE ARE RESISTANT TO LEPTIN

Introduction

It is well recognized that undernutrition can impact negatively reproduction in mammals. Reduced secretion of LH, likely resulting from decreased release of GnRH into the hypothalamic-adenohypophyseal portal system, is a prominent manifestation of decreased nutrient availability (Kile *et al.,* 1991; I'Anson *et al.,* 2000). However, the physiological mechanisms through which nutrition mediates its effects are not well characterized. A variety of metabolic fuels, hormones, and neurotransmitters have been implicated in this process (Keisler and Lucy, 1996; Wade *et al.,* 1996). Leptin, a protein hormone synthesized and secreted mainly by adipocytes, has been shown to communicate nutritional status to the central nervous system (Ahima *et al.,* 2000), rescue leptin-deficient (*ob/ob*) mice from infertility (Barash *et al.,* 1996), stimulate earlier puberty in female rats (Ahima *et al.,* 1997), and prevent fasting-mediated reductions in

frequency of LH pulses in rats (Nagatani *et al.,* 1998), castrated male sheep (Nagatani *et al.,* 2000), and prepubertal heifers (Maciel *et al.,* 2003a).

In full-fed rats, leptin increases the release of GnRH from rat hypothalamic explants and LH and FSH from hemi-adenohypophysis (Yu *et al.,* 1997), demonstrating that it can act at both levels. However, in the mature, ovariectomized cow leptin appears to increase mean concentrations of LH only in fasted animals (Amstalden *et al.,* 2002). Therefore, across species, a striking dichotomy appears to exist with respect to conditions under which leptin is capable of stimulating the hypothalamic-pituitary axis. The current studies were designed to determine more directly whether leptin can stimulate the release of GnRH from bovine hypothalamic-infundibular (HYP) explants and gonadotropins from bovine adenohypophyseal cells obtained from normal-fed cattle.

Materials and Methods

All animal-related procedures in these experiments were approved by the Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System.

Experiment 1

Twenty-four, normal-fed beef bulls (n = 17) and steers (n = 7) of various breeds were used in this experiment. Animals were maintained in feedlot conditions to promote weight gain. Bulls were confirmed sexually mature (2 consecutive ejaculations of $\geq 500 \times 10^6$ sperm with $\geq 50\%$ motility) before used in this experiment. Animals were

slaughtered after an overnight fast at the Rosenthal Meat Science Center, Texas A&M University, College Station, Texas. After removal of the heads, crania were cut caudally in an oblique plane beginning at the dorsal aspect of the orbit of the eyes and continuing to the dorsal aspect of the occipital condyles. Diencephalons were removed after disconnection of infundibuli from hypophyses. To isolate the medial basal hypothalamus (MBH), the entire hypothalamic-infundibular complex was transected sagittally into equal halves. A cut was made approximately 2 mm lateral to the median sagittal cut for each half, followed by an anterior coronal cut extending from the optic chiasm to the anterior commissure. A posterior coronal cut and a transverse cut were made resulting in a section containing approximately half of the mamillary body.

Each half of the MBH connected to its infundibular half was incubated in 0.7 ml Krebs-Ringer bicarbonate buffer (KRB) containing glucose (1.8g L^{-1}) and bovine serum albumin (1 g L^{-1}) for 3 h at 37ºC. At 3 h of incubation, one half of the HYP explants from each animal were incubated for 30 minutes with one of the following: 1) Control: KRB alone (n = 6); 2) Leptin 10 ng ml^{-1}: KRB containing 10ng ml^{-1} recombinant ovine leptin (oleptin; n = 6) (Gertler *et al.,* 1998); 3) Leptin 100 ng ml^{-1}: KRB containing 100 ng ml^{-1} oleptin (n = 5); 4) Leptin 1000 ng ml^{-1}: KRB containing 1000 ng oleptin (n = 7). The other half of each HYP explant was incubated KRB containing 60 mM K^{+} to test tissue viability, based on nonspecific stimulation of GnRH release (Waters *et al.,* 1998). Incubation of HYP explants continued for additional 30 min. Medium was collected and replaced every 30 min (Fig. 7A), boiled for 3 min immediately after collection to destroy potential GnRH-degrading enzymes, and stored at -20 ºC until assayed for GnRH.

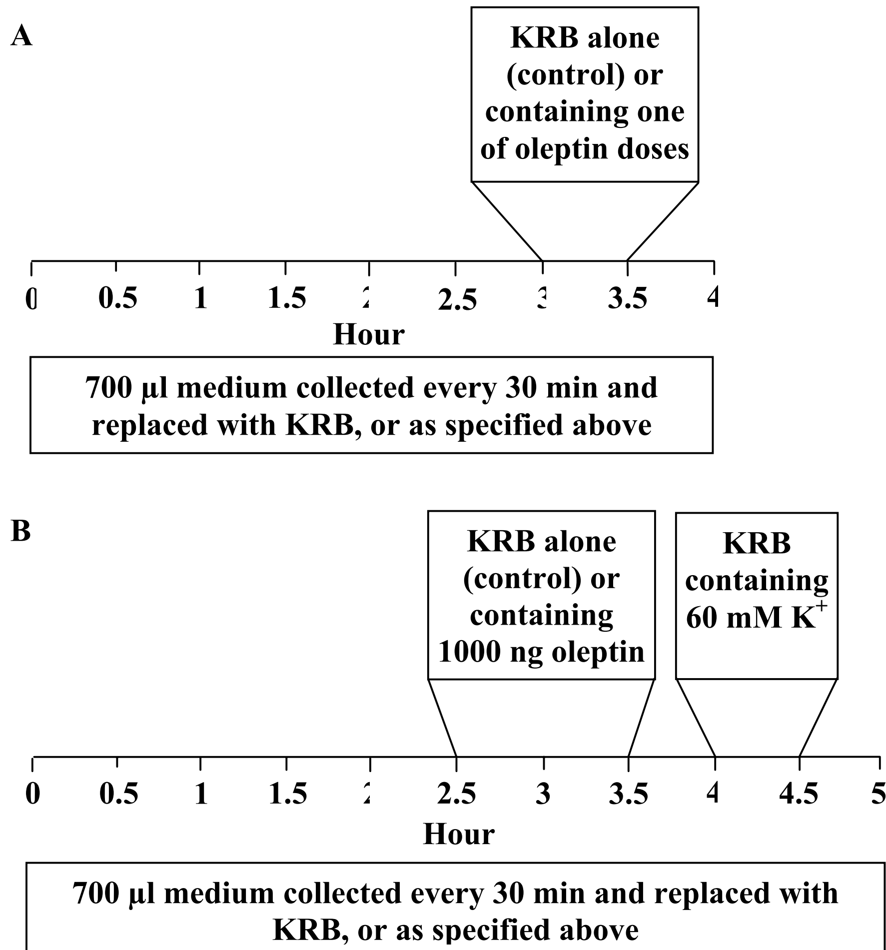


Fig. 7. Timeline for experimental procedures for HYP explant incubations in Experiments 1 (A) and 2 (B). In Experiment 1, HYP explants were incubated in KRB for 4 h. Medium was replaced every 30 min. At 3 h, medium was replaced with KRB alone (control) or KRB containing 10, 100, or 1000 ng ml^{-1} oleptin. In Experiment 2, HYP explants were incubated in KRB for 5 h. Medium was replaced every 30 min. Beginning at 2.5 h, explants were incubated in KRB alone or containing 1000 ng oleptin for 1 h. Explants were treated at 4 h of incubation with KRB containing 60 mM K^+ to induce release of GnRH.

Experiment 2

Hypothalamic-infundibular explants were collected from six steers and processed as described in Experiment 1. Each half of the MBH connected to its infundibular half was incubated in 0.7 µl of KRB for 5 h. Medium was collected and replaced every 30 min. After 2.5 h of equilibration, HYP explants from each steer were incubated with KRB containing either 0 (control) or 1000 ng of oleptin (Gertler *et al.,* 1998) for 1 h, so that one-half served as a control for the corresponding half (Fig. 7B). At 4 h of incubation, all explants were incubated with KRB containing 60 mM K^+ to test tissue viability, based on nonspecific stimulation of GnRH release (Waters *et al.,* 1998). Medium was boiled for 3 min immediately after collection to destroy potential GnRH-degrading enzymes, and stored at -20 ºC until assayed for GnRH.

Experiment 3

Adenohypophyses from six steers were collected at slaughter at the Rosenthal Meat Science Center, Texas A&M University, College Station, Texas. After removal of diencephalons as described in Experiment 1, hypophyses were removed from the sella turcica and kept on ice until tissue processing. Adenohypophyses were dissected from the neurohypophyses and AP cells were dispersed enzymatically as described previously (Tanner *et al.,* 1990). Dispersed cells from 2 steers were combined, plated in 6-well plates, and cultured in Dulbeccos's modified Eagle's medium (DMEM; Gibco Biologicals, Santa Barbara, CA) containing 10% fetal calf serum for 4 days. On Day 4, cells were incubated with serum-free DMEM overnight. On the next day, cells were

treated with DMEM alone (control) or DMEM containing 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8} M of oleptin (Gertler *et al.,* 1998), 10^{-9} M GnRH (Sigma, St. Louis, MO), 10^{-5} M forskolin (CalbioChem, La Jolla, CA), and 10^{-6} M phorbol 12-myristate 13-acetate (TPA; Sigma, St. Louis, MO) for 4 h. Forskolin (activator of adenyl cyclase) and TPA (activator of protein kinase C) are known to activate cellular mechanisms that induce gonadotropin release (Welsh *et al.,* 1986). Each treatment was applied to 6 wells. At the end of the 4-h incubation, culture media were harvested and stored at -20ºC until hormone assays. Three independent replications were performed.

Radioimmunoassays

Concentrations of GnRH in HYP culture media were determined by a validated assay as described previously (Waters *et al.,* 1998). Intra- and interassay coefficients of variation for these assays averaged 6.8 and 9.5%, respectively. Concentrations of LH and FSH in medium samples collected from primary culture of adenohypophyseal (AP) cells were determined in a single assay as described by McVey and Williams (1991) and Krystek et al. (1985), respectively. In the FSH assay, a highly-purified ovine FSH (AFP 5679C; NHPP, Harbor-UCLA Medical Center, Torrance, CA) was used as both the reference preparation and as iodinated tracer. Antiserum produced in rabbits immunized against oFSH was utilized as the primary antiserum. This antiserum shows similar cross-reactivity between partially purified preparations of ovine (oFSH S8 and S9; NHPP) and bovine (bFSH B1; NHPP) FSH and does not cross-react with other pituitary

hormones (Krystek *et al.,* 1985). Intraassay coefficients of variation for LH and FSH assays were 14.7 and 8.1%, respectively.

Statistical analysis

Gonadotropin-releasing hormone data obtained from Experiments 1 and 2 were analyzed by general linear mixed models for repeated measures using the Mixed procedure (PROC MIXED) of the Statistical Analysis System (SAS 8.1; SAS Institute Inc., Cary NC). Sources of variation in Experiment 1 were leptin treatment dose, period, reproductive status, and treatment x period interaction. Sources of variation in Experiment 2 were treatment, period, and treatment x period interaction. Period was used as repeated variable and animal was used as the subject. Concentrations of GnRH in media collected prior to leptin or control treatments (Hour 3 and 2.5 for Experiments 1 and 2, respectively) were used as covariate to adjust for differences in basal release of GnRH among explants. The Least Squares Means procedure was used to compare means when significant differences were detected.

Luteinizing hormone and FSH data obtained from cultures of AP cells (Experiment 3) were analyzed by Analysis of Variance (ANOVA) using the general linear models procedure (PROC GLM) of SAS. Sources of variation were treatment, replication, well(replicate), and treatment x replication interaction. The Least Squares Means procedure was used to compare means when significant differences were detected.

Results

Experiment 1

Because reproductive status had no effect ($P > 0.05$) on the release of GnRH from HYP explants, data obtained from explants collected from bulls and steers were combined for further analysis and representation. None of the doses of leptin tested affected ($P > 0.05$) the release of GnRH from HYP explants (Fig. 8).

Experiment 2

Release of GnRH from HYP explants was not affected ($P > 0.05$) by leptin treatment (Fig. 9). As expected, 60 mM K^+ increased ($P < 0.001$) the release of GnRH from HYP explants, but K^+-stimulated GnRH release was not affected ($P > 0.05$) by prior treatment with leptin (Fig. 9).

Experiment 3

Differences in the magnitude of release of LH and FSH into the media among replicates resulted in a significant replicate effect ($P < 0.001$). Therefore, analyses of treatment effects were performed within each replicate. None of the doses of leptin tested affected ($P > 0.05$) the release of LH from AP cells (Fig. 10). In replicate 1, leptin at 10^{-11}, 10^{-10}, and 10^{-9} M increased ($P < 0.03$) slightly the release of FSH compared to control-treated cells (Fig. 11). However, this effect was inconsistent and not confirmed in replicates 2 and 3. As expected, 10^{-9} M GnRH, 10^{-5} M forskolin, and 10^{-6} M TPA induced a marked release ($P < 0.001$) of LH (Fig. 10) and FSH (Fig. 11) from AP cells.

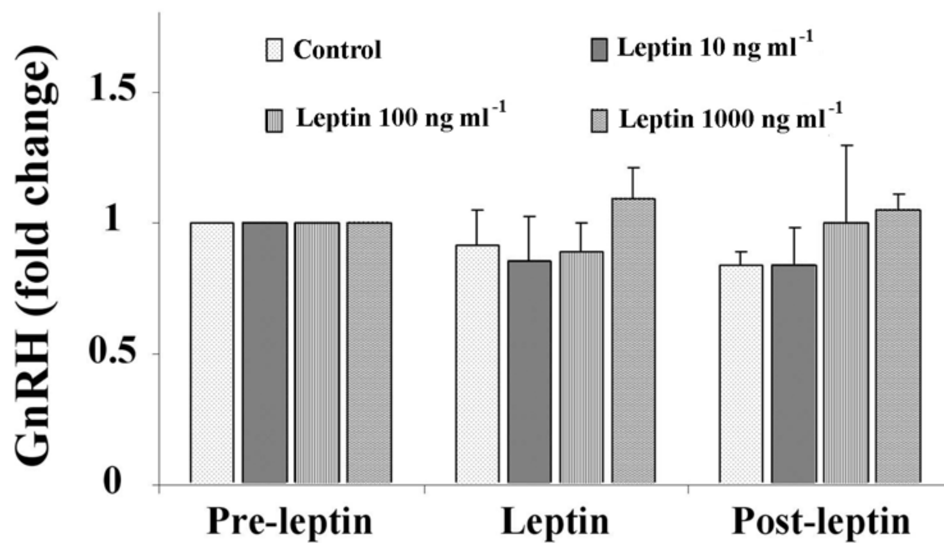


Fig. 8. Fold change in mean concentrations of GnRH in media collected from HYP explants in Experiment 1 before (Pre-leptin; 2.5 to 3 h), during (Leptin; 3 to 3.5 h), and after (Post-leptin; 3.5 to 4 h) control or leptin treatment. Leptin did not ($P > 0.05$) affect the release of GnRH into the media.

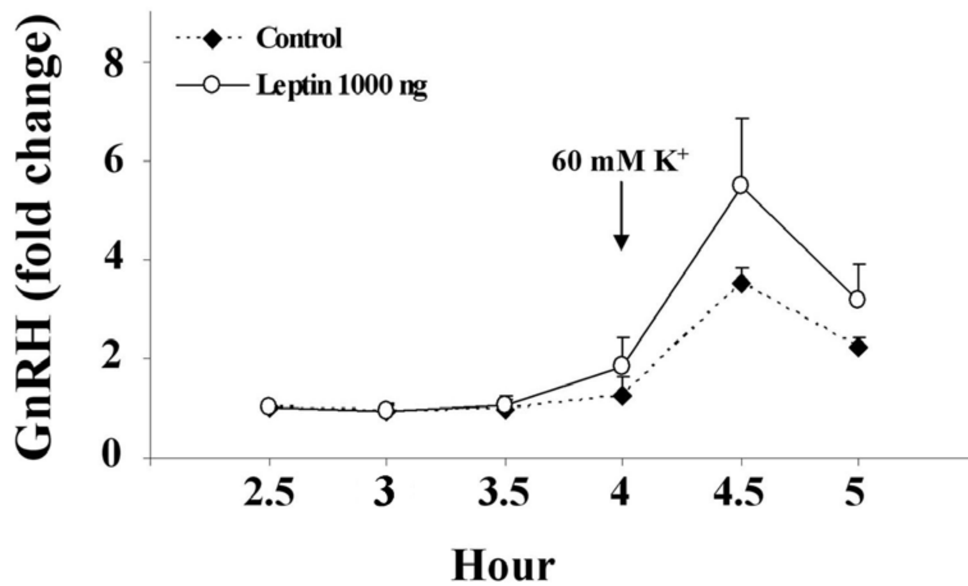


Fig. 9. Fold change in mean concentrations of GnRH in media collected from HYP explants in Experiment 2. Hypothalamic-infundibular explants were incubated in 0.7 µl of KRB either alone (control) or containing 1000 ng oleptin at 2.5 and 3 h. Explants were incubated with KRB containing 60 mM K^+ at 4 h of incubation. No effects (P > 0.05) of leptin were observed on basal or K^+-stimulated GnRH release.

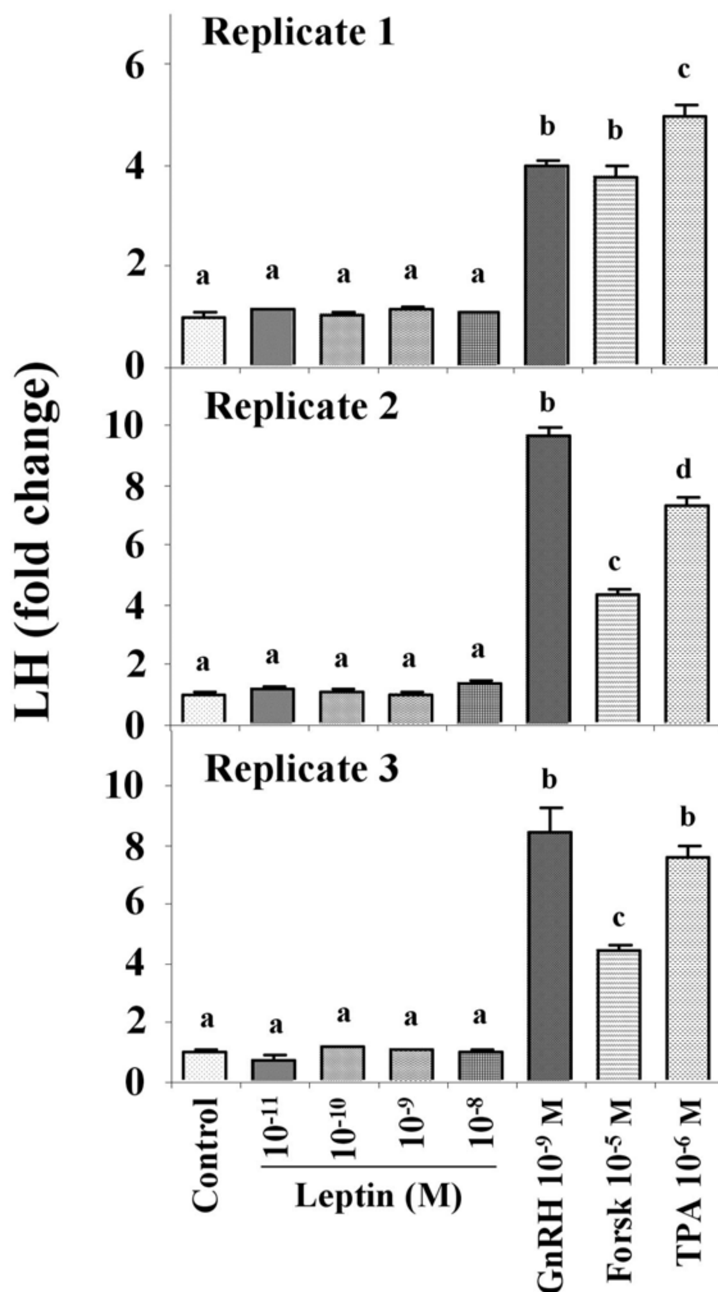


Fig. 10. Fold changes in mean concentrations (± SEM) of LH released into the media by primary AP cell cultures relative to controls (Experiment 3). None of the doses of leptin stimulated the release of LH. Gonadotropin-releasing hormone, forskolin, and TPA stimulated a marked release of LH in all 3 replications. Each value represents the mean of 6 wells. Different letters for each experimental group denote significant differences ($P < 0.001$).

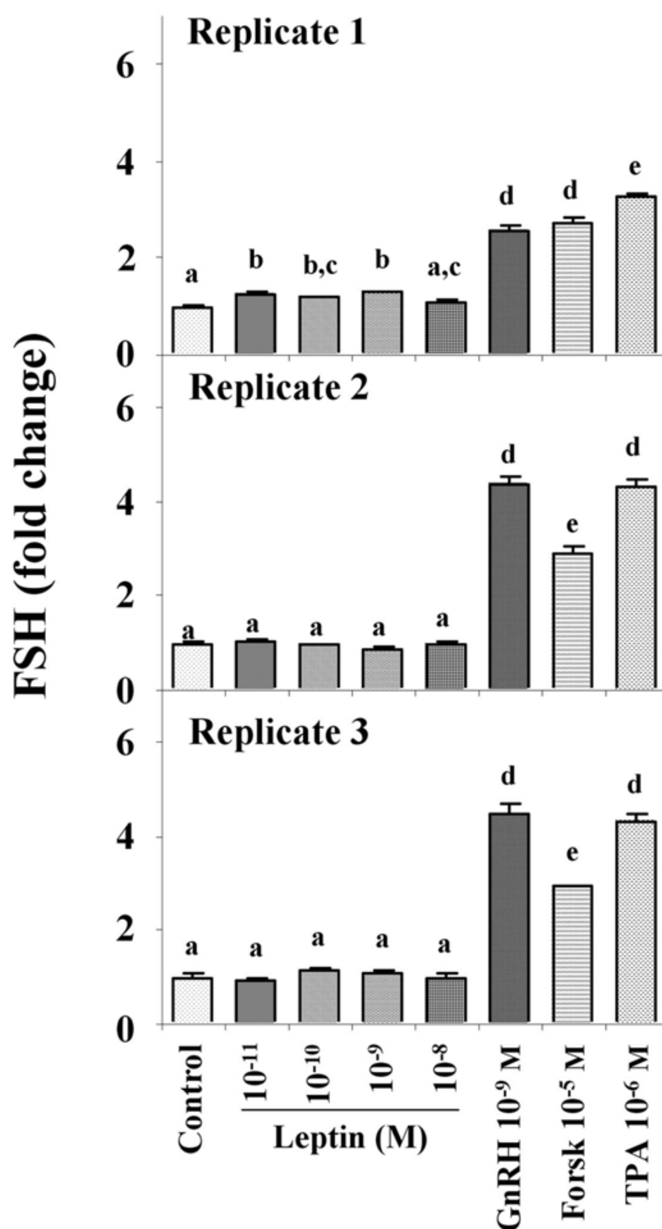


Fig. 11. Fold changes in mean concentrations (± SEM) of FSH released into the media by primary AP cell cultures relative to controls (Experiment 3). Release of FSH was increased slightly by 10^{-11}, 10^{-10}, and 10^{-9} M doses of leptin in Replicate 1, but not in replicates 2 and 3. Gonadotropin-releasing hormone, forskolin, and TPA stimulated a marked ($P < 0.001$) release of FSH in all 3 replications. Each value represents the mean of 6 wells. Different letters for each experimental group denote significant differences (a,b differ, $P < 0.03$; a,d; a,e; and d,e differ; $P < 0.001$).

Discussion

Using in vitro approaches, the current studies examined whether recombinant oleptin has direct actions at the medial basal hypothalamus-infundibular complex and adenohypophysis of fully-nourished cattle. We were unable to detect effects of leptin at any dose tested on the release of GnRH from HYP explants, nor on the release of LH and FSH from AP cells cultured under optimal nutrient conditions. This is in contrast to observations reported for rodent and pig tissues. In rodents and pigs, leptin stimulates the release of GnRH from HYP explants (Yu *et al.,* 1997; Barb *et al.,* 1999; Woller *et al.,* 2001) and from enzimatically dispersed hypothalamic neurons (Woller *et al.,* 2001), LH and FSH from rat hemi-adenohypophyseal explants (Yu *et al.,* 1997; De Biasi *et al.,* 2001), and LH from rat (Ogura *et al.,* 2001) and pig (Barb *et al.,* 1999) AP cells. With the exception of a single report by Watanobe (2002) in fasted rats, apparent contrasts in the ability of cattle and monogastrics to respond to leptin suggest important species differences in its effects on the hypothalamic-gonadotropic axis. In essentially all reports to date, the stimulatory effects of leptin on the release of LH in ruminants have been restricted to animals under acute or chronic nutrient restriction. Leptin stimulates the secretion of LH in fasted, but not in the normal-fed, ovariectomized, estradiol-implanted cow (Amstalden *et al.,* 2002) and in the chronically feed-restricted, but not *ad libitum*-fed, ovariectomized ewe (Henry *et al.,* 2001). In addition, leptin prevents the fasting-mediated reduction of pulsatility of LH in the castrated, estradiol-implanted ram (Nagatani *et al.,* 2000) and intact, prepubertal heifer (Maciel *et al.,* 2003a). However, leptin failed to stimulate release of LH in fully-fed ovariectomized, mature ewes (Henry

et al., 1999) and in growing, prepubertal ewes (Morrison *et al.,* 2002) and heifers (Maciel *et al.,* 2003b).

Mechanisms explaining the differential ability of leptin to stimulate LH in fed and feed-restricted sheep and cattle are not fully understood. Resistance to leptin has been observed in obese humans (Caro *et al.,* 1996; Considine *et al.,* 1996) and rodents (Halaas *et al.,* 1997; Widdowson *et al.,* 1997), and cellular mechanisms have been proposed. The development of resistance to leptin may involve excessive activity of suppressor of cytokine signaling (SOCS)-3 (Bjorbaek *et al.,* 1998; Bjorbaek *et al.,*1999; Emilsson *et al.,* 1999), which inhibits leptin receptor signal transduction by inhibiting or suppressing activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Bjorbaek *et al.,* 1998; Bjorbaek *et al.,*1999). Moreover, a decrease in the abundance of leptin receptors in the hypothalamus of obese rats has been associated with the development of leptin resistance (Scarpace *et al.,* 2001). If similar mechanisms are operative in cattle and other ruminants, it would suggest that leptin plays a permissive role in the control of gonadotropin secretion, but is more active during periods of starvation when leptin concentrations fall bellow a threshold. The mass of hormone used to evaluate exogenous effects of leptin must also be considered. In ovariectomized, estradiol-implanted cows fasted for 60 h, leptin stimulates the release of LH at low (0.2 $\mu g\ kg^{-1}$) and moderate (2 $\mu g\ kg^{-1}$), but not at high (20 $\mu g\ kg^{-1}$) doses (Zieba *et al.,* 2003). Moreover, the length of exposure to leptin may also influence responses. Mean concentrations of LH increased after a 3-day treatment with recombinant human leptin in chronically undernourished ewes (Henry *et al.,* 2001).

This effect was not observed in ewes treated with increasing doses of recombinant ovine leptin for 8 days (Morrison *et al.,* 2001). Therefore, it is possible that resistance to leptin develops in ruminants exposed to high concentrations of leptin for an extended period. These effects are evident in rats treated with high and moderate doses of leptin for 28 days (Martin *et al.,* 2000). In that study, rats developed resistance to the anorectic effects of leptin and leptin receptor mRNA and protein were down-regulated by prolonged leptin treatment.

Several studies have provided evidence that leptin's effects on gonadotropin secretion are mediated at the level of the central nervous system. However, leptin has been demonstrated to stimulate both GnRH release from the hypothalamus (Yu *et al.,* 1997; Barb *et al.,* 1999; Woller *et al.,* 2001; Watanobe, 2002) as well as LH from AP explants (Yu *et al.,* 1997; De Biasi *et al.,* 2001) and AP cells (Barb *et al.,* 1999; Ogura *et al.,* 2001), suggesting that leptin can act at both sites to stimulate gonadotropin secretion. Moreover, the leptin receptor is present at hypothalamic and adenohypophyseal loci (Zamorano *et al.,* 1997; Dyer *et al.,* 1997; Iqbal *et al.,* 2000; Lin *et al.,* 2001). Recently, we have demonstrated a direct action of leptin on AP explants of female cattle (Amstalden *et al.,* 2003) where leptin stimulated a higher basal release of LH from AP explants obtained from fasted cows. However, no effects of leptin on the release of GnRH from hypothalamic-infundibular explants were evident (Amstalden *et al.,* 2003). Therefore, leptin can probably act at the central nervous system to prevent fasting-mediated reductions in the frequency of LH pulses in animal models in which the pulse generator is impaired by fasting (e.g., castrated rams; Nagatani *et al.,* 2000; and

peripubertal heifers; Maciel *et al.,* 2003a). In contrast, direct effects of leptin at the adenohypophysis are most evident in mature, fasted animals that respond to leptin but in which the pulse generator is not affected by fasting (Amstalden *et al.,* 2002, 2003).

In conclusion, results from the current experiments suggest that leptin does not affect the release of GnRH from hypothalamic-infunbidular explants obtained from full-fed bull and steers, nor gonadotropins from bovine adenohypophyseal cells maintained under optimal nutrient conditions. This is in contrast to the majority of observations in rodents and pigs. This supports the view that cattle are physiologically resistant to leptin when they are under neutral or positive energy balance. Mechanisms controlling this resistance remain to be determined.

CHAPTER V

LEPTIN ACTS AT THE BOVINE ADENOHYPOPHYSIS TO ENHANCE BASAL AND GONADOTROPIN-RELEASING HORMONE-MEDIATED RELEASE OF LUTEINIZING HORMONE: DIFFERENTIAL EFFECTS ARE DEPENDENT UPON NUTRITIONAL HISTORY*

Introduction

Leptin, a 16 kDa product of the *ob* gene, plays a major role in communicating nutritional status to the central nervous system, including centers that control reproduction (Cunningham *et al.,* 1999; Ahima *et al.,* 2000; Smith *et al.,* 2002; Williams *et al.,* 2002). The leptin receptor (LR) is present at both hypothalamic and adenohypophyseal (AP) loci (Dyer *et al.,* 1997a; Zamorano *et al.,* 1997; Iqbal *et al.,* 2000; Lin *et al.,* 2001), and in vitro studies using explants collected from normal-fed rodents indicate that leptin can act directly at both sites (Yu *et al.,* 1997; Woller *et al.,* 2001) to stimulate the release of GnRH and LH, respectively. However, in domestic

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* Reprint with permission from "Leptin acts at the bovine adenohypophysis to enhance basal and gonadotropin-releasing hormone-mediated release of luteinizing hormone: differential effects are dependent upon nutritional history" by Amstalden *et al.,* 2003. *Biology of Reproduction* 10.1095/biolreprod 103.018119. Copyright 2003 by the Society for the Study of Reproduction, Inc.

ruminants, leptin has been shown repeatedly to stimulate LH secretion only during periods of negative energy balance. In addition, leptin receptor mRNA increases in feed-restricted ewes (Dyer *et al.,* 1997a). In this context, restriction of nutrient intake for 2-3 d reduces concentrations of leptin concurrent with reductions in the frequency of LH pulses in castrated male sheep (Nagatani *et al.,* 2000), and peripubertal heifers (Amstalden *et al.,* 2000). Leptin treatment prevents these reductions, suggesting an effect at hypothalamic centers that regulate pulsatile GnRH secretion (Nagatani *et al.,* 2000; Maciel *et al.,* 2003a).

Importantly, leptin also stimulates an increase in LH secretion in animals in which short-term restrictions in nutrient intake does not measurably alter the pattern of LH release. For example, 2-3 d of fasting reduces expression of the leptin gene and markedly diminishes circulating concentrations of leptin, insulin, and insulin-like growth factor (IGF)-I in mature ruminants, without reducing mean concentrations of LH or the frequency of LH pulses (McCann and Hansel, 1986; Kadokawa and Yamada, 1999; Nagatani *et al.,* 2000; Amstalden *et al.,* 2002). Nonetheless, 2-3 d of total feed restriction clearly hypersensitizes the mature cow to leptin, resulting in acute increases in the mean baseline and the magnitude of individual pulses of LH (Amstalden *et al.,* 2002; Zieba *et al.,* 2003). This would suggest that the primary effects of leptin in this animal model are at the AP level. To test this hypothesis further, we examined in vitro the stimulatory effects of leptin on basal and GnRH-mediated secretion of LH from AP explants obtained from fasted and non-fasted cows. To assess coincident hypothalamic

responses, the effects of leptin on GnRH secretion from hypothalamic-infundibular (HYP) explants were also examined.

Materials and Methods

All animal-related procedures used in these studies were approved by the Institutional Agricultural Animal Care and Use Committee of The Texas A&M University System.

Animal model and procedures

Eleven mature, ovariectomized cows, each bearing an estradiol implant to maintain circulating estradiol at 2 - 5 pg ml^{-1}, were used. This animal model has been used extensively by our laboratory (Gazal *et al.,* 1998; Amstalden *et al.,* 2002; Zieba *et al.,* 2003) and others (Day *et al.,* 1986b) to study the effects of nutrition on neuroendocrine control of gonadotropin secretion in cattle. With this approach, implants provide a constant level of steroid (estradiol) negative feedback without the complications associated with ovarian cyclicity. Cows used in the current experiments had mean (±SEM) concentrations of estradiol of 4.35 ± 0.45 pg ml^{-1}.

Cows, in moderately thin body condition (BC = 4; scale of 1-9), were fed once daily at 0700 h a diet formulated to provide 100% of the National Research Council (NRC) (1996) requirements for maintenance for at least 2 weeks before the beginning of the experiment. Each cow was assigned randomly to one of two dietary groups: 1) Normal fed, in which cows were fed 100 % of the NRC requirements to maintain body weight (*n*

= 6); and 2) Fasted, in which cows were fasted for 72 h with free access to water (*n* = 5). On the day before the start of dietary treatments (Day –1), cows were fitted with jugular catheters (polyethylene tubing, 1.4 mm inside diameter, 1.9 mm outside diameter; Becton Dickinson, Parsippany, NJ) for intensive blood sampling. Cows were placed in stanchions and blood was collected at 10-min intervals for 6 h on Days 0 and 3 of the experiment via an extension connected to the jugular catheter. Blood samples were dispensed into tubes containing 150 µl of a solution containing heparin (1000 IU ml^{-1}) and 5% EDTA and placed immediately on ice. Plasma was harvested by centrifugation and stored at –20°C until hormone analysis. At the end of the intensive blood sampling on Day 3, cows were humanely euthanized by exsanguination following captive bolt stunning. Diencephalons were removed after disconnection of infundibuli from adenohypophyses. Adenohypophyses were removed from the sella turcica and kept on ice until tissue processing.

Effects of leptin on basal and GnRH-mediated release of LH from AP explants

Adenohypophyses collected from each cow were dissected and sliced sagittally into approximately 0.5 X 2-mm strips. Four AP strips from each adenohypophysis were selected randomly, placed into each of five perifusion chambers, and perifused with Krebs-Ringer bicarbonate buffer (KRB) for 6.5 h using a multiple micro chamber perifusion system (Endotronics Inc., Coon Rapids, MN). Thus, AP explants from each cow were perifused with each dose of leptin tested or control media. In preliminary studies, it was determined that basal release of LH by AP explants was attained within

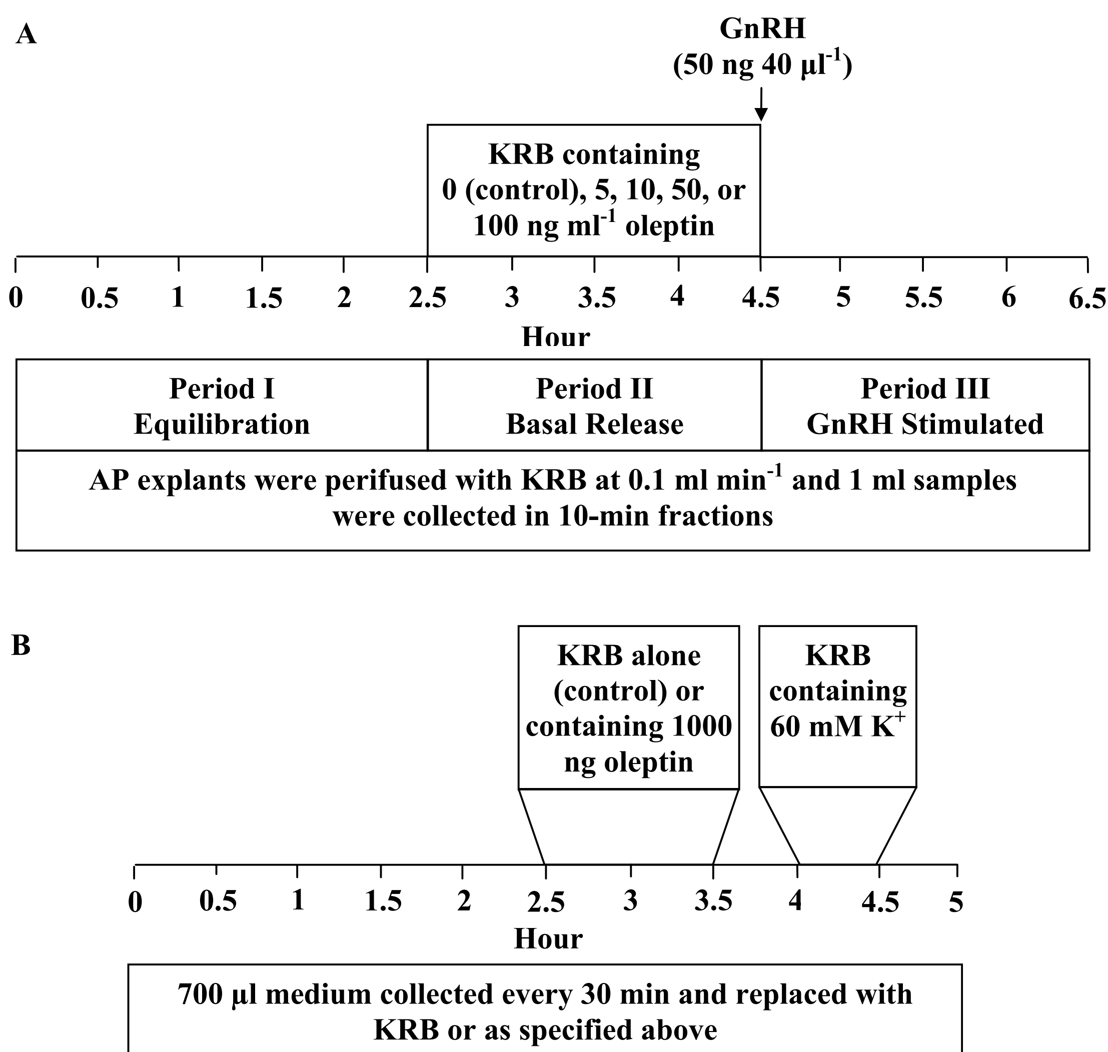

Fig. 12. Timeline for experimental procedures for AP (A) and HYP (B) explant incubations. Adenohypophyseal explants were perifused with KRB for 6.5 h. The first 2.5 h of perifusion served as an equilibration period (Period I). Beginning at 2.5 h, explants were perifused with KRB containing 0, 5, 10, 50, or 100 ng/ml oleptin for 2 h (Period II – Basal Release). At 4.5 h, explants were challenged with 50 ng of GnRH and perifused for an additional 2 h (Period III – GnRH Stimulated). Hypothalamic-infundibular explants were equilibrated in 0.7 ml KRB for 2.5 h. Beginning at 2.5 h, explants were incubated in KRB alone or containing 1000 ng oleptin for 1 h. Explants were treated at 4 h of culture with KRB containing 60 mM K^+ to induce release of GnRH as a test of viability.

the first 2.5 h of perifusion. At 2.5 h, AP explants were perifused for 2 h with KRB containing 0 (control), 5, 10, 50, or 100 ng ml^{-1} of oleptin (Gertler *et al.,* 1998). After 4.5 h of perifusion, AP explants were challenged with 50 ng of GnRH (Sigma, St. Louis, MO) via an injection port in the chamber (Fig. 12A). In addition to investigating the effects of leptin on GnRH-mediated release of LH, the stimulation of AP explants by GnRH also made it possible to verify the viability of tissues based on specific stimulation of gonadotropin release. Approximately 1-ml fractions of perifused media were collected at 10-min intervals and stored at –20° C until analysis for LH. In a separate study, we have reported simultaneous effects of leptin on basal and growth hormone (GH)-releasing hormone-mediated GH release from these same explants (Zieba *et al.,* 2003b)

Effects of leptin on GnRH secretion from the isolated medial basal hypothalamus-infundibular complex

To isolate the medial basal hypothalamus (MBH), the entire hypothalamic-infundibular complex was transected sagittally into equal halves. A cut was made 2 mm lateral to the median sagittal cut for each half, followed by an anterior coronal cut extending from the optic chiasm to the anterior commissure. A posterior coronal cut was made resulting in a section containing approximately half of the mamillary body. A final transverse cut containing two-thirds of the mamillary body was performed. Each half of the MBH connected to its infundibular half was incubated in 0.7 ml KRB for 5 h. Medium was collected and replaced every 30 min. After a 2.5 h period of equilibration,

HYP explants from each cow were incubated with KRB containing either 0 or 1000 ng of oleptin for 1 h, so that one-half served as a control for the corresponding half (Fig. 12B). At 4 h of incubation, all explants were incubated with KRB containing 60 mM K^+ to test tissue viability, based on nonspecific stimulation of GnRH release (Waters *et al.,* 1998).

Doses of recombinant oleptin used to treat HYP explants were chosen empirically based on quantities shown to have positive effects in comparable tissue collected from rodents (Yu *et al.,* 1997) and on previous experiments using tissues collected from bulls and steers at slaughter. In these preliminary experiments, GnRH released from HYP explants collected from full-fed cattle was not affected by leptin (1 to 1000 ng of recombinant oleptin for 30 to 60 min). This observation was not totally surprising, since the stimulatory effects of leptin on LH release observed in vivo (Amstalden *et al.,* 2002) were limited to fasted cows. Thus, we chose to use 1000 ng of oleptin in the current experiment.

Radioimmunoassays

Concentrations of GnRH in HYP culture media were determined by a validated assay as described previously (Waters *et al.,* 1998). Plasma concentrations of LH and concentrations of LH in AP perifused media were determined as reported previously (McVey and Williams, 1991). Intra- and interassay coefficients of variation for these assays averaged 6.2 and 10.7 %, respectively. Serum estradiol was assayed in extracted samples in a single assay as reported previously (Talavera *et al.,* 1985).

Statistical analysis

Circulating concentrations of LH were analyzed by general linear mixed models for repeated measures using the Mixed procedure (PROC MIXED) of the Statistical Analysis System (SAS 8.1; SAS Institute Inc., Cary, NC). The frequency and amplitude of LH pulses were determined using both visual inspection and a pulse-detection algorithm, Pulsefit 1.2 (Kushler *et al.,* 1991). Sources of variation were diet, day, and diet X day. Day was used as the repeated variable and cow(diet) was used as the subject.

To test the effects of diet, leptin, and GnRH treatments on the release of LH from AP explants in vitro, the 6.5-h period of perifusion was subdivided in three periods (I-III), corresponding to Equilibration (0 to 2.5 h), Basal Release (2.5 to 4.5 h), and GnRH Stimulation (4.5 to 6.5 h), respectively. Hormone data obtained from perifused media were analyzed by the general linear mixed model (PROC MIXED) procedure of SAS. Sources of variation were diet, leptin treatment, period, and all interactions. Period was used as the repeated variable and cow(diet) was used as subject. The Least Squares means procedure was used to compare means when a significant F value was obtained. Because of differences in the release of LH by AP explants among perifusion chambers at the end of the Equilibration period, covariate analyses were performed to test main effects during Period II (basal release). Mean concentrations of LH in the last 3 samples collected during equilibration were used as the covariate (Covariate 1). To test main effects during Period III (GnRH Stimulation), another covariate analysis was performed, using the mean of the last 3 samples of Period II (Basal) as covariate (Covariate 2). To test cumulative (Basal + GnRH) effects of leptin and GnRH-stimulation on the amount

of LH released by AP explants, an analysis was performed using Covariate 1 to test main effects during Period III.

To test the effects of leptin on GnRH release from HYP explants, data were analyzed by the general linear mixed model procedure of SAS (PROC MIXED). Sources of variation were diet, leptin treatment, period, and all possible interactions. Period was used as the repeated variable and cow(diet) was used as subject.

Results

Does leptin modulate basal or GnRH-mediated LH release from the adenohypophysis?

As expected, mean plasma concentrations and mean frequency and amplitude of LH pulses did not differ ($P > 0.1$) between Fasted and Normal-fed groups before euthanasia (Table 1). Using covariate analysis to adjust for differences in basal release of LH among perifusion chambers, it was observed that dietary treatment interacted with in vitro leptin treatments to influence the release of LH from AP explants. Leptin-treated AP explants from fasted cows had a higher ($P < 0.02$) basal release of LH than control-treated explants. Because effects of individual doses of leptin on the basal release of LH from AP explants of fasted cows did not differ, all doses of leptin were combined for presentation (Fig. 13). In explants of normal-fed cows, leptin did not affect ($P > 0.1$) basal release of LH (Fig. 13).

In contrast to observations made for basal release, all doses of leptin increased ($P < 0.001$) GnRH-mediated release of LH from AP explants of normal-fed cows, but not of fasted cows (Fig. 14). In fact, in the Fasted group, AP explants treated with 5 ng ml^{-1}

Table 1. Mean concentrations of LH, and mean frequency and amplitude of LH pulses in normal-fed and fasted cows

	Normal Fed		Fasted	
Parameter	Day 0	Day 3	Day 0	Day 3
Concentration (ng/ml)	5.2± 0.1	5.9 ± 0.1	4.2 ± 0.1	5.3 ± 0.2
Frequency (pulses/6 h)	8.3 ± 1.2	10 ± 1.0	5.0 ± 1.4	7.0 ± 1.6
Amplitude (ng/ml)	1.6 ± 0.6	2.0 ± 0.5	1.7 ± 0.8	1.3 ± 0.2

No dietary effect was observed. Values are presented as mean ± SEM.

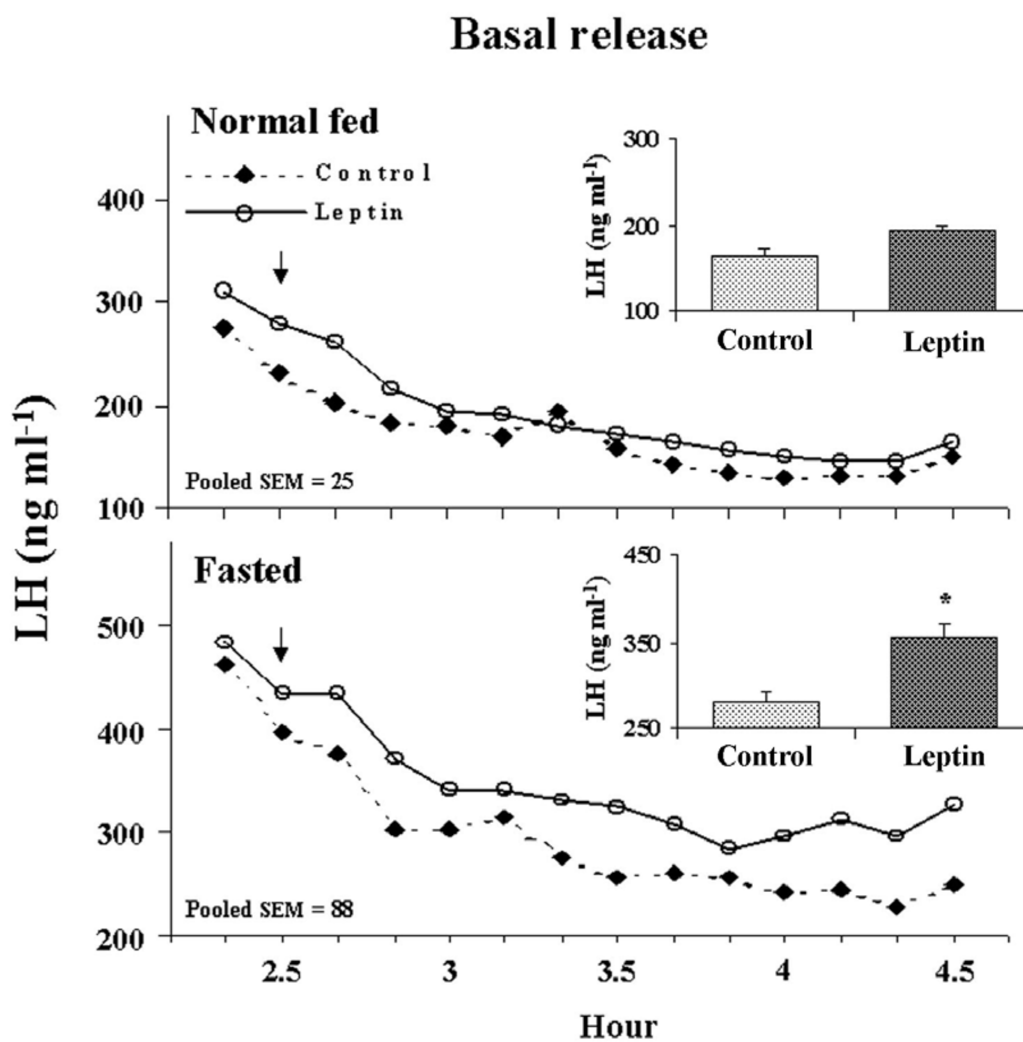

Fig. 13. Mean concentrations of LH in media collected from AP explants obtained from normal-fed (top) and fasted (bottom) cows and perifused (2.5 to 4.5 h) with KRB alone (control) or KRB containing 5, 10, 50, and 100 ng ml^{-1} oleptin (combined). No effect (*P* > 0.1) of leptin was observed on the release of LH (Basal Release) from AP explants from normal-fed cows (top inset). However, basal release of LH was elevated (* $P <$ 0.02) in leptin-treated AP explants from fasted cows (bottom inset). Arrows indicate the beginning of control (KRB alone) or leptin treatment.

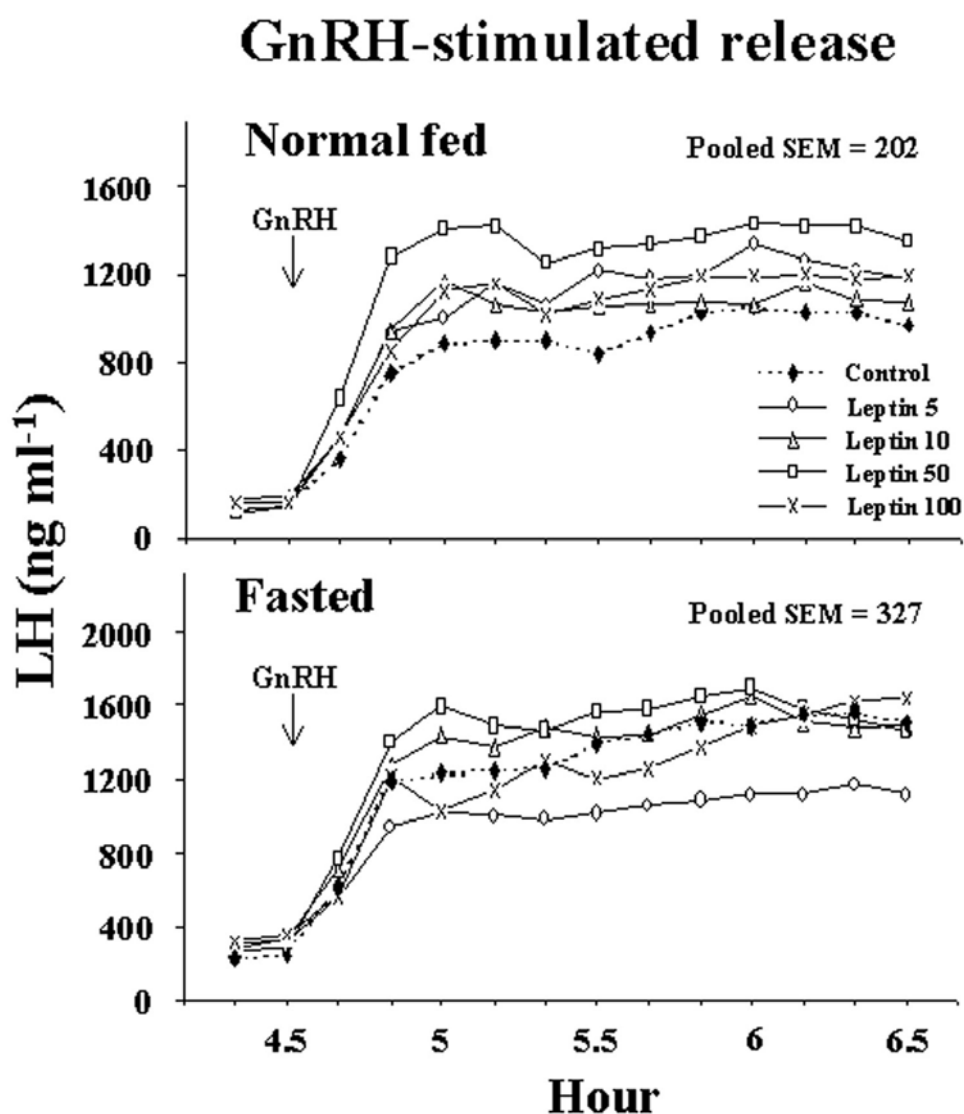

Fig. 14. Mean concentrations of LH in media after GnRH stimulation of AP explants of normal-fed and fasted cows perifused in the presence of either KRB alone (control) or KRB containing 5, 10, 50, and 100 ng ml^{-1} oleptin. All doses of leptin increased ($P < 0.001$) GnRH-stimulated release of LH in AP explants collected from normal-fed (top), but not fasted (bottom) cows. Adenohypophyseal explants from fasted cows perifused with 5 ng ml^{-1} oleptin released less ($P < 0.001$) LH in response to GnRH than control-treated explants. Note the elevated baseline for leptin-treated compared to control-treated AP explants from fasted cows at the time of GnRH stimulation (4.5 h).

of oleptin released less ($P < 0.001$; 1059 ± 55.4 ng ml^{-1}) LH in response to GnRH than control-treated (1133 ± 62.6 ng ml^{-1}) explants, and all other doses had no effect on this variable. Because leptin increased basal release of LH from AP explants obtained from fasted cows before GnRH treatment, followed by non-significant numerical increases after GnRH, we examined the combined effects of leptin on basal and GnRH-stimulated release of LH. Leptin at doses of 50 (1416 ± 87.6 ng ml^{-1}) and 100 ng ml^{-1} (1455 ± 89.1 ng ml^{-1}) increased ($P < 0.001$) the overall (basal plus GnRH-stimulated) mean concentrations of LH compared to control-treated explants.

Effects of leptin on the secretion of GnRH from the MBH-infundibular complex

Data from one normal-fed cow were not included in the analysis due to a lack of response to 60 mM K^+, suggesting a loss of tissue viability. Neither fasting nor leptin affected ($P > 0.1$) basal or potassium-stimulated release of GnRH from HYP explants (Fig. 15).

Discussion

In the current study, we examined the hypothesis that the hypersecretion of LH induced by leptin in vivo in fasted, mature cows (Amstalden *et al.,* 2002) could be explained by effects at the AP level. Significant effects of leptin on basal release of LH were observed in AP explants from fasted cows, on AP responsiveness to GnRH in normal-fed cows, and on overall release (basal plus GnRH-stimulated release of LH) in AP explants of fasted cows perifused with the highest doses of leptin (50 and 100 ng ml^{-1}).

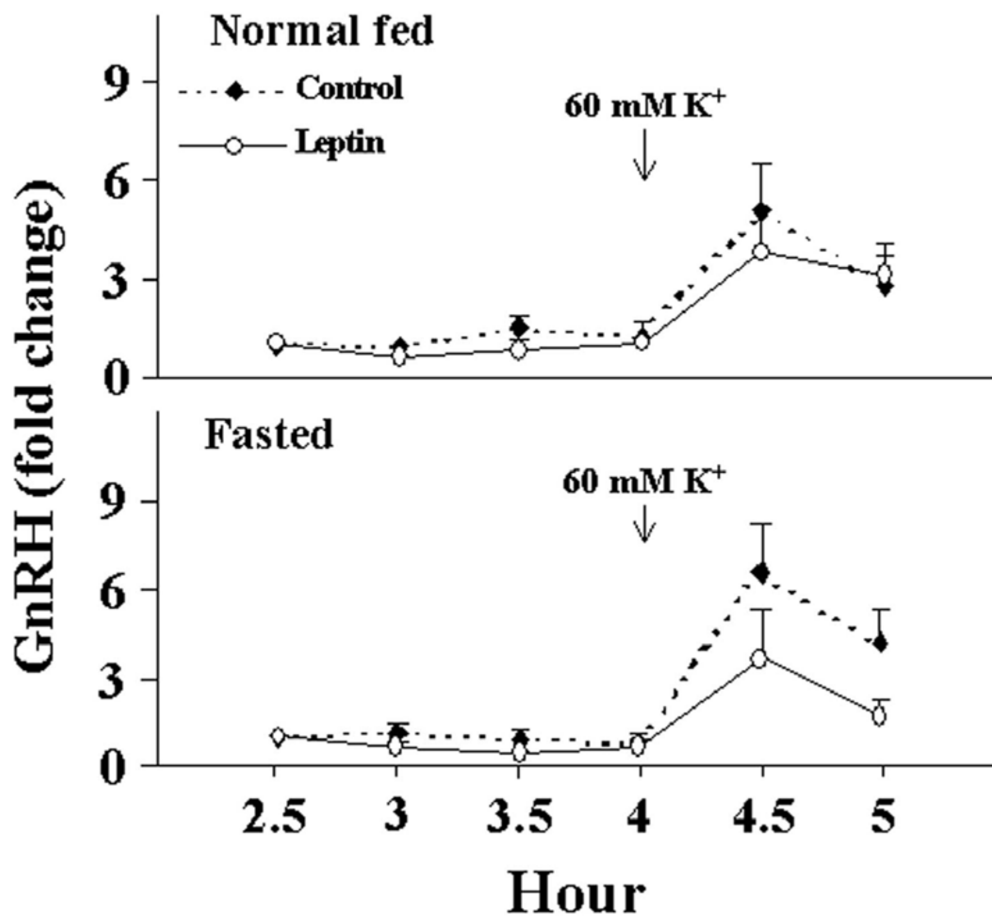

Fig. 15. Fold change in mean concentrations of GnRH in media collected from HYP explants of normal-fed and fasted cows. Hypothalamic-infundibular explants were incubated in 0.7 ml of KRB either alone or containing 1000 ng oleptin at 2.5 and 3 h. Explants were incubated with KRB containing 60 mM K^+ at 4 h of incubation to test viability of tissue. No effects of diet or leptin were observed ($P > 0.1$).

In contrast, we were unable to detect effects of leptin on the release of GnRH from isolated HYP explants obtained from either normal-fed or fasted cows.

As noted previously, 2-3 d of restriction of feed intake does not affect mean concentrations of LH or its secretory patterns in mature ewes or cows (McCann and Hansel, 1986; Kadokawa and Yamada, 1999; Nagatani *et al.,* 2000; Amsatlden *et al.,* 2002), although signs of metabolic stress, including decreased circulating insulin, IGF-I, and leptin, are clearly present. Similar metabolic constraints are observed in fasted heifers (Amstalden *et al.,* 2000; Maciel *et al.,* 2003a) and estradiol-implanted wethers (Nagatani *et al.,* 2000), but in these ruminant animal models, short-term fasting is able to reduce the frequency of LH pulses similar to that observed for monogastric species (Cagampang *et al.,* 1990; Cameron and Nosbish, 1991; Samuels and Kramer, 1996). Nonetheless, feed-restricted, mature cows are responsive to leptin treatment and mean concentrations, mean baseline, and the magnitude of pulses of LH are increased (Amstalden *et al.,* 2002). Questions then arise as to whether stimulation of LH release by leptin is accounted for by effects at hypothalamic and/or AP levels. Most studies have proposed that leptin stimulates secretion of LH via interaction with neuronal systems and ultimately, stimulation of GnRH secretory neurons. It is likely that, in models in which the frequency of LH pulses is diminished by feed restriction (Nagatani *et al.,* 2000; Maciel *et al.,* 2003a), leptin stimulates GnRH release to prevent the effects of fasting. Alternatively, diminished release of LH during fasting could be explained by changes in the amplitude of GnRH pulses and its ability to trigger LH release, as observed previously in this laboratory (Gazal *et al.,* 1998) and others (I'Anson *et al.,*

2000) in other physiological contexts. However, under feed-restricted conditions, in which frequency of LH pulses is not affected, as observed in mature female ruminants (Amstalden *et al.,* 2002), the stimulatory effects of leptin on LH secretion are more plausibly explained by a direct action at the adenohypophysis. In addition to the current results, evidence supporting a direct action of leptin at the level of the adenohypophysis includes the localization of leptin receptors on gonadotropes of ewes (Iqbal *et al.,* 2000) and leptin stimulation of LH release from rat anterior pituitaries in vitro (Yu *et al.,* 1997). However, in contrast to the study performed using rat tissue (Yu *et al.,* 1997), we observed no effect of leptin on basal release of LH from AP explants from normal-fed cows, suggesting important species differences in the acute interaction of nutritional status and leptin with gonadotropes in cattle. This view is supported further by our observation that LH release from primary cultures of bovine adenohypophyseal cells, maintained under optimal nutrient conditions, was not affected by leptin at doses of 1 to 1000 ng ml^{-1}.

Increases in basal release of LH from AP explants from fasted cows treated with leptin appear differently than what would be expected from a classical secretagogue. In fact, after the period of equilibration (initial 2.5 h of perifusion) and before GnRH stimulation (last 2 h of perifusion), basal release of LH tended to continue to decrease in all treatments, independent of dietary group. This steady decline in the release of LH during the Basal-Release period may reflect the absence of stimulation by endogenous GnRH. However, the decrease in LH release by AP explants from fasted cows treated with leptin was significantly smaller than control-treated explants. This effect was not

observed in explants from normal-fed cows. It is possible that leptin may have stimulated an increase in basal metabolism of gonadotropes from fasted cows, resulting in an elevated release of LH compared to gonadotropes of control-treated explants. In support of this concept, increased glucose uptake has been observed in muscle and adipose tissue treated with leptin (Kamohara *et al.,* 1997).

Mechanisms involved in the leptin-mediated increase in responsiveness to GnRH of AP explants from normal-fed cows are unknown. However, there are several potential pathways through which these effects could occur, including effects on Ca^{2+} ion channels, an increase in the releasable pool of LH, and/or GnRH receptor desensitization. All of these are important for cellular exocytosis of gonadotropins (McArdle *et al.,* 2002). Leptin receptor signaling involves activation of Janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathways (Bjorbaek *et al.,* 1997). In addition, mitogen-activated protein kinase (Bjorbaek *et al.,* 1997) and phosphatidylinositol-3 kinase (Niswender *et al.,* 2001) have also been involved in leptin receptor signaling. In porcine chromaffin cells, leptin caused a sustained increase of intracellular Ca^{2+} and activated inositol 1,4,5-triphosphate production (Takekoshi *et al.,* 2001), which are intracellular factors known to be associated with GnRH-receptor signaling and release of LH (Zorec, 1996).

The lack of increased responsiveness to GnRH in AP explants from fasted cows treated with leptin is unclear. However, it is well established that GnRH stimulation of gonadotropes is necessary for synthesis of gonadotropins (Haisenleder *et al.,* 1994). Thus, it is possible that the increased basal release of LH observed in leptin-treated

explants of fasted cows and lack of any GnRH stimulation during this period caused a diminution of readily releasable pools of LH that were not replenished.

Experimental models describing the temporal secretion pattern of GnRH by the hypothalamus have been developed in several large species. In sheep, this has been accomplished through collection of hypophyseal-portal blood (Skinner *et al.,* 1997). In the mature cow, the anatomy of the cranium impairs practical application of this technique. However, collection of cerebrospinal fluid from the third ventricle has been shown by our laboratory and others to be a useful approach for determining patterns of GnRH secretion in cattle (Gazal *et al.,* 1998) and sheep (Skinner *et al.,* 1997), and studies are currently underway to investigate the effects of leptin using this approach. Incubation of HYP explants has also been used extensively to study the neuroendocrine regulation of GnRH release in several species (Convey *et al.,* 1981; Barb *et al.,* 1994; Fowler *et al.,* 1994). In vitro incubations of isolated HYP tissue allows the investigation of factors acting directly at the hypothalamus. Hypothalamic explants used in the current experiment included the MBH connected to the infundibulum. The arcuate nucleus, which rests within the MBH, contains leptin receptors (Dyer *et al.,* 1997) as well as neuronal cell bodies that express mediators of the effects of leptin within the central nervous system, such as neuropeptide Y, agouti-related peptide, and POMC (precursor of α-melanocyte-stimulating hormone) (Ahima *et al.,* 2000). Thus, explants used in the current experiment contain at least part of the neuronal systems that potentially mediate the effects of leptin on GnRH neurons. Using this approach, we were unable to detect effects of leptin on GnRH secretion from hypothalamus harvested

from either normal-fed or fasted cows. In contrast, Yu et al. (1997) have reported that leptin stimulates GnRH release from rat hypothalamic explants. It is not clear whether the discordant results are due to species differences or experimental methodology used to detect leptin effects.

Mechanisms that explain the inability of leptin to independently stimulate an increase in circulating concentrations of LH in cattle and sheep that are fed either maintenance (Henry *et al.,* 1999; Nagatani *et al.,* 2000; Morrison *et al.,* 2001) or growing (Morrison *et al.,* 2002) diets are not understood. However, the inability of leptin to stimulate directly basal secretion of LH in AP explants of normal-fed cows in the current study supports those findings. Syndromes involving the development of resistance to leptin have been observed in obese humans (Caro *et al.,* 1996; Considine *et al.,* 1996) and rodents (Halaas *et al.,* 1997; Widdowson *et al.,* 1997), and there is evidence that excessive activity of suppressor of cytokine signaling (SOCS)-3 activity is involved in leptin resistance (Bjorbaek *et al.,* 1999; Emilsson *et al.,* 1999). Inhibition of leptin signaling by SOCS-3 seems to involve suppression of JAK2 activation (Bjorbaek *et al.,* 1999). Whether a leptin-resistant condition occurs in ruminants that are under neutral or positive energy balance has not been determined; however, leptin is able to decrease feed intake in sheep (Henry *et al.,* 1999; Morrison *et al.,* 2001). Moreover, the mass of ligand reaching target tissues must also be considered. We have reported recently that intravenously-injected recombinant oleptin causes an inverse, dose-related increase in basal plasma concentrations of LH in ovariectomized, estradiol-implanted cows fasted for 60 h (Zieba *et al.,* 2003). A dose of 0.2 μg kg^{-1} maximized the increase

in LH, whereas doses of 2 and 20 μg kg^{-1} caused lower and no responses, respectively. Reports in rodents (Yu *et al.,* 1997) confirm that AP explants treated with high concentrations of leptin (10^{-5} M) lose their ability to respond with an increase in LH release. Therefore, the duration and/or amount of exposure to the hormone likely determines the development of resistance.

In conclusion, results from the current experiment support the hypothesis that leptin affects the secretion of LH in the mature cow, in part, by its direct action at the adenohypophysis. Leptin maintained higher basal AP release of LH in tissues obtained only from fasted cows, whereas increased responsiveness to GnRH was evident in AP explants obtained from normal-fed cows. This indicates that, while leptin acts on gonadotrope function under both normal and fasting conditions, the manifestations of these effects are expressed differentially and will require further study at the cellular level to characterize in detail.

CHAPTER VI

DETERMINATION OF THE LEPTIN RECEPTOR, SUPPRESSOR OF CYTOKINE SIGNALING-3, AND PHOSPHORYLATED SIGNAL TRANSDUCER AND ACTIVATORS OF TRANSCRIPTION-3 IN THE ADENOHYPOPHYSIS OF NORMAL-FED AND FASTED COWS

Introduction

Leptin, a metabolic hormone synthesized and secreted mainly by adipocytes, has been shown to communicate nutritional status to the central nervous system, including centers that control reproduction (Cunningham *et al.,* 1999; Ahima *et al.,* 2000). *In vitro* studies with rat (Yu *et al.,* 1997, Woller *et al.,* 2001) and pig (Barb *et al.,* 1999) tissues have shown that leptin stimulates the release of GnRH from the hypothalamus and LH from the adenohypophysis. *In vivo* studies in the rat (Nagatani *et al.,* 1998), castrated ram (Nagatani *et al.,* 2000), and peripubertal heifer (Maciel *et al.,* 2003a) have demonstrated the ability of leptin to prevent fasting-induced reductions in the frequency of pulses of LH, suggesting effects at the level of hypothalamic neurons. However, in the mature cow, 2 to 3days of fasting does not affect the frequency of pulses of LH, yet leptin increases mean concentrations of LH (Amstalden *et al.,* 2002, Zieba *et al.,* 2003) without effects on the frequency or amplitude of LH pulses. This suggests that the stimulatory effects of leptin on the release of LH in the mature, fasted cows occur at the adenohypophyseal (AP) level. More recent observations in our laboratory support this

concept, and demonstrate that leptin can act directly at the adenohypophysis to increase basal and GnRH-stimulated release of LH from AP explants of fasted cows (Amstalden *et al.,* 2003).

That leptin stimulates the hypothalamic-adenohyphyseal axis of nutritionally restricted sheep (Nagatani *et al.,*2000; Henry *et al.,* 2001) and cattle (Amstalden *et al.,* 2002; Zieba *et al.*. 2003), but not those that are in neutral or positive energy balance (Henry *et al.,* 1999; Morrisson *et al.,* 2001; Amstalden *et al.,* 2002; Morrison *et al.,* 2002), suggests that physiological resistance to leptin may occur in well-nourished animals. Resistance to leptin is observed in humans (Caro *et al.,* 1996) and rodents (Halaas *et al.,* 1997) and has been speculated to involve decreased numbers of leptin receptor (LR) and a diminished ability to stimulate downstream cellular signals, such as activation of signal transducers and activators of transcription (STAT) pathway (Scarpace *et al.,* 2001). Moreover, excessive activity of suppressor of cytokine signaling (SOCS)-3 has been implicated in the development of leptin resistance syndromes (Bjorbaek *et al.,* 1998, 1999; Emilsson *et al.,* 1999). The LR is present in the hypothalamus and adenohypophysis, and the expression of LR is increased in the hypothalamus of nutritionally-restricted female (Dyer *et al.,* 1997a) and fasted male (Adam *et al.,* 2002) sheep. In the current studies, we investigated whether alterations in the expression of LR and SOCS-3 in the adenohypophysis could account for the increased responsiveness of the hypothalamic-adenohypophyseal axis to leptin observed in fasted cows. In addition, we investigated the ability of leptin to stimulate phosphorylation of STAT-3 in AP explants of fasted and control-fed cows.

Materials and Methods

All animal-related procedures used in the present study were approved by the Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System.

Animal model and procedures

Ten mature, ovariectomized cows, each bearing a subcutaneous estradiol implant to maintain circulating estradiol at 2 to 5 pg ml^{-1}, were used. Cows in moderately thin body condition (BC = 4; scale of 1 to 9) were fed once daily at 0700 h a diet formulated to provide 100% of the National Research Council (NRC) (1996) requirements for maintenance for at least 2 weeks before the beginning of the experiment. Each cow was assigned randomly to one of two dietary groups: 1) Normal-fed, in which cows were fed 100% of the NRC requirements to maintain body weight (n = 5); and 2) Fasted, in which cows were fasted for 72 h with free access to water (n = 5). At the end of the 72-h period, cows were euthanized by exsanguination following captive bolt stunning. Crania were cut caudally in an oblique plane beginning at the dorsal aspect of the orbit of the eyes and continuing to the dorsal aspect of the occipital condyles. After removal of diencephalons, each adenohypophysis was removed from the sella turcica and dissected from the neurohypophysis. The adenohypophysis was cut coronaly, resulting in a section approximately 3-mm thick that was fixed in 4% paraformaldehyde. Another coronal section of similar size was cut, embedded in medium for frozen tissue (OCT, Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA), and snap frozen in liquid

nitrogen. Approximately 0.2 g of AP tissue was snap frozen in liquid nitrogen for isolation of RNA. The remaining AP tissue was dissected sagittally and sliced into approximately 0.5 X 2-mm strips. Four strips from each adenohypophysis were selected randomly, placed into each of four perifusion chambers, and perifused with Krebs-Ringer bicarbonate buffer (KRB) for 2.5 h using a multiple microchamber system (Endotronics, Inc., Coon Rapids, MN). At 2.5 h, AP explants were perifused for 2 h with KRB containing 0 (control) or 10 ng ml^{-1} of recombinant ovine leptin (Gertler *et al.* 1998). At the end of the 2-h perifusion, explants were removed from each chamber and snap frozen in liquid nitrogen.

Real-time polymerase chain reaction

Total cellular RNA was isolated from approximately 0.1 g of AP tissue from normal-fed and fasted cows using TRIzol® reagent (Gibco-BRL, Gaithersburg, MD) as described previously (Spencer *et al.,* 1999). Subsequently, RNA was treated with RNase-free DNase (Promega Co., Madison, WI) to remove contaminating DNA. The quantity of RNA was determined spectrophotometrically and integrity of RNA was examined by gel electrophoresis in a denaturing 1% agarose gel and transillumination of ethidium bromide-stained RNA. Total RNA (5 μg) was reverse transcribed to cDNA using SuperScript II reverse transcriptase (Gibco BRL, Rockville, MD) and oligo dT_{14} and random primers. Complementary DNA was precipitated with acid ethanol, diluted in sterile water, and stored at -20° C.

Real-time polymerase chain reaction (PCR) was performed in 20-µl reactions containing 10 µl of 2 X SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1 µl cDNA template diluted 1:10 using the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems), according to manufacturer's instructions. Reactions were performed in triplicate for each cDNA sample. Primers were designed using the Primer Express® software (Applied Biosystems). Primer sequences and concentrations used in the reactions are summarized on Table 2. After initial incubation at 95° C for 10 min to activate AmpliTaq Gold® DNA polymerase (Applied Biosystems), samples were amplified for 40 cycles at 95° C for 15 sec, followed by 60° C for 1 min. Immediately after the real-time PCR run, samples were incubated in one cycle of 50° C for 2 min, 95° C for 10 min and 15 sec, 60° C 15 sec, a final increase in temperature up to 95° C to estimate melting temperature of PCR products and confirm the absence of nonspecific amplification. In addition, no-template control was used to verify the absence of primer-dimer formation and amplification. Isolated RNA samples used to generate cDNAs were diluted at 1:40 and used to confirm absence of genomic DNA carryover. Threshold cycle (CT) for each reaction was determined using the ABI Prism® SDS 2.1 analysis software (Applied Biosystems).

Table 2. Sequence of primers used for real-time PCR amplification of LR, SOCS-3, and cyclophilin

Gene		Oligonucleotide sequence (5' – 3')	Genbank accession no.
Leptin receptor	(75 bp product)		U62385
	Forward primer	CAATGCAGCAGTGCTCAATTC	
	Reverse primer	GGGCTGTCTCCTGCTCTCAT	
SOCS-3	(68 bp product)		NM_174466
	Forward primer	CCAGCCTGCGCCTCAA	
	Reverse primer	CTTGCGCACTGCGTTCAC	
Cyclophilin	(64 bp product)		D14074
	Forward primer	CCAACGGCTCCCAGTTCTT	
	Reverse primer	ACTACGTGCTTCCCATCCAAA	

In situ *hybridization*

Detection of LR mRNA in AP tissue was performed by an *in situ* hybridization procedure as described previously (Spencer *et al.* 1999). Briefly, 7-µm AP tissue sections were deparaffinized in xylene and rehydrated with phosphate buffered saline (PBS) through a graded series of ethanol solutions and post-fixed in 4% paraformaldehyde in PBS. Tissue sections were digested with proteinase K (20 µg ml^{-1} in 50 mM Tris, 5 mM EDTA) for 7.5 min at 37° C, refixed in 4% paraformoldehyde for 5 min, rinsed in PBS, dehydrated through a graded series of ethanol solutions and air dried. Sections were hybridized overnight with sense or antisense radiolabeled cRNA probes for LR at 55° C in a humidified chamber. Radiolabeled cRNA probes were synthesized from a linearized ovine LR plasmid (Dyer *et al.*, 1997a), graciously provided by Dr. Duane Keisler (University of Missouri, Columbia, MO), using *in vitro* transcription with [^{35}S]UTP. Before applying to each slide, probes (5 X 10^6 cpm in 75 µl hybridization buffer) were denatured at 70° C for 10 min. After hybridization and a series of washes, sections were treated with ribonuclease to remove non-specific bound probe followed by another series of washes and dehydration. Liquid film emulsion autoradiography was performed using Kodak NTB-2 liquid photograph emulsion. Slides were stored at 4° C for 7 weeks, developed in Kodak D-19 developer, and counterstained in Harris' modified hematoxylin. Bright and dark field microscopy (Zeiss Photo microscopy III; Carl Zeiss Inc., Thornwood, NY) was used to evaluate slides.

Western blot analysis

Total protein was isolated from approximately 0.1 g of AP explants from normal-fed and fasted cows perifused in the presence or absence of leptin. Tissue was thawed and immediately homogenized in 1 ml of lysis buffer (60 mM Tris, 1 mM Na_3VO_4, 10% glycerol, 2 % SDS, containing 44 μg ml^{-1} aprotinin and 100 μg ml^{-1} PMSF) using a PRO250 (PRO Scientific Inc., Monroe, CT) homogenizer and incubated on ice for 5 min. Homogenates were ground using a Dounce homogenizer (Kontes Co., Vineland, NJ) and centrifuged at 16,000 X g for 15 min at 4° C. Supernatant was collected and concentration of protein in extracts was determined using a Bradford protein assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin (BSA) as standard.

Twenty μg of protein isolated from perifused explants were denatured and separated in 10% SDS-PAGE gels and transferred onto nitrocellulose membranes as described previously (Johnson et al. 1999). Blots were blocked in Tris-buffered saline and 0.1% Tween 20 (TBST) containing 5% BSA and incubated with polyclonal rabbit anti mouse phospho-Tyr705 STAT-3 antiserum (1:1000; Cell Signaling Technology, Beverly, MA) in TBST containing 5% BSA overnight at 4° C. Blots were washed in TBST and incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, Bethesda, MD) in 2% non-fat dried milk-TBST for 1 h at room temperature while rocking. Immunoreactive proteins were detected using enhanced chemiluminescence (SuperSignal® West Pico, Pierce Chemical Co., Rockford, IL) according to the manufacturer's recommendations and X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

After exposure to film, blots were blocked with TBST containing 5% non-fat dried milk for 6 h at room temperature and incubated with monoclonal mouse IgG anti STAT-3 (1:3000; BD Biosciences, San Jose, CA) in TBST containing 2% milk overnight at 4° C. Blots were then washed and incubated with goat anti-mouse IgG-horseradish peroxidase conjugated (Kirkegaard and Perry Laboratories, Bethesda, MD) for 1 h at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence (SuperSignal) and X-OMAT AR film (Kodak).

Statistical analysis

Real-time PCR. Data for LR and SOCS-3 gene expression in adenohypophysis were analyzed using the Q-Gene software application (Muller *et al.,* 2002). Initially, the efficiency of PCR amplification for targets (LR and SOCS-3) and reference (Cyclophilin) genes were estimated based on amplification of serial dilutions of a reference cDNA sample. Threshold cycle values for various (1:5, 1:10, 1:20, 1:50, and 1:100) dilutions of the reference cDNA were regressed against the logarithmic transformation of proportions of cDNA in each reaction. The slope of the regressed equation was used to estimate the efficiency of amplification for target and reference genes for each PCR run, such as efficiency is equal to $10^{(-1/slope)}$. Mean normalized gene expression for LR and SOCS-3 for each adenohypophyseal sample was calculated as described previously by Muller *et al.,* 2002. Data were then analyzed by the *t*-test procedure of SAS (SAS 8.1; SAS Institute, Inc., Cary, NC) to compare Normal-fed and Fasted groups.

Results

Expression of LR gene in adenohypophysis of normal-fed and fasted cows

Amplification curves for LR and cyclophilin to determine expression of LR are shown (Fig. 16). Dissociation curves for LR and cyclophilin confirmed the absence of nonspecific amplification (Fig. 17). Regression of CT values determined for amplification of a reference cDNA against the amount of cDNA in the reaction indicated a linear relationship ($P < 0.0001$) for both LR ($R^2 = 0.9792$) and cyclophilin ($R^2 = 0.996$) (Fig. 18). Estimated efficiency of amplification for LR and cyclophilin was 2.46 and 2.43, respectively. Comparison of normalized expression between Normal-fed and Fasted groups indicated that dietary treatment did not ($P > 0.1$) affect the expression of LR in adenohypophysis (Fig. 19). Although positive amplification signals in no-template and RNA controls were detected (Fig. 20), dissociation curves suggested that they were most likely not the result of specific primer amplifications (lower melting temperature than expected for specific products; compare to Fig. 17).

In situ hybridization analysis demonstrated that LR mRNA is present in the adenohypophysis in very low amounts, with no apparent pattern of distribution or distinction between normal-fed and fasted cows (data not shown). Although minimal or no signal was detected on adenohypophyseal sections incubated with sense cRNA probe for LR, the time required for exposure of slides in film emulsion increased the background signal in both sense and antisense slides. This impaired our ability to perform a more detailed evaluation of LR expression in the adenohypophysis.

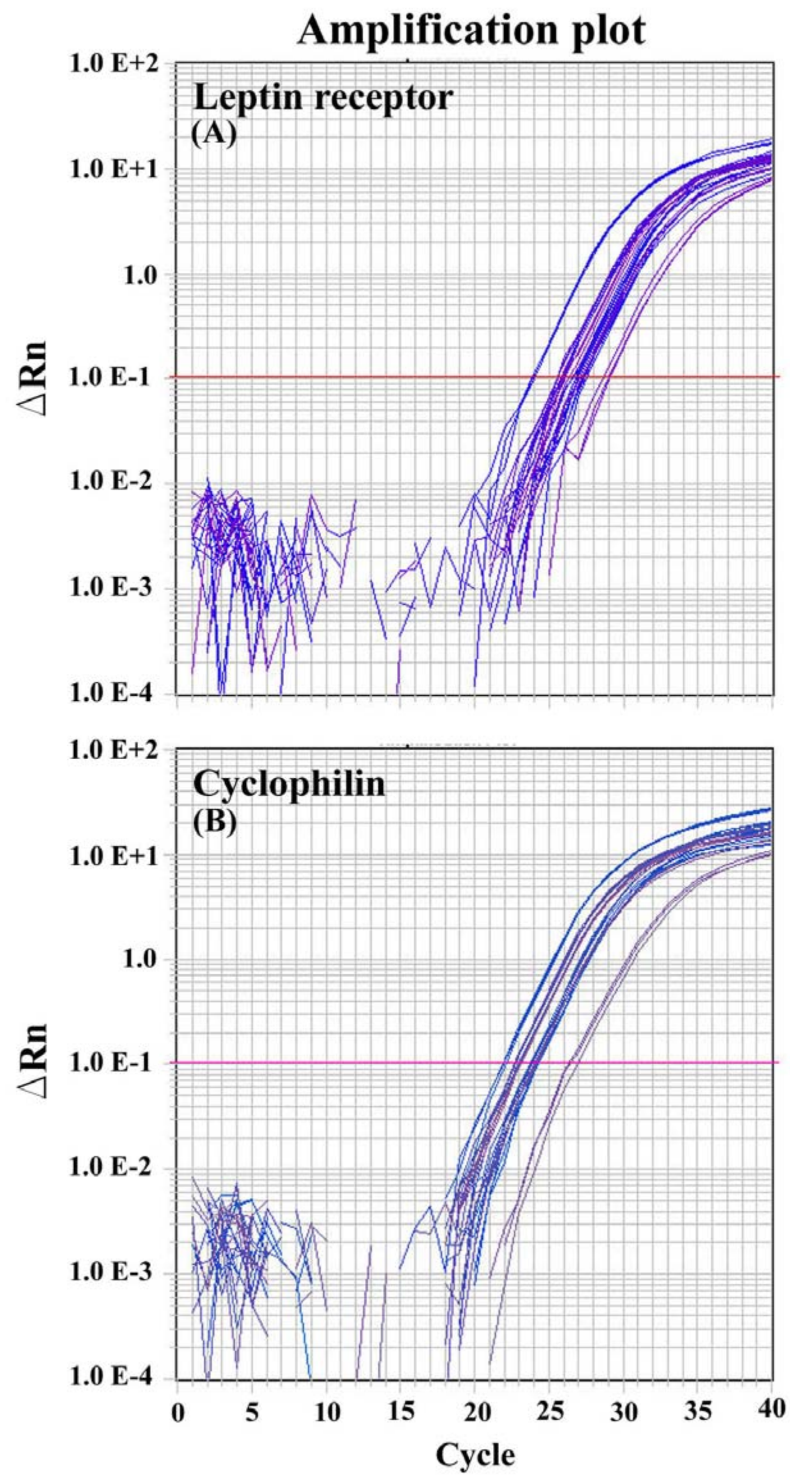

Fig. 16. Amplification plots of real-time PCR for LR (A) and cyclophilin (B).

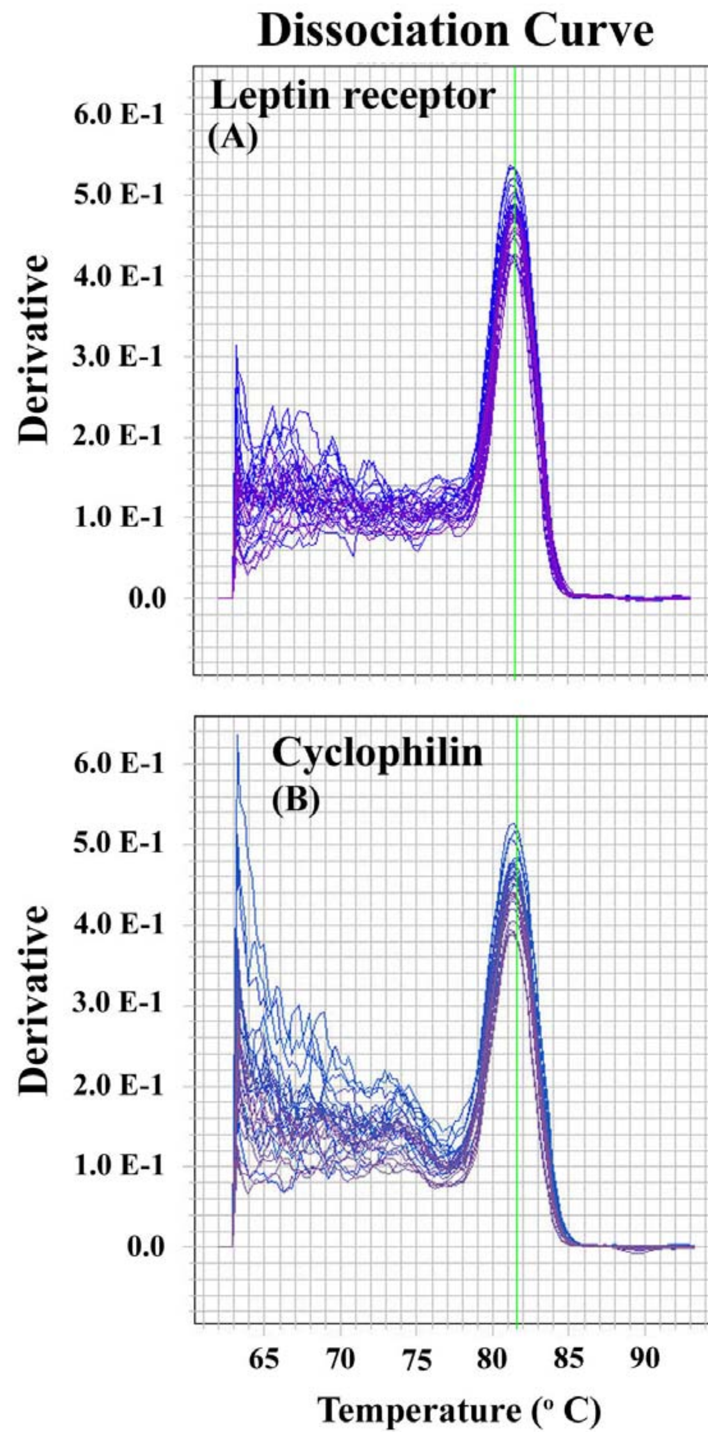

Fig. 17. Dissociation curves of real-time PCR products amplified using primers for LR (A) and cyclophilin (B). Note the consistent peak dissociation at approximately 81.5^{o} C.

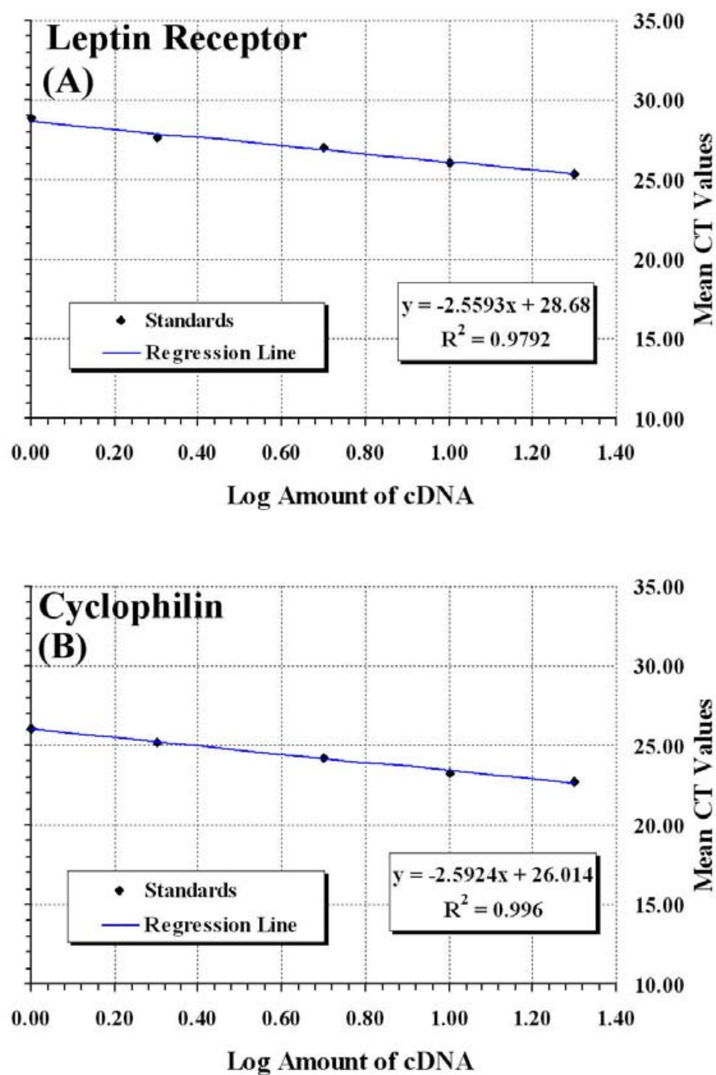

Fig. 18. Amplification efficiency plot for LR (A) and cyclophilin (B) of a reference cDNA. Regression of CT values against the amount of cDNA in the reaction resulted in a linear relationship ($P < 0.0001$) for both LR and cyclophilin.

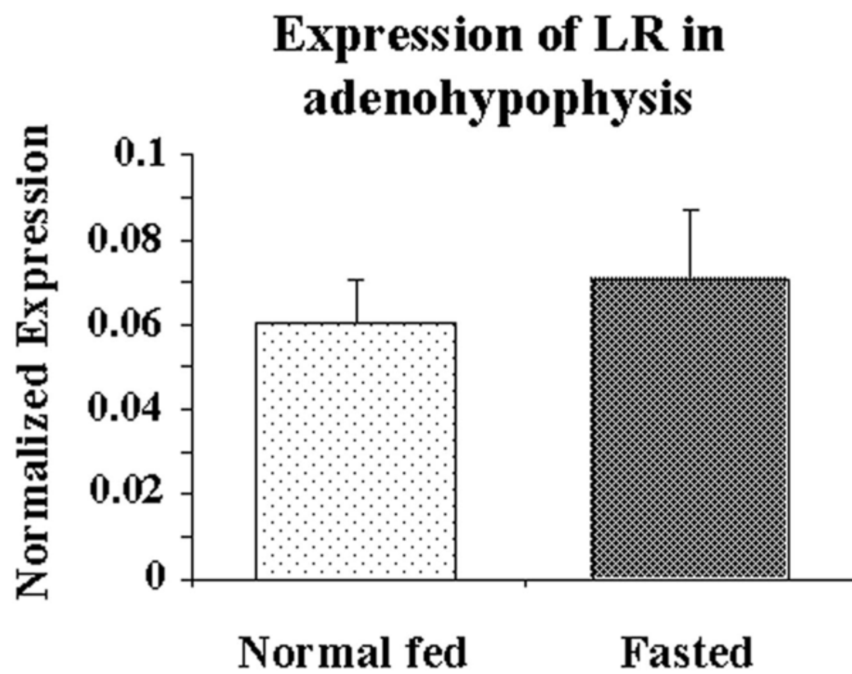

Fig. 19. Normalized mean expression of LR in adenohypophyses of normal-fed and fasted cows. Three days of fasting did not affect ($P > 0.1$) the expression of leptin receptor gene.

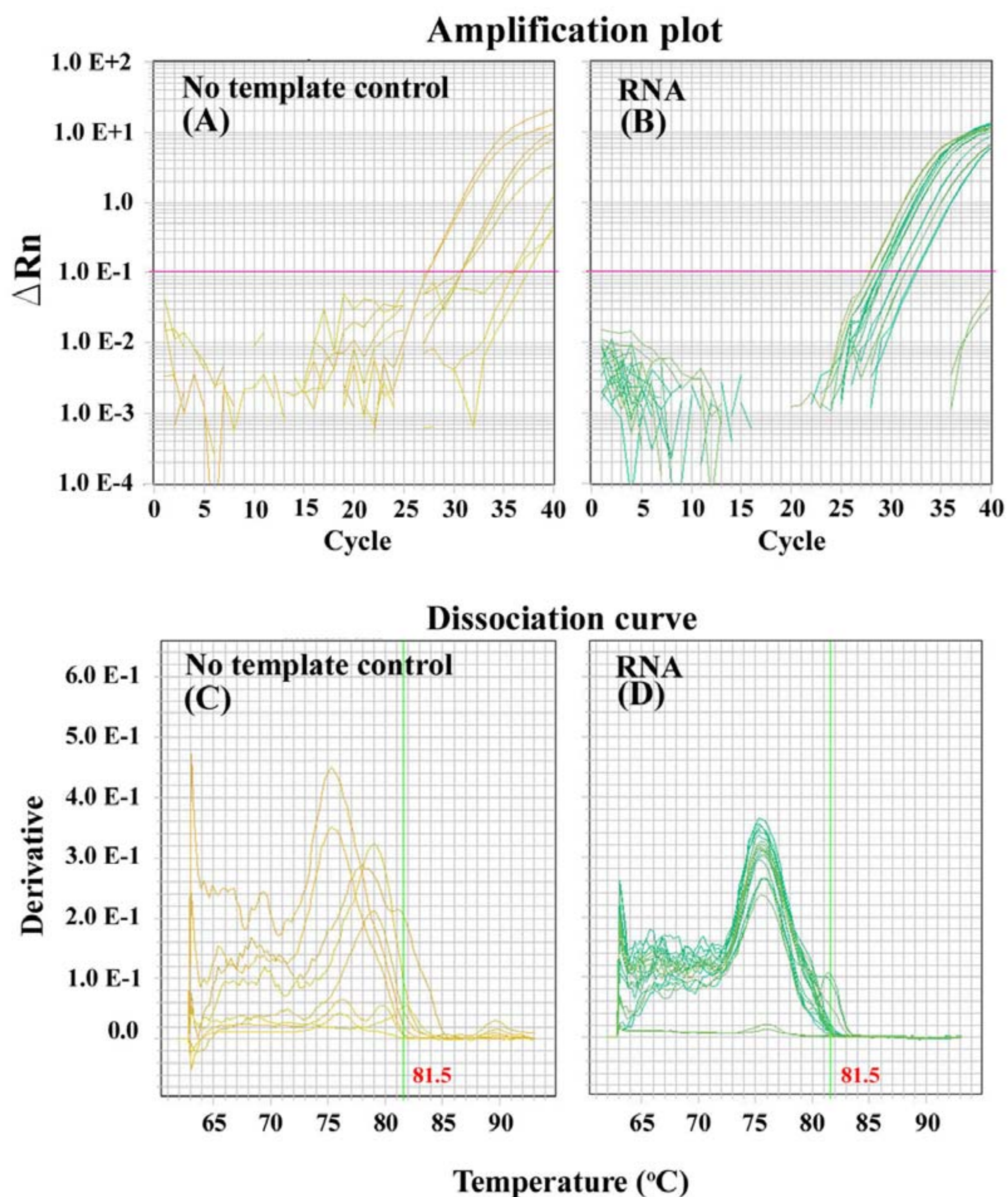

Fig. 20. Amplification plots (A, B) and dissociation curves of amplified products (C, D) using primers for cyclophilin for no-template and RNA controls. Amplification signals were detected in no-template (A) and RNA (B) controls. However, based on dissociation curves, signals detected were generated by products of smaller size (based on melting temperature of products) than expected for specific products, suggesting that signals were not the result of specific primer amplifications.

Expression of SOCS-3 gene in adenohypophysis of normal-fed and fasted cows

Amplification curves for SOCS-3 and cyclophilin to determine expression of SOCS-3 are shown in Figure 21. Dissociation curves confirmed the absence of nonspecific amplification (data not shown). Regression of CT values determined for amplification of reference cDNA against the amount of cDNA in the reaction indicated a linear relationship ($P < 0.0001$) for both SOCS-3 ($R^2 = 0.9786$) and cyclophilin ($R^2 = 0.992$) (Fig. 22). Estimated efficiency of amplification for SOCS-3 and cyclophilin in this assay was 3.35 and 2.22, respectively. Comparison of normalized expression between Normal-fed and Fasted groups indicated that fasting increased ($P < 0.052$) the expression of SOCS-3 in the adenohypophysis (Fig. 23).

Stimulation of phosphorylation of STAT-3 by leptin in perifused AP explants from normal-fed and fasted cows

Western blot analysis demonstrated that phosphorylated STAT-3 was present in large amounts in both treated and non-leptin treated AP explants of normal-fed and fasted cows (Fig. 24). The observation of large amounts of phosphorylated STAT-3 in AP explants not treated with leptin prevented us from performing a more detailed evaluation of leptin-induced activation of STAT-3 phosphorylation.

Discussion

In the present study, we tested the hypothesis that increased expression of LR and diminished expression of SOCS-3 could account for the increased responsiveness to

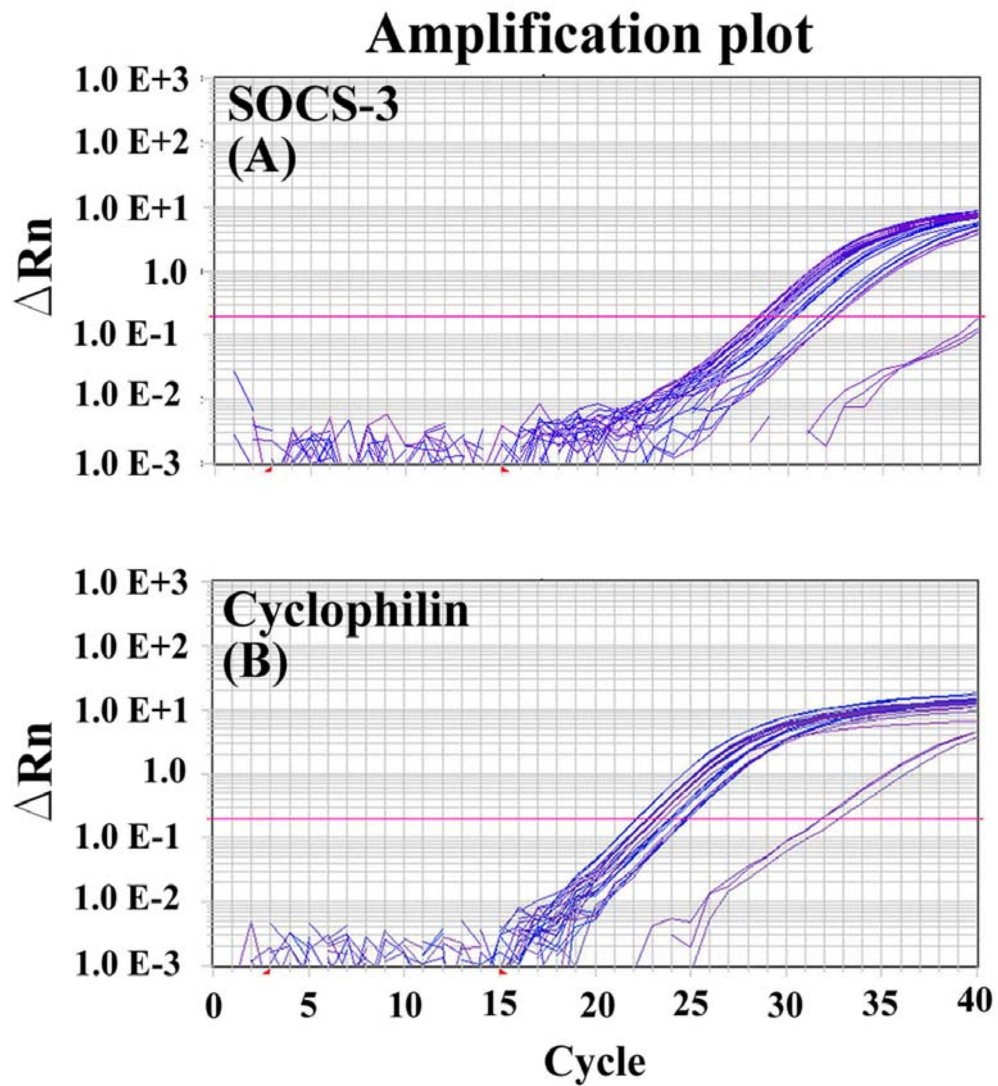

Fig. 21. Amplification plots of real-time PCR for SOCS-3 (A) and cyclophilin (B).

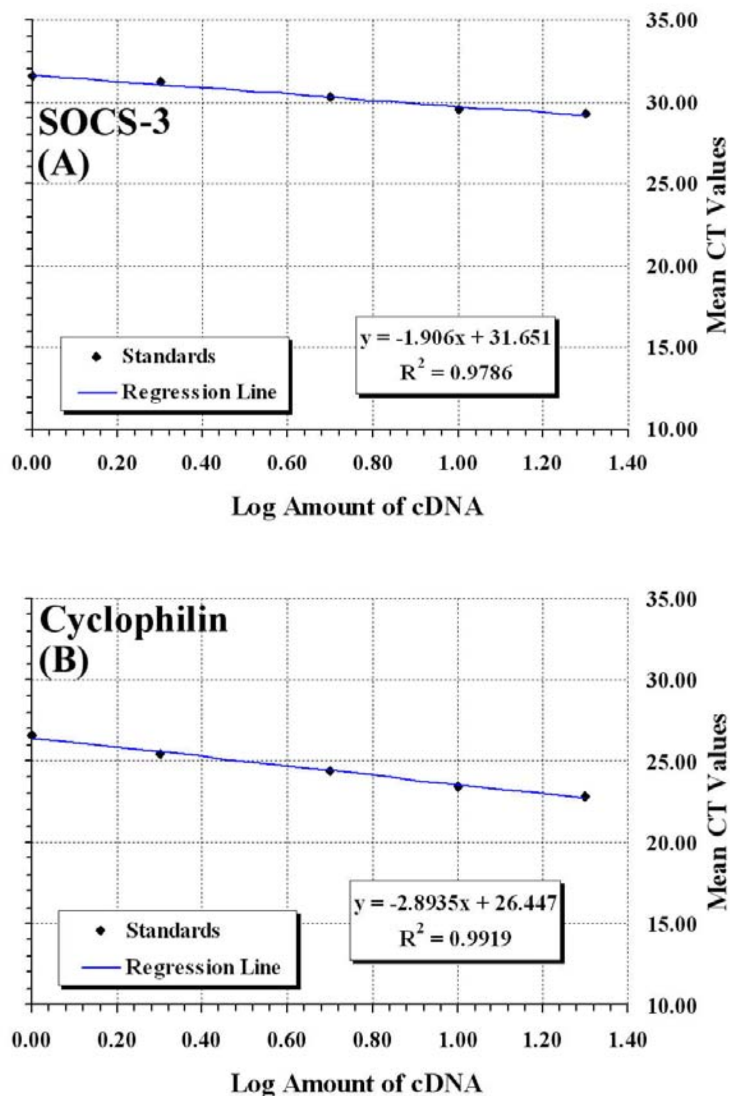

Fig. 22. Amplification efficiency plot for SOCS-3 (A) and cyclophilin (B) of a reference cDNA. Regression of CT values against the amount of cDNA in the reaction indicated a linear relationship ($P < 0.0001$) for both SOCS-3 and cyclophilin.

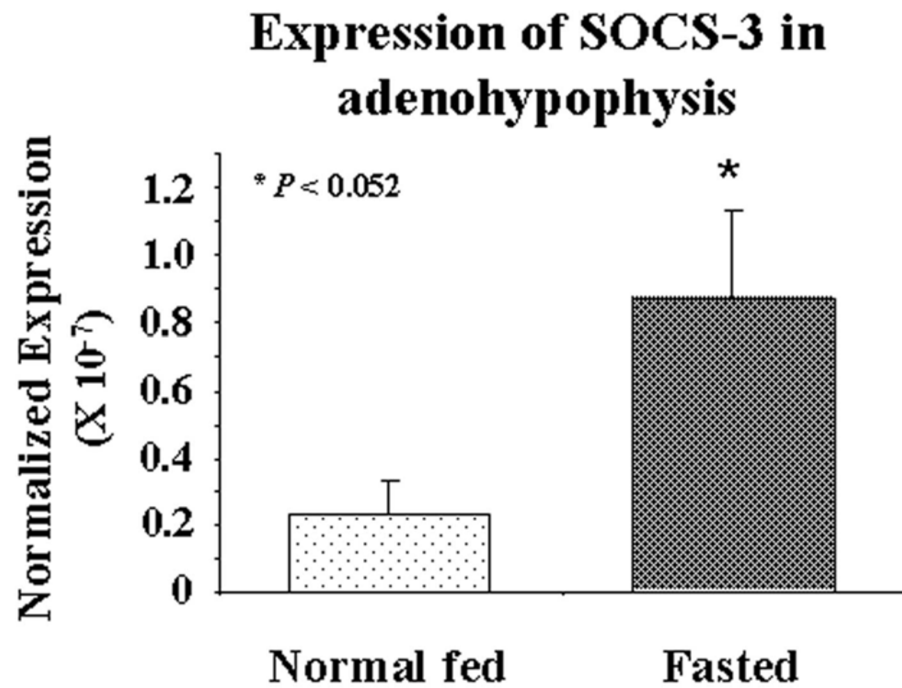

Fig. 23. Normalized mean expression of SOCS-3 in adenohypophyses of normal-fed and fasted cows. Three days of fasting increased ($P < 0.052$) the expression of SOCS-3 in fasted cows.

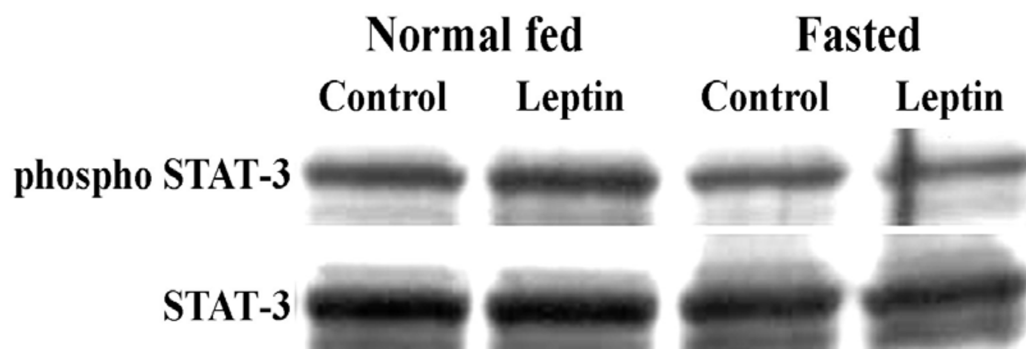

Fig. 24. Detection of phosphorylated STAT-3 and total STAT-3 in AP explants of normal-fed and fasted cows perifused with media alone (control) or media containing 100 μg ml^{-1} oleptin (Leptin) using Western blotting. High levels of phosphorylated STAT-3 in control-treated explants impaired more detailed analysis on the leptin-induced activation of STAT-3 phosphorylation in the adenohypophysis.

leptin in fasted, mature cows (Amstalden *et al.,* 2002, 2003). Although LR mRNA was detected in the adenohypophysis using PCR, no difference in the expression of LR gene was observed between normal-fed and fasted cows. Moreover, contrary to our hypothesis, expression of SOCS-3 gene was increased in adenohypophysis of fasted cows, suggesting that fasting or fasting-induced mechanisms actually stimulate expression of SOCS-3.

Acute or chronic restrictions of food intake increase expression of LR in the hypothalamus in rodents (Baskin *et al.,* 1998; Bennett *et al.,* 1998) and sheep (Dyer *et al.,* 1997a; Adam *et al.,* 2002). In aged, obese, leptin-resistant Norway rats, LR protein is diminished in the hypothalamus compared to levels observed in young, lean, leptin-sensitive rats (Scarpace *et al.,* 2001). Moreover, in the aged, leptin-resistant Wistar rat, chronic feed restriction increases LR in the hypothalamus and restores responsiveness to centrally-infused leptin (Fernandez-Galaz *et al.,* 2002). In our studies, 3 days of fasting did not affect expression of LR in the adenohypophysis of cows. Therefore increased expression of LR in the adenohypophysis does not seem to account for the ability of leptin to increase basal release of LH from perifused AP explants of fasted cows (Amstalden *et al.,* 2003), nor the ability of leptin to increase mean concentrations of circulating LH after central (Amstalden *et al.,* 2002) or peripheral (Zieba *et al.,* 2003) administration in fasted cows. These finding are in contrast to reports in the mouse showing that 48 h of fasting increased expression of the long form of the LR in the adenohypophysis (Cai and Hyde, 1999).

Although expression of LR did not differ between normal-fed and fasted cows, alterations in expression within an individual AP cell type may occur. Luteinizing hormone-synthesizing cells form only approximately 12% of the endocrine cell types of the adenohypophysis in cattle (Bastings *et al.,* 1991). Iqbal *et al.* (2000) reported that LR is observed by immunohystochemistry in less than 30% of gonadotropes of the pars distalis of the ovine adenohypophysis. Therefore, alterations in proportions of LH-synthesizing cells expressing LR gene may not be reflected in determinations of overall gene expression in the adenohypophysis. In the ewe, the proportion of gonadotropes containing LR did not differ among various phases of the estrous cycle (Iqbal *et al.,* 2000).

We also used *in situ* hybridization to colocalize LR with specific cell types within the adenohypophysis. Expression signals of LR were very low in the adenohypophysis of both normal-fed and fasted cows, which impaired our ability to perform colocalization studies. In addition, our laboratory has explored the use of immunohistochemistry for colocalization of LR in LHβ-containing gonadotropes. However, none of the anti-LR antibodies or antisera tested produced unambiguous identification of bovine LR using Western blotting or immunohistochemistry. To our knowledge, there are no antibodies currently available that recognize specifically the bovine LR.

Binding of leptin to the long form of the LR stimulates activation of receptor-associated Janus kinases (JAK) (White *et al.,* 1997) and STAT proteins, a family of transcription factors targets of JAK proteins (Bjorbaek *et al.,* 1997). The major class of

STAT proteins activated by leptin is the STAT-3 class, although STAT-5 and -6 have also been reported to be activated by leptin (Bjorbaek *et al.,* 1997). Upon activation, STAT-3 becomes phosphorylated, dimerizes, translocates to the nucleus, and modulates transcription of target genes (White *et al.,* 1997). One of the genes stimulated by STAT-3 is the SOCS-3 gene, which suppresses JAK-2 activation. Excessive activity of SOCS-3 is involved in leptin resistance (Caro *et al.,* 1996; Considine *et al.,* 1996). Studies by Baskin *et al.* (2000) demonstrated that SOCS-3 mRNA detected by *in situ* hybridization was decreased in the arcuate and dorsomedial nucleus of rats fasted for 48 h. Therefore, we hypothesized that diminished expression of SOCS-3 in fasted cows could account for an increased responsiveness to leptin. However, we observed that expression of SOCS-3 was in fact increased in the adenohypophysis of fasted cows. Although SOCS-3 expression is induced by other members of the cytokine family in addition to leptin (Starr *et al.,* 1997), it is not clear whether they are influenced by nutrient restriction.

Increased expression of SOCS-3 gene in adenohypophysis of fasted cows suggests that leptin-induced effects through STAT-3 could actually be diminished. However, observations from our previous studies demonstrate that fasted cows are more responsive to leptin. Therefore, it is possible that leptin acts through alternate cellular mechanisms to exert its effects at the level of the adenohypophysis. Leptin has also been shown to activate mitogen-activated protein kinase (MAPK) (Bjorbaek *et al.,* 1997) and phosphatidylinositol-3 kinase (Niswender *et al.,* 2001) pathways. However, the involvement of those signaling pathways on mediating leptin's effects at the adenohypophysis remains to be determined.

Basal release of LH from AP explants from fasted cows treated with leptin is higher than control-treated explants (Amstalden *et al.,* 2003). Therefore, we have attempted to investigate the involvement of activation of STAT-3 as a potential mediator of actions of leptin at the adenohypophysis in cows. We hypothesized that phosphorylation of STAT-3 would be enhanced in AP explants from fasted cows. However, AP explants from both normal-fed and fasted cows perifused with control media exhibited high levels of phosphorylated STAT-3, which prevented an evaluation of the effects of leptin on activation of STAT-3. Culture of tissue explants requires maintaining tissue and cells viable during the period of the experiment. However, it is likely that tissue collection, slicing, and incubation in an oxygenated buffer causes a significant amount of damage to the tissue. There is evidence that activation of STAT-3 is involved in cell survival and apoptosis (Groner and Hennighausen, 2000). Although bovine AP explants can be maintained viable for several hours in perifusion (Amstalden *et al.* 2003), cells are possibly under distress. Therefore, the elevated levels of phosphorylated STAT-3 observed in control-treated explants may reflect activation of endogenous mechanisms for cell survival, and may not be suitable for determining the effects of leptin on activation of STAT-3.

Results of the present studies demonstrated that LR is present in the adenohypophysis of cattle. Moreover, although leptin is effective in stimulating the release of LH in fasted cows, changes in the expression of LR and SOCS-3 mRNA do not appear to account for their increased responsiveness to leptin. Whether leptin

activates STAT-3 and/or other signaling mechanisms in the adenohypophysis remains to be determined.

CHAPTER VII

CONCLUSIONS

Studies reported herein demonstrate that 2 to 3 d of total feed restriction reduces expression of the leptin gene in subcutaneous adipose tissue and circulating leptin in mature cows. These findings are in concordance with those reported for the peripubertal heifer and animals of other species. Although this nutritional insult does not restrain the secretion of LH in mature cows, fasted cows are responsive to leptin and secretion of LH is enhanced after central or peripheral treatment with leptin. Importantly, the increased release of LH is not manifested by changes in frequency or amplitude of LH pulses. Rather, hypersecretion of LH after central infusion of leptin is manifested by augmentation of the mean baseline and magnitude of individual LH pulses, suggesting an effect at the adenohypophyseal level. This is in contrast to observations in peripubertal heifers, in which leptin prevents the fasting-induced reduction in the frequency of LH pulses, which suggests an effect at the hypothalamic level. In addition, the lack of response to ICV infusions of leptin in cows maintained under a normal nutritional regimen suggests that cows in neutral energy balance are either less sensitive or resistant to leptin.

Results of studies with hypothalamic explants suggested that leptin does not directly affect the release of GnRH from the medial basal hypothalamus-infundibular complex of normal-fed cattle. In addition, leptin does not affect the release of LH or FSH from adenohypophyseal cells cultured under optimal conditions, suggesting further that

cattle under neutral or positive energy balance are resistant to the effects of leptin. This is in contrast to observations in rodents and pigs, in which leptin has been shown to stimulate the release of GnRH from the hypothalamus and LH from the adenohypophysis of normal fed animals.

The site of leptin's action in stimulating the release of LH in mature cows appears to be, at least in part, at the adenohypophyseal level. The release of GnRH from hypothalamic-infundibular explants collected from fasted cows was not affected by leptin treatment. This is in contrast to observations in the peripubertal heifer and castrated ram, in which leptin appears to act within the central nervous system to prevent fasting-mediated reductions in the frequency of LH. However, AP explants from fasted cows treated with leptin had higher basal release of LH than control-treated explants. Interestingly, the mechanism through which basal release of LH is stimulated by leptin may differ from that typical of a classical secretagogue, as adenohyphyseal effects appear to occur by increasing the basal metabolism of gonadotropes.

Although basal release of LH from AP explants from normal-fed cows was not affected by leptin, the GnRH-stimulated release of LH was enhanced in explants perifused with leptin. Therefore, although *in vivo* studies indicate that the hypothalamic-gonadotropic axis of ruminants maintained under neutral or positive energy balance are less sensitive, or perhaps resistant to leptin, some aspects of the response (e.g. GnRH-mediated release) may be modulated apart from nutritional status.

Resistance to leptin has been observed in humans and in rodents. Some of the possible mechanisms leading to resistance may involve the number of LR in leptin-

responsive tissues, and the ability of LR to trigger intracellular signaling responses. Although decreased numbers of LR in the hypothalamus and increased activity of a intracellular factor that suppresses LR signaling, SOCS-3, are observed with leptin resistance in rodent models, they do not appear to play a role in the increased responsiveness to leptin observed in mature, fasted cows. Expression of LR in the adenohypophysis did not differ between normal-fed and fasted cows. Moreover, expression of SOCS-3 is increased in fasted cows, which are more responsive to leptin. Whether other components of LR signaling or alternate signaling pathways are involved in the increased responsiveness to leptin of cows under negative nutrient balance and resistance to leptin in cows under neutral and/or positive nutrient balance remain to be determined.

Development of obesity and infertility in rodents lacking functional leptin or leptin receptors indicates that leptin is essential for normal body homeostasis, and accumulating evidence indicates that leptin acts as a permissive hormone or potentiates the action of other factors. However, leptin has stimulatory effects on reproductive hormones in chronically and acutely undernourished animals, in which circulating leptin is diminished. Therefore, under conditions of negative energy balance, such as those commonly observed during the postpartum period in cows, exogenous leptin could potentially have beneficial effects as therapeutic agent. Further examination of the mechanisms of action of leptin, particularly during undernutrition, is warranted and practical applications for using leptin to control reproductive function in cattle will require additional investigations.

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APPENDIX

PROCEDURES

Luteinizing Hormone RIA

1. Iodination: Iodination grade bLH (USDA–bLH-I-1; AFP 6000)
   Reaction: 5 µg of hormone, 0.5 mCi of ^{125}I, 90 µg chloramine T, 2 min

2. Antibody: Anti-ovine LH (rabbit anti-oLH – TEA #35; obtained from Dr. Jerry Reeves)
   Dilution: 1:100,000

3. Standards: Biological grade bLH (NIH bLH-B-10)
   Range: 0.25 – 100 ng/ml

4. Reference preparation: bLH added to cow serum

5. RIA procedure
   (Davis et al. 1971; Biol Reprod 4:415 and Williams and Ray 1980; J. Anim Sci 50: 906)
   a) Label assay sheets and polypropylene or borosilicate glass tubes
      4 NSB, 9 TC, 3 "0", standards in triplicate, 2 X references in duplicate, and unknown samples in duplicate

   b) Day 1: Pipette the following into each tube
      NSB: 500 µl PBS-1% EW
      0 std.: 500 µl PBS-1% EW
      Stds.: 200 µl std + 300 µl PBS-1% EW
      Ref.: 200 µl reference + 300 µl PBS-1% EW
      Unknowns: 200 µl sample + 300 µl PBS-1% EW
      Store at 4° C until next step

      Pipette 200 µl PBS-EDTA + 1:400 NRS without 1st Ab into the NSB tubes
      Pipette 200 µl anti-oLH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes
      Vortex briefly and incubate for 2 h at 4° C

      Pipette 100 µl ^{125}I-bLH (20,000 cpm/tube diluted in PBS-1% EW) into all tubes, vortex briefly, and incubate for 24 h at 4° C

   c) Day 2: Pipette 200 µl of sheep-anti-rabbit gamma globulin (SARGG) diluted in PBS-EDTA into all tubes except TC
      Vortex and incubate 48-72 h at 4° C

   d) Day 4: Add (per spin basis) 3 ml ice-cold 0.01M PBS into all tubes except TC
      Centrifuge tubes for 1 h at 3600 rpm at 4° C
      Decant supernatant
      Count radioactivity associated with the pellet in gamma counter

Follicle-Stimulating Hormone RIA

1. Iodination: Iodination grade oFSH (USDA–oFSH-I-1; AFP 5679C)
   Reaction: 2 µg of hormone, 0.5 mCi of ^{125}I, 18 µg chloramine T, 1 min

2. Antibody: Anti-ovine FSH (rabbit anti-oFSH - JAD 17-679; obtained from Dr. James Dias)
   Dilution: 1:12,000

3. Standards: Biological grade oFSH (NIAMDD oFSH-RP-1)
   Range: 0.25 – 50 ng/ml

4. Reference preparation: oFSH added to cow serum

5. RIA procedure
   (Krystek SR Jr et al. 1985 Endocrinology 117: 1125)
   a) Label assay sheets and polypropylene or borosilicate glass tubes
      4 NSB, 9 TC, 3 "0", standards in triplicate, 2 X references in duplicate, and unknown samples in duplicate

   b) <u>Day 1</u>: Pipette the following into each tube
      NSB: 500 µl PBS-1% EW
      0 std.: 500 µl PBS-1% EW
      Stds.: 200 µl std + 300 µl PBS-1% EW
      Ref.: 200 µl reference + 300 µl PBS-1% EW
      Unknowns: 200 µl sample + 300 µl PBS-1% EW
      Store at 4° C until next step

      Pipette 200 µl PBS-EDTA + 1:400 NRS without 1st Ab into the NSB tubes
      Pipette 200 µl anti-oFSH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes
      Vortex briefly and incubate for 2 h at 4° C

      Pipette 100 µl ^{125}I-oFSH (20,000 cpm/tube diluted in PBS-1% EW) into all tubes, vortex briefly, and incubate for 24 h at 4° C

   c) <u>Day 2</u>: Pipette 200 µl of sheep-anti-rabbit gamma globulin (SARGG) diluted in PBS-EDTA into all tubes except TC
      Vortex and incubate 48-72 h at 4° C

   d) <u>Day 4</u>: Add (per spin basis) 3 ml ice-cold 0.01M PBS into all tubes except TC
      Centrifuge tubes for 1 h at 3600 rpm at 4° C
      Decant supernatant
      Count radioactivity associated with the pellet in gamma counter

Gonadotropin-Releasing Hormone RIA

1. Iodination: GnRH (Sigma, St. Louis, MO; Catalog # L-7134)
   Reaction: 2.5 µg of hormone, 1 mCi of ^{125}I, 10 µg chloramine T, 10 sec

2. Antibody: Anti-GnRH (rabbit anti-GnrH – R1245; obtained from Dr. Terry Nett)
   Dilution: 1:16,000

3. Standards: GnRH (Sigma, St. Louis, MO Catalog # L-7134)
   Range: 1 – 1000 pg/ml

4. Reference preparation: GnRH added PBS-0.1% gelatin

5. RIA procedure

   a) Label assay sheets and polypropylene or borosilicate glass tubes
      4 NSB, 9 TC, 3 "0", standards in triplicate, 2 X references in duplicate, and unknown samples in duplicate

   b) Day 1: Pipette the following into each tube
      NSB: 500 µl PBS-0.1% gel
      0 std.: 500 µl PBS-0.1% gel
      Stds.: 500 µl (std + PBS-0.1% gel)
      Ref.: 500 µl (reference + PBS-0.1% gel)
      Unknowns: 500 µl (sample + PBS-0.1% gel)
      Store at 4° C until next step

      Pipette 50 µl PBS-EDTA + 1:400 NRS without 1st Ab into the NSB tubes
      Pipette 50 µl anti-GnRH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes
      Vortex briefly and incubate for 24 h at 4° C

   c) Day 2: Pipette 50 µl ^{125}I-GnRH (11,000 cpm/tube diluted in PBS-0.1% gel) into all tubes, vortex briefly, and incubate for 24 h at 4° C

   d) Day 3: Add 1.5 ml ice-cold 95% ethanol into all tubes except TC
      Vortex and centrifuge at 3000 rpm (2000 X g) for 20 min at 4° C
      Decant supernatant
      Count radioactivity associated with the pellet in gamma counter

Estradiol 17-β RIA

1. Tracer: ^{3}H-estradiol 17-β

2. Antibody: Anti-estradiol (Eli Lilly, E_2-6-#3; Lot # 0403061873)
   Dilution: 1:100,000

3. Standards: Estradiol 17-β
   Range: 12.5 – 2500 pg/ml

4. Reference preparation: add E_2 17-β into charcoal-stripped serum (CSS) to final concentration of 4 pg/ml and 30 pg/ml

5. RIA procedure
   (Britt et al. 1974; J Anim Sci 39: 915 and Williams and Ray 1980; J. Anim Sci 50: 906)

   a) Day 1: Extraction
      Label scintillation vials (7 ml plastic vials) for unknowns in duplicate, blanks (4 total), recoveries (R1-R4), total counts for recoveries (TCR1-TCR6), and TC (TC1-TC9)
      Label 2 extraction vials (glass vials; 25 x 150 mm) for each sample and reference
      Label borosilicate glass tubes (4 NSB, 3 "0", standards in triplicate, 4 blank, 2 x references in duplicate, and unknown samples in duplicate
      Pipette 1 ml of sample and reference into corresponding extraction vials (in duplicate)

      Pipette 1 ml of CSS into extraction vial and add 1500 cpm of ^{3}H-E_2 17-β, vortex, and let equilibrate at 4° C for at least 1 h (recovery estimates R1-R4)
      Pipette 1500 cpm ^{3}H-E_2 17-β into 6 scintillation vials labeled TCR1-TCR6, add 5 ml of scintillation fluid into them, cap vials, and vortex

      Add 4 ml of redistilled or fresh benzene into extraction vials and place caps
      Shake extraction vials for 45 min in the end-to-end shaker
      Remove extraction vials from shaker and allow to equilibrate at room temperature for at least 20 min
      Transfer extract into the corresponding labeled glass tube and R1-R4 into corresponding scintillation vials (be consistent in transfer process)
      Dry down partially with air

      Repeat the extraction and transfer and add extracts into the corresponding glass tubes
      Dry off benzene with air (be sure no benzene is left)

Estradiol 17-β RIA (cont.)

b) Day 2: : Pipette the following into each tube
NSB: 700 µl PBS-0.1% Gel
0 std.: 500 µl PBS-0.1% Gel
Stds.: 200 µl std + 300 µl PBS-0.1% Gel

Once tubes containing extracts are dried, normalize volume to 500 µl using PBS 0.1% Gel
Vortex to redissolve extract dried to the walls of the tubes

Pipette 200 µl E2 anti-E2 into all tubes except NSB
Incubate for 30 min on ice

Pipette 100 µl ^3H-E_2 17-µ (5,000 cpm/tube) into all tubes plus TC scintillation vials.
Vortex and incubate for 16-24 h at 4° C

Add 5 ml scintillation fluid into TC vials

c) Day 3: Add (per spin basis) 1 ml ice-cold charcoal-dextran suspension (kept in an ice bath and constantly stirred) into all tubes
Vortex and incubate on ice for 30 min
Centrifuge tubes for 45 min at 3200 rpm at 4° C
Decant supernatant into corresponding scintillation vial
Add 5 ml scintillation fluid to each vial, place cap, and vortex
Count radioactivity associated with the supernatant in Beta Counter

Insulin RIA

1. Iodination: Etherton et al. 1987, J. Anim. Sci. 64: 433
   Iodination grade b-insulin (Eli Lilly, Lot # 615-70N-80, 26.6 U/mg)
   Reaction: 10 µg of hormone, 0.5 mCi of ^{125}I, 10 µg chloramine T, 50 sec

2. Antibody: Anti-bovine Insulin (guinea pig anti-bInsulin; Miles Laboratory/ICN Biomedicals 65-101-GP616)
   Dilution: 1:30,000

3. Standards: Purified bInsulin (Lilly Research Laboratories)
   Range: 0.1 – 2.5 ng/ml

4. Reference preparation:
   Bovine Insulin added to charcoal-stripped serum (CSS)

5. RIA procedure (Albano et al. 1972; ACTA Endocrinologica 70: 487-509)
   a) Label assay sheets and polypropylene or borosilicate glass tubes
      4 NSB, 9 TC, 3 "0", standards in triplicate, 2 X references in duplicate, and unknown samples in duplicate

   b) Day 1: Pipette the following in each tube
      NSB: 300 µl PBS-1% BSA + 200 µl CSS
      0 std.: 300 µl PBS-1% BSA + 200 µl CSS
      Stds.: 200 µl std + 100 µl PBS-1% BSA + 200 µl CSS
      Ref.: 300 µl PBS-1% BSA + 200 µl reference
      Unknowns: 300 µl PBS-1% BSA + 200 µl sample
      Store at 4° C until next step

      Pipette the following in each tube
      NSB: 200 µl PBS-EDTA + 1:400 NGPS without 1st Ab
      All tubes except NSB and TC: 200 µl anti-binsulin in PBS-EDTA + 1:400 NGPS
      Vortex briefly and incubate 90 min at room temperature
      Pipette 100 µl ^{125}I-bInsulin (11,000 cpm/tube diluted in PBS-1% BSA) to all tubes
      Vortex briefly and incubate for 24 h at 4° C

   c) Day 2: Place tubes on ice and pipette 1 ml of Charcoal (50 mg/ml diluted in PBS-0.3% BSA kept stirring during pipetting) using an 1-ml selectapette pipette. Add charcoal only in tubes that will be centrifuged at the same time
      Vortex tubes briefly and centrifuge for 30 min at 3600 rpm at 4° C
      Decant supernatant into pre-labeled corresponding tube
      Count radioactivity associated with the supernatant in gamma counter

Isolation of RNA and Northern Blotting

Isolation of RNA

Pulverize 0.5 g of adipose tissue with a mortar and pestle with the tissue covered with liquid N_2

Let the N_2 evaporate, transfer the pulverized tissue into a polypropylene tube containing denaturing solution (Guanidine thiocyanate/β mercaptoethanol solution; 1 ml/150 mg tissue) – starting volume

Homogenize in a polytron at 60% of maximum speed for 20 sec

Place homogenized samples on ice for 15 min

Centrifuge for 15 min at 4,000 rpm (3,000 X g)

Push aside the fat cake and decant into a new polypropylene tube

Add one starting volume of Phenol/Chloroform/Isoamyl (25:24:1)

Vortex for 1 min and place on ice for 10 min

Centrifuge for 30 min at 9,000 X g

Remove aqueous phase and place in new polypropylene tube (be sure not to take up any of the interphase or the organic phase)

Add 1/10 vol. Sodium Acetate (3M; pH 4.5) and vortex

Add 1 vol. Acid Phenol/Chloroform (5:1), vortex for 1 min, and place on ice for 10 min

Centrifuge for 30 min at 9,000 X g

Remove aqueous phase and place in a new polypropylene tube (be sure not to take up any of the interphase or the organic phase)

Add 1 vol. of isopropanol, vortex and place on -20° C overnight

Briefly vortex isopropanol solution and transfer as much as possible to a 1.7-ml microcentrifuge tube

Spin at 14,000 rpm for 20 min

Discharge supernatant, add remaining isopropanol solution, and repeat centrifugation until all of the isopropanol containing RNA solution is incorporated into a single pellet in one microcentrifuge tube.

Add 500 µl 75% ethanol to the final pellet, vortex briefly, and let stand at room temperature for 10 min

Spin for 20 min, pour off ethanol, spin briefly, and carefully remove remaining ethanol with vacuum and a gel loading tip

Let RNA pellet dry for 3-5 min and add 12 µl of H2O/0.1mM EDTA per 400 mg of adipose tissue

Add 2 µl of RNA solution to 900 µl 1mM $NaHPO_4$ and read at 260 and 280 nm

Electrophoresis

1. Gel formulation: 1% agarose, 10% 10X Mops, 5% formaldehyde
2. Sample preparation:

   15 µg of RNA - the sample volume (SV) will depend on the concentration of the least concentrate sample. Adjust the volume of the sample with RNase free water to the same SV

Isolation of RNA and Northern Blotting (cont.)

Calculate the sample buffer volume (SBV) and total sample volume (TV) as follow:
SBV=SV*4.5
TV=SBV+SV

Prepare sample buffer:	Deionized formamide =	SBV*0.555 µl
	Formaldehyde =	SBV*0.2 µl
	10X Mops =	SBV*0.1185
	Loading buffer =	SBV*0.111
	Ethidium bromide =	SBV*0.015

(loading buffer = 50% glycerol, 0.25% bromophenol blue, 0.25% sylene cyanol)

Mix RNA and sample buffer and incubate in heat block for 20 min at 68° C
Incubate on ice for 10 min

3. Load samples in the gel and run at 100 volts for 2.5 h or until bromophenol blue is about 1 cm from the bottom of the gel

Transfer into membrane

Set up transfer with components in the following order (bottom up):
(between each layer roll a 30 ml test tube to remove bubbles)

Fill reservoir with 400 ml of 10X SSC
Wet 11 x 23 cm filter paper with 10X SSC
Gel
Membrane (10.8 x 13.8 cm Genescreen plus membrane)
One piece (10.8 x 13.8 cm) of wet filter paper
One piece (10.8 x 13.8 cm) of dry filter paper
Stack of paper towels cut to size (15 cm high)
Place weight on top
Let transfer for 24 h

Once transfer is complete, rinse membrane for 20 min in 2X SSC while rocking
Place membrane in wet filter paper and UV cross-link (energy set at 12,000)

Wrap membrane in plastic wrap and place in freezer

Isolation of RNA and Northern Blotting (cont.)

Riboprobe transcription

Add the following to a microfuge tube on ice and make up to final volume 20 µl:
500 ng antisense cDNA
2 µl 10 X transcription buffer
1 µl 10 mM ATP
1 µl 2 mM modified CTP (Strip EZ® RNA; Ambion, Austin, TX)
1 µl 10 mM GTP
2.5 µl ^{32}P-UTP (800 Ci/mmol, 20 mCi/ml)
2 µl T7 polymerase
H_20 to final volume (20 µl)

Incubate at 37° C for 1 h
Add 1 ul DNase I
Incubate at 37° C for 15 min

Remove non-incorporated ribonucleoside triphosphates (Micro-Bio Spin column P-30®; BioRad Laboratories, Hercules, CA)

Add 1 µl 0.5 M EDTA (Store at -20° C overnight if necessary)

Determine specific activity in 2 µl ribroprobe solution using scintillation β counter

Confirm quality of ribroprobe by gel (4% acrylamide-urea) electrophoresis

Hybridization

Pre-hybridize membrane in ULTRAhyb® buffer for 30 min at 64° C

Denature radioactive-labeled cRNA in heat block at 95° C for 10 min
Incubate on ice for 10 min

Add $1x10^6$ cpm specific activity per ml of hybridization solution (ULTRAhyb®)
Hybridize overnight with rotation at 40° C

Discard hybridization buffer
Add 50 ml of 2X SSC-0.1% SDS (wash I) at 38° C, swish 20 times, and discard
Remove membrane and place in a RNase free dish
Wash 2 times with wash I solution for 10 min in hybridization oven at 40° C
Wash 2 times with wash II solution (0.1X SSC-0.1% SDS) at 50° C

Wrap membrane in plastic wrap and place on imager
(more wash II may be needed)

Isolation of RNA and Northern Blotting (cont.)

Stripping radioactive probe off of membrane

Stripping solution: 0.1X SSC-2% SDS, at 100° C

Place membrane in *Pyrex* dish and pore boiling stripping solution into the dish
Place dish into hybridization oven and rock at maximum speed for 10 min
Pore solution into radioactive waste
Repeat 3 times

Check membrane for radiation – counts should be near zero
Wrap membrane in plastic wrap and store in freezer

Real-Time Polymerase Chain Reaction

Isolation of RNA

Add 1 ml TRIzol® (Gibco-BRL, Gaithersburg, MD) to a conical tube and place on ice
Add approximately 0.1 g of adenohypophyseal tissue (kept frozen) and grind tissue using PRO 250 homogenizer
Place homogenized samples on ice for 5 min
Add 0.2 ml of Chloroform, vortex for 1 min, and place on ice
Centrifuge at 12,000 X g for 10 min
Remove aqueous phase and place in new polypropylene tube (be sure not to take up any of the interphase or the organic phase)
Add an equal volume of isopropanol, vortex
Incubate at RT for 10 min
Spin at 12,000 X g for 10 min at 4° C
Discharge supernatant
Add 1 ml 70% ethanol to the pellet, vortex briefly
Spin at 7,500 X g for 5 min at 4° C
Pour off ethanol, and carefully remove remaining ethanol with pipette tip
Let RNA pellet dry for 3-5 min and add 30 µl of depc-treated, RNase-free, autoclaved H2O
Incubate samples for 5 min at 70° C and vortex

Determine concentration of RNA in spectrophotometer (read at 260 and 280 nm): use 2 µl sample in 900 µl H20 (1:450 dilution)

Determine quality of RNA by denaturing agarose-gel electrophoresis (Northern gel)

Reverse transcriptase reaction

Combine 5 µg total RNA with sterile water to final volume of 10.2 µl in a microcentrifuge tube
Add 1µl oligo dT primers (0.2 µg/µl) and 1 µl random primers (1 µg/µl)
Incubate at 70° C for 10 min
Place at room temperature for 2 min

Spin tube briefly and add the following:
- 0.3 µl RNasin
- 4 µl 5X 1st Strand buffer (Gibco-BRL, Gaithersburg, MD)
- 1 µl 100 mM dNTPs
- 2 µl 100 mM DTT
- 0.5 µl SupersScript II RTase (Gibco-BRL, Gaithersburg, MD)

Incubate at 42° C for 60 min
Incubate at 95° C for 2 min, spin briefly, and place on ice
Add 1 µl 0.5 M EDTA and 20 µl Phenol/Chloroform/Isoamyl alcohol (25:24:1)

Real-Time Polymerase Chain Reaction (cont.)

Vortex well and spin for 2 min
Remove aqueous layer and place in a clean tube

Add 22 µl 4 M ammonium acetate
1 µl glycogen
88 µl ethanol
Vortex and place at -80° C for 1 h

Centrifuge at 21 X g for 15 min at 4° C

Remove all supernatant and resuspend pellet in 20 µl of sterile water

Store at -20° C

Real-time PCR reaction

Add to each well on reaction plate the following:
10 µl 2X SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA)
1 µl cDNA template diluted 1:10
1 µl forward primer at appropriate concentration
1 µl reverse primer at appropriate concentration
7 µl of sterile water

Perform amplification reaction using the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems), at the following conditions:
Initial incubation at 95 ^{o} C for 10 min (Applied Biosystems) to activate AmpliTaq Gold® DNA polymerase
40 cycles of 95° C for 15 sec followed by 60° C for 1 min

Perform dissociation curve
One cycle of 50° C for 2 min, 95° C for 10 min and 15 sec, 60° C for 15 sec, and a final increase in temperature up to 95° C

Determine threshold cycle using the ABI Prism® SDS 2.1 analysis software (Applied Biosystems)

In Situ Hybridization Histochemistry

Linearization of plasmids for in vitro transcription

Add the following to a microcentrifuge tube:

DNA plasmid	20 µg
10X restriction enzyme buffer (specific for enzyme of interest)	20 µl
Restriction enzyme (specific to plasmid of interest)	10 µl (use at least 1 U per µg DNA)
Water	to 200 µl final volume

Incubate 37° C for 2 h (or appropriate temperature for the enzyme)

Add 200 µl PCI (phenol:chloroform:isoamyl alcohol); vortex
Centrifuge at 21,000 X g for 2 min

Transfer supernatant to a clean tube
Add 200 µl chloroform; vortex
Centrifuge at 21,000 X g for 2 min

Transfer supernatant to a clean tube
Add
3 volumes (approximately 600 µl) 100% ethanol
1/10 volume (approximately 20 µl) 3M NaOAc
5 µl Dextrane T500 (10 mg/ml)
Vortex well

Place at -80° C for 1 h

Centrifuge at 21,000 X g for 10 min at room temperature

Discharge supernatant and wash pellet with 150 µl 70% ethanol
Centrifuge at 21,000 X g for 2 min

Resuspend pellet in 20 µl of RNase-free water
DNA is at approximately 1 µg/µl

Store at -20° C

In Situ Hybridization Histochemistry (cont.)

Ribroprobe transcription

Add the following to a microcentrifuge tube (enough for about 10 slides):

DNA template	1.0 µl (approximately 1 µl)
5X transcription buffer	2.5 µl (Promega)
100 mM DTT	1.25 µl (Promega)
2.5 mM rACG	1.25 µl (Promega)
[^{35}S] UTP	1.25 µl (40 mCi/ml)
RNasin	0.5 µl (Promega)
RNase-free water	4.0 µl
Polymerase (T7, T3, SP6)	0.75 µl (Promega)

Incubate at 37° C for 2.5 h

Add 3µl RQ1-DNase I (Promega) and 0.5 µl RNasin
Incubate at 37° C for 15 min

Add 20 µl yeast tRNA (10 mg/ml) and 40 µl PCI; vortex
Centrifuge at 21,000 X g for 5 min and transfer supernatant to a clean tube
Add 40 µl chloroform; vortex
Centrifuge at 21,000 X g for 2 min

Transfer supernatant to equilibrated column (Centri-Sep; Princeton Separations Inc., Adelphia, NJ) and follow manufacture's instructions

After column purification add:
60 µl 3M NaOAc (pH 5)
1 µl yeast tRNA
300 µl ethanol (RNA grade)

Place at -80° C for at least 20 min
Centrifuge 21,000 X g for 10 min at 4° C

Discharge supernatant and wash pellet with 70% ethanol (RNA grade)
Centrifuge 21,000 X g for 3 min

Dissolve pellet with 50 µl 100 mMDTT

Determine specific activity in 1 µl ribroprobe solution using scintillation β counter

In Situ Hybridization Histochemistry (cont.)

Preparation of slides

Process slides through:
Xylene or substitute, 5 min, 3 changes (agitate every 2-3 min)
100 % ethanol, 1 min, 2 changes
95% ethanol, 1 min, 2 changes
70% ethanol, 3 min, 1 change
Phosphate-buffered saline (PBS), 5 min, 2 changes
Fresh 4% paraformaldehyde, 20 min
PBS, 5 min, 2 changes
Proteinase K, 7.5 min (20 µg/ml in 50 mM Tris, 5 mM EDTA)
4% paraformoldehyde, 5 min (can reuse solution used in previous step)
Double-distilled water, 1 min
PBS, 5 min, 2 changes
70% ethanol, 3 min
95% ethanol, 1 min, 2 changes
100% ethanol, 1 min, 2 changes

Dry slides on air at room temperature

Hybridization

Denature radiolabeled cRNA probeat 70° C for 10 min in hybridization solution containing 100 mM DTT (need approximately 70 µl hybridization solution/slide

Add 5 X 10^{6} cpm/slide of radiolabeled probe in hybridization solution
Place coverslip on each slide gently; avoid bubbles

Incubate overnight at 55° C in humidified chamber containing Whatman 3MM paper wetted with 50% formamide/5X SSC

Washing

Wash hybridized slides using the following procedure:
5X SSC/10 mM β-mercaptoethanol, 30 min, at 55° C
50% formamide/2X SSC/50 mM β-mercaptoethanol, 20 min at 65° C
TEN (0.5 M NaCl/10 mM Tris (pH 8)/5 mM EDTA), 10 min, RT
TEN, 10 min, at 37° C, 3 changes
RNase (10µg/ml) in TEN, 30 min, 37° C
TEN, 15 min, at 37° C
50% formamide/2X SSC/50 mM β-mercaptoethanol, 20 min, at 65° C
2X SSC, 15 min, at RT
0.1X SSC, 12 min, at RT
70% ethanol/0.3 M ammonium acetate, 5 min, 2 changes, at RT
95% ethanol/0.3 M ammonium acetate, 1 min, at RT
100% ethanol, 1 min, 2 changes at RT

In Situ Hybridization Histochemistry (cont.)

*Note: dispose the first 2 washes in the radioactive waste

Dry slides on air

Expose slides to film overnight to estimate autoradiography time or expose to phospho-image screen a few hours

Autoradiography

Thaw Kodak NTB2 emulsion at 42° C in a light tight container

Under a Safelight:
Mix the emulsion with an equal volume of 42° C water and pour into slide holder
Dip slide in emulsion solution, wipe off excess emulsion on slide back
Dry slides in a light tight box (containing desiccant) for 3 to 6 hours
Add desiccant to slide boxes and wrap boxes in foil
Store boxes at 4° C for appropriate amount of time

Developing slides
Allow slides to warm to RT

Develop and stain using the following procedure:
Kodak D-19 developer (diluted 1:1 with water), chilled to 15° C, 4 min (dip 10 times/min)
Water, 0.5 min
Fixer, 5 min (use fixer with hardener)
Water, 5 min, 2 changes (after this can turn lights on)
Hematoxylin, 1 min or less (check under microscope to verify intensity of staining)
Water until clear
70% ethanol, 5 min
95% ethanol, 1 min, 2 changes
100% ethanol, 1 min, 2 changes
Xylene, 1 min, 3 changes

Coverslip slides using Permount

Protein Isolation and Western Blotting

Isolation of protein

Homogenize 100 mg of tissue in 1 ml lysis buffer (60 mM Tris, 1 mM Na_3VO_4, 10% glycerol, 2 % SDS, containing 44 µg/ml aprotinin and 100 µg/ml PMSF) using a 5-ml polypropelene tube and the PRO250 homogenizer (PRO Scientific)

Incubate on ice for 5 min

Grind homogenate 30 times using a Dounce homogenizer with pestol “B”

Centrifuge homogenate at 16,000 X g for 15 min at 4° C

Collect supernatant in a clean tube

Determine concentration of protein using the Bradford protein assay

Bradford protein assay

Use a 96-well plate

Add standards (BSA; stock 0.2 mg/ml) in duplicate as follow

A1-A2	0 µl
B1-B2	2 µl standard (0.4 µg/200 µl)
C1-C2	3 µl standard (0.6 µg/200 µl)
D1-D2	4 µl standard (0.8 µg/200 µl)
E1-E2	5 µl standard (1 µg/200 µl)
F1-F2	7.5 µl standard (1.5 µg/200 µl)
G1-G2	10 µl standard (2 µg/200 µl)
H1-H2	12.5 µl standard (2.5 µg/200 µl)

Add 3 µl of each sample diluted 1:10 with ddH_2O in duplicate

Add 200 µl Bradford reagent (Bio-Rad Laboratories, Richmond, CA) diluted 1:5 with ddH_2O to each well

Allow reaction to proceed for 10 min at RT

Read plate at OD 562

Electrophoresis

1. Gel formulation
   a. Resolving gel: 8% acrylamide (SDS-PAGE) in separating buffer
   b. Stacking gel: 4% acrylamide (SDS-PAGE) in stacking buffer

2. Sample preparation:
   Add 20 µg of protein to 2X volume of SDS-PAGE loading buffer; vortex
   Incubate at 70° C for 10 min
   Spin briefly and place on ice

3. Load samples and standards on the gel and run at 100 volts for approximately 1.5 h or until standards have moved far enough down to allow good resolution of target protein

Protein Isolation and Western Blotting (cont.)

Transfer into membrane

Set up transfer with components in the following order using the Bio-Rad transfer apparatus
- Black plastic
- Fabric filter
- Whatman paper (10.5 X 8 cm); 4 layers
- Gel
- Nitrocellulose membrane
- Whatman paper (10.5 X 8 cm); 4 layers
- Fabric filter
- Clear plastic

Place the sandwiche in the transfer unit with black facing black
Add a stir bar to the bottom of the tank and an ice container
Fill the tank with cold 1X transfer buffer (Tris-glycine, containing 20% methanol)
Place on a stir plate
Transfer at 100 V for 1.25 h while stirring tank buffer (replace ice container after 45 min)

Once transfer is complete, rinse nitrocellulose membrane in TBST while rocking

Blocking of blots

Incubate membranes in 5% non-fat dried milk in TBST for 1 h at RT or 4 h at 4° C while rocking (or 5% BSA in TBST for phospohotyrosine antibodies)

Immunodetection of target proteins

Add primary antibody at appropriate concentration diluted in 2% milk-TBST (or 5% BSA for phosphotyrosine Ab)
Incubate overnight at 4° C while rocking

Wash blots with TBST for 10 min, at RT in rocker. Repeat 2 X

Add peroxidase-conjugated secondary antibody at appropriate concentration diluted in 2% milk-TBST
Incubate for 1 h at RT while rocking

Wash blots with TBST for 10 min, at RT in rocker. Repeat 2 X

Add 5 ml SuperSignal detection reagent (Pierce Chemical, Rockford, IL) according to manufacture's recommendations
Incubate for 5 min at RT
Place blots on cardboard and cover with plastic wrap
Expose to X-OMAT AR filme (Kodak, Rochester, NY) for 1 min to determine appropriate exposure time
Develop and repeat exposure until desired image is attained

VITA

Marcel Amstalden was born on March 9, 1970 in Marília, São Paulo, Brazil and grew up in Assis, São Paulo, where he was involved with his family business and cattle operation. In December, 1992, Marcel graduated from the Universidade Estadual Paulista, Botucatu, São Paulo, Brazil with a Degree in Veterinary Medicine. From 1992 to 1997, Marcel worked for several ranches in the state of Mato Grosso do Sul, Brazil practicing herd health and reproductive management in beef cattle. From 1995 to 1997, he worked for the São Bernardo Veterinary Clinic, Campo Grande, Mato Grosso do Sul, Brazil practicing large animal medicine and surgery. In August 2000, Marcel graduated with a Master of Science degree in physiology of reproduction from the Texas A&M University, College Station, Texas, under the direction of Dr. Gary L. Williams. In September 2000, Marcel began a Doctor of Philosophy program in physiology of reproduction at the Texas A&M University.

While pursuing graduate degrees, Marcel served as graduate research assistant and graduate teaching assistant for laboratory sections of Reproduction in Farm Animals (Dr. David Forest), Animal Reproduction Management (Dr. Paul Harms), and Molecular Genetics (Dr. Nancy Ing) courses. Marcel was honored by the College of Veterinary Medicine, Universidade Estadual Paulista, Brazil, for outstanding performance in Large Animal Medicine, and by the Physiology of Reproduction Section, Texas A&M University, as recipient of the Mauro Procknor Memorial Awards (2000, 2001, and 2002) and the Dr. A.M. "Tony" Sorenson Jr. Achievement Award (2003). Marcel has authored or co-authored 13 refereed articles, and 23 abstracts.

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