EFFECT OF GNRH AND LEPTIN ON LUTEINIZING HORMONE RELEASE FROM DISPERSED BOVINE ANTERIOR PITUITARY CELLS IN VITRO

By

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CELLS IN VITRO

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CHAPTER I

INTRODUCTION

Reproductive efficiency is of major economic concern in the beef cattle industry, accounting for up to 10% of a farm's income (Johnson and Gentry, 2000). To maximize reproduction, one must look beyond genetic potential, to nutrition and management practices. It is known that inadequate nutrition is associated with anestrous in cattle (Connor et al., 1990) and body fat stores regulate reproduction (Selk et al., 1988; Vizcarra, et al., 1998; Morrison et al., 1999). Leptin has been proposed to be a metabolic signal that links the amount of body fat to reproduction in several species. Leptin is a protein produced by adipose tissue and is responsible for the regulation of food intake, body composition, and may have a role in reproduction (Dyer et al., 1997; Carro et al., 1997; Ahima et al., 1997). The leptin gene is expressed in the hypothalamus and adipocytes of rats (Morash et al., 1999) and receptors for leptin are localized in the paraventricular and arcuate nuclei of the hypothalamus (Yu et al., 1997; McCann et al., 1998; Lin et al., 2000).

The hypothalamus secretes gonadotropin-releasing hormone (GnRH) which controls synthesis and secretion of luteinizing hormone (LH) by the pituitary (Anderson et al., 1981). Secretion of LH in the bovine occurs in pulses (Rahe et al., 1980) and pulses of LH are associated with pulses of GnRH in

sheep (Moenter et al., 1992a) and cattle (Williams et al). Luteinizing hormone is synthesized and released from the pituitary as a result of GnRH stimulation (Moenter et al., 1992a; Rahe et al., 1980). Exogenous GnRH results in secretion of LH in vivo (Zolman et al., 1973; Kaltenbach et al., 1974; Foster, 1978; Jagger et al., 1987) and in vitro (Padmanabhan and Convery, 1978; Baratta et al., 1997; Hall et al., 1988).

Fasting reduces the expression of the leptin gene in fat cells and decreases serum concentrations of leptin in prepubertal heifers (Amstalden et al., 2000). Short term feed restriction in sheep also leads to decreased serum leptin and LH pulse frequency (Blache et al., 2000). Leptin may be a signal by which body fat and/or nutrient intake, regulate LH secretion and reproduction.

Factors affecting pituitary hormone release have been extensively studied in vitro using several animal models. These models differ in the type of culture system used, perifusion or static culture, and whole glands or dispersed cells. Dispersed cell cultures are preferred because they yield a more uniform population of cells and animal variation is removed since pituitaries can be pooled from several animals (Nakano et al., 1976). Dispersed cell culture systems are also considered less labor intensive and easier to maintain (Rodgers-Neame et al., 1984).

The objective of this experiment was to develop a static culture system of dispersed cells to evaluate the control of pituitary cells and to determine if GnRH, leptin and serum have direct effects on bovine pituitary cells to influence release of LH.

CHAPTER II

REVIEW OF LITERATURE

FACTORS CONTROLLING SECRETION OF LH

GnRH

The hypothalamus secretes the decapeptide, gonadotropin releasing hormone (GnRH) (Schally et al., 1971a). This hormone is known by several names, including LHRH, LHF and LHFSH, which are used to describe the LH stimulating portion of the compound GnRH. Therefore, when speaking of GnRH as strictly a stimulating hormone, it is generally referred to as GnRH (Schally et al., 1971b, Schally, 2000). Secretion of LH is episodic and depends on stimulation from the hypothalamus (McCann et al., 1973; Anderson et al., 1981). Luteinizing hormone is released from the pituitary as a result of GnRH stimulation from the hypothalamus (McCann and Ramirez, 1964; Rahe et al., 1980; Moenter et al., 1992a). The pattern of LH release is pulsatile and is characterized by sharp increases followed by a slow decline (Rahe et al., 1980; Fox and Smith, 1985).

Secretion of LH mimics that of GnRH. GnRH secretion in sheep is characterized by a sharp increase in concentration followed by an elevated plateau. This elevated period is completed by an abrupt decrease (Moenter et al., 1992a). Moenter et al. (1992b) found that during anestrous in ewes, GnRH was highly episodic with one pulse occurring every 6 min and each pulse lasting

4 to 5 min in length. However, the pattern of GnRH release switches from highly episodic to that of a maintained elevated level over baseline prior to the LH surge. During the LH surge, both GnRH and LH surge simultaneously, but the LH surge ended while GnRH levels remain elevated. This decrease in LH could be due to the pituitary becoming unresponsive to constant stimulation by maximal amounts GnRH (Moenter et al., 1992b; Evans et al., 1996). Blocking GnRH action in ewes at any time during the LH surge, with a receptor antagonist, causes premature regression of the LH surge (Evans et al., 1996).

Continuous administration of exogenous GnRH, to cattle results in a depression of LH released and number of GnRH receptors (Lamming and McLeod, 1988; Ronayne et al., 1993; Vizcarra et al, 1997; Vizcarra et al., 1999). Clarke et al (1986) found similar results when sheep where continuously infused with GnRH. Kakar et al. (1993) found that the pituitary is the primary location for bovine mRNA GnRH receptor is located. Thus the continuous administration of exogenous GnRH could cause a decrease in LH secretion by down regulating the high concentration of receptors in the pituitary. Nett et al. (1987) found that the number of GnRH receptors in the anterior pituitary of cattle remains unchanged until d 19 of the estrous cycle. GnRH content in the pre-optic area is positively correlated to the number of GnRH receptors present in the anterior pituitary on d 19. On the other hand, LH increases in the anterior pituitary until d 19 when concentrations decrease abruptly. Exogenous GnRH results in the synthesis and release of LH in animals and in vitro (Hall et al., 1988). Repeated injections of postpartum cows with GnRH, at a low dose, increased plasma LH

concentrations and caused pulse frequency to increase. This is similar to the LH pattern observed during the preovulatory period of cycling cows. During repeated injections, LH pulse amplitude is dependent on the dose of GnRH (Jagger et al., 1987).

A factor that should be considered when studying control of secretion of anterior pituitary hormones is the type of releasing factors that will be used, whether they are synthetic or extracts from the hypothalamus (HE). Kao et al (1977) found that whole rat pituitaries perifused with 10 ng/mL or 80 ng/mL of synthetic GnRH for 10 min, at hourly intervals for 8 to 9 h, responded just as rapidly with LH release as pituitaries perifused with hypothalamic extracts (GnRH in 1 HEq/mL ranged from 3 to 5 ng/mL). However the return to base line was less rapid after GnRH treatment than after treatment of pituitaries with HE. The difference in response could be related to the dose of GnRH used.

Synthetic GnRH is a very effective stimulator of LH release in several species. A rapid increase in serum LH follows an i.m. injection of GnRH. LH concentrations increased 15 min after the injection (Kaltenbach et al., 1974). Foster (1978) found that two injections with synthetic LHRH (1.5 h apart) to dairy cows, caused a significant increase in LH release. Giving a single injection of LHRH of the same total dose did not elicit a significant increase in LH. However, every injection of GnRH induced a LH release. Injection of bulls with 160 ug of synthetic LHRH increased LH release from 1.1 ng/mL to 39.1 ng/mL post injection (Zolman et al., 1973).

Leptin

It has been hypothesized for 40 yr that a feedback mechanism links body composition, feed intake and reproduction (Barb, 1999). Leptin, a 350-bp protein, has been proposed to be involved in this link (Dyer et a., 1997). Leptin is produced by adipose tissue and is responsible for regulation of food intake, body composition and possibly reproduction (Dyer et al., 1997; Carro et al., 1997; Ahima et al., 1997; Zamorano et al., 1997; Barash et al., 1996; Kiess et al., 1998; Cheung et al., 1997; Karlsson et al., 1997; Mantzoros et al., 1997; Satoh et al., 1997; Chehab et al., 1997).

Messenger RNA for leptin receptors has been found in fat, cerebellum, cerebral cortex, hypothami, pineal glands and pituitaries of rats (Morash et al., 1999). In pigs, mRNA for the leptin receptor is present in the cerebral cortex, amygdala, thalamus, cerebellum, area postrema, anterior pituitary and most abundantly in the hypothalamus (Lin et al., 2000). Leptin could have a critical role in metabolic control of body function, because these areas have major roles in controlling feed intake, energy balance and reproduction. Barb et al. (1998) found that intracerebroventricular injection of recombinant porcine leptin in pigs caused an increase in serum GH and decreased feed intake by up to 90%.

Leptin receptor has been found in the anterior pituitary and hypothalamus, especially in the ventromedial and acruate nuclei regions of ewes, based on the presence of nRNA. It is also found in lesser quantity in adipose tissue. The amount of mRNA for leptin receptor is much greater in feed restricted ewes than in well-fed ewes (Dyer et al., 1997). Fasting heifers for 2 d reduces expression of

leptin mRNA in adipose tissue by 42% (Amstalden et al., 2000). Leptin appears to be positively correlated with body weight, and more strongly linked to body condition score and body fat in sheep. Concentrations of leptin in plasma were constant in ewes fed a maintenance diet in which their body weight did not change (Delavaud et al., 2000). However, when ewes were fed a restricted diet, in which a significant loss of body weight and fat mass occurred, concentrations of leptin in plasma decreased.

Injection of human recombinant leptin into the arcuate nucleus (Arc), ventromedial hypothalamus (VMH) and the lateral hypothalamus (LH) of rats altered feeding activity. Food intake of the rats was reduced by 93% when leptin was injected into the Arc, but reduced by only 52% and 57% when leptin was injected into the VMH and LH, respectively. Body weight was also significantly reduced in animals injected with leptin in the Arc, VMH and LH. These findings suggest that the arcuate nucleus could play a role in satiety (Satoh et al., 1997). Yu et al. (1997) found that after a 30-min incubation of rat arcuate nucleus with a 10^{-12} or 10^{-10} M leptin, LHRH was significantly increased. Leptin also produced a positive dose response in LH release when rat anterior pituitaries were incubated with 10^{-11}, 10^{-9} and 10^{-7} M leptin. Leptin at 10^{-11} M was the minimum effective dose, and concentrations greater than 10^{-7} M did not significantly alter LH release. Similarly, McCann et al. (1998) found that LHRH release from rat anterior pituitaries responded in a dose response fashion to leptin concentrations between 10^{-7} and 10^{-11} M. LH release was not altered during the first 30 min of incubation with leptin. However, leptin at 10^{-10} and 10^{-12} M showed a tendency to

increase LHRH after 30 min (McCann et al., 1998), even though an increase in LH was not seen. In contrast to other studies, Gonzalez et al. (2000) found that female rats injected with leptin antiserum during proestrus, had increased serum concentrations of LH and advanced LH peaks. Incubation of hemipituitaries with leptin, for 60 and 120 min, resulted in a decrease in LH concentration. Leptin at 10^{-6} M decreased GnRH induced LH release, when leptin and GnRH were co-incubated.

Mutant ob/ob mice do not produce a functional form of leptin and do not reproduce. However, treatment with leptin can restore reproductive function in ob/ob females (Zamorano et al., 1997). Intraperitoneal injections of leptin given to mutant ob/ob males and females resulted in a significant weight loss of 29% and 17%, respectively. Leptin treatment significantly increased both ovarian and uterine weight in ob/ob females when compared with non treated females and also increased the number of follicles on ovaries of ob/ob females. Leptin treated males had greater testes and seminal vesicle weights when compared with non treated males and had fewer seminiferous tubule abnormalities (Barash et al., 1996).

Leptin also enhances the onset of puberty and reproductive function in mice. Chahab et al. (1997) found that injection of mice with human recombinant leptin resulted in females mating significantly earlier than control females. Leptin treatment resulted in vaginal opening (sexual maturation) 1 to 4 d earlier than control mice. Leptin also increased the weights of the uteri, ovaries and oviducts by 53%, 37.5% and 43.8% respectively, when compared with control females at

29 d of age. Leptin may be linked with puberty in human. Mean concentrations of leptin increased by approximately 100% in boys, during the months immediately proceeding the time testosterone signals puberty. After this initial increase in testosterone, concentrations of leptin decrease to near baseline concentrations of prepubertal boys (Mantzoros et al., 1997).

The attainment of body fat may also have a major role in the initiation of puberty. In girls, the increase in serum leptin was significantly associated with total body fat (Kiess et al., 1998). Ahima et al. (1997) determined that an injection of leptin to mice, at a dose that was not great enough to effect body weight, resulted in an earlier onset of puberty when compared with saline injected controls. Leptin treatment advances reproductive maturation in female rats even though it resulted in a decrease in food intake and thus body weight (Cheung et al., 1997).

Leptin, in pregnant and lactating humans, is positively correlated with body weight, body mass index, fat mass and percentage fat mass (Maffei et al., 1995; Considine, et al., 1996; Butte et al., 1997). Women that gained more weight during gestation had greater concentrations of leptin levels in plasma than women that gained less weight. Postpartum weight was also positively correlated with concentrations of leptin in plasma. A 6% decrease in fat mass during 3 months postpartum resulted in a 61% decrease in concentrations of leptin. Leptin did not differ among lactating women at 3 and 6 months postpartum, however prolactin did differ. It has been suggested that leptin can affect milk production indirectly through concentrations of prolactin in plasma

(Butte et al., 1997). Leptin receptor mRNA has been found in granulosa cells, thecal cells and interstitial cells of the human ovary. Leptin levels in follicular fluid are similar to serum levels, however there is no evidence that leptin in produced by the ovary (Karlsson et al., 1997). Leptin receptors for leptin have not yet been identified in bovine granulosa cells. However, these particular cells appear to bind leptin readily. This is evidenced by leptin suppressing steriodogenesis in bovine granulosa cells in vitro (Spicer and Francisco, 1997).

Body Condition

A major management factor associated with anestrous in cattle is less than adequate nutrition and decreased body fat reserves (Richards et al., 1989; Connor et al., 1990; Bossis et al., 1999). Cows with body condition scores less than 5 at calving have extended postpartum anestrous intervals and decreased pregnancy rates (Selk et al., 1988). Cows that calve with a body condition score of 3.0 (BSC 1 = very thin, 3 = moderate, 5 = very fat) or higher that have been allowed to gain weight for 30 d postpartum have higher pituitary LH than cows in lower body condition and thus re-initiate estrous earlier than thinner cows (Connor et al., 1990). Cows consuming a maintenance ration before and after calving have greater pregnancy rates than cows allowed to lose weight prior to calving and then allowed to either gain or maintain weight after calving (Selk et al., 1988). Inactivity of the ovary in anestrous cows could be due to decreased GnRH release (Rasby et al., 1992). During nutrient restriction, estradiol may play

a role in causing the bovine hypothalamus to become less responsive to LHRH (Imakawa et al., 1986).

MODELS TO STUDY SECRETION OF PITUITARY HORMONES

Several in vitro models have been used to study control of secretion of pituitary hormones. These models range from perifusion to static culture, and from whole glands to dispersed cells. Models have been developed for several animals including rats (Loughlin et al., 1981; Vale et al., 1972; Chao et al., 1987; Nakano et al., 1976; Walker and Hopkins, 1978; Reel et al., 1978), cattle (Padmanabhan et al., 1978; Chao et al., 1987), sheep (McIntosh and McIntosh, 1983), quail (Smith and Follett, 1972) and humans (Rodgers-Neame et al., 1984). Each system or model has advantages and disadvantages. One considerable disadvantage with the use of explants or slices of pituitary tissue in culture is that the architecture of the pituitary is not homologous. Zolman and Convey (1972) found that tissue from the central portion of the bovine pituitary released 17% more luteinizing hormone (LH) in the perifusion culture system than tissue cultured from the outer portion of the gland.

Synthesis and release of many regulatory hormones, including gonadotropin releasing hormone (GnRH), have been studied using cell culture. It has been demonstrated that dispersed pituitary cells from cattle and other species, have increased LH release when treated with GnRH and/or luteinizing hormone releasing hormone (LHRH) (Padmanabhan and Convey, 1978, Baratta et al., 1994).

The effect of leptin on secretion of LH by pituitary cell culture has been studied to determine if it is a link between body composition and reproduction. Leptin increases release of LH from cultured anterior pituitaries of rats (Yu et al., 1997; McCann et al., 1998). Leptin also restores reproductive function in ob/ob mice and reduces feed intake in mice (Zamorano et al., 1997) and pigs (Barb et al., 1998).

The objective of this study was to determine if GnRH, leptin and constituents in serum have direct effects on the release of LH in vitro from dispersed bovine anterior pituitary cells.

DEVELOPMENT OF PITUITARY CULTURE SYSTEMS

Culture of Dispersed Cells

Nakano et al. (1976) suggested that use of a dispersed cell culture system for anterior pituitaries of rats resulted in a more uniform population of cells and removed individual animal variation since pituitaries can be pooled. Enzymatic dispersion of pituitary cells alters cellular function, but this effect is overcome with a short time in culture (Chao et al., 1987). Maintenance of dispersed cell cultures is also considered to be more simplified than perifusion cultures (Rodgers-Neame et al., 1984).

Several criteria should be considered when searching for a suitable model to study secretion of pituitary hormones, particularly when studying LH. First the model should allow GnRH to be pulsed into the cell culture. Second, a base line of LH secretion must be determined. This base line must be constant, since it is

used as a comparison to determine an alteration in LH secretion. Third, LH response to identical doses of GnRH must be repeatable. Next, a dose response of LH to GnRH treatment must be apparent with both decreasing and increasing dosages of GnRH. Lastly, the model system must allow for several days of treatment and testing of the same cell population (Loughlin et al., 1981).

Enzymatic dissociation of rat pituitaries yields a homogenous cell suspension that remains viable and retains its responsiveness to stimulatory hormones (Vale et al., 1972; Nakano et al., 1976). Approximately 95% of the cells present in the suspension remained viable. This retention of responsiveness was observed in static culture by the administration of synthetic LHRH (Nakano et al., 1976) or synthetic luteinizing hormone releasing factor (LRF) (Vale et al., 1972). Cells began producing LH immediately after enzymatic dispersion, without stimulation with LHRH (Vale et al., 1972 and Nakano et al., 1976). The administration of LHRH for a 3-h incubation period had a specific and dose dependent effect on LH release. A 10^{-8} M concentration of LHRH provided maximal release of LH. This increase in LH release was approximately seven-fold that of the control cells and accounted for about 90% of the total LH present in the cells. After a 3-h incubation period, none of the concentrations of LHRH studied changed the total LH released into the cell media (Nakano et al., 1976). Vale (1972) saw similar results when a 10^{-9} M concentration of ovine LRF was administered to a cell culture every 12 h for 72 h. Cells cultures were incubated for 3 d to allow the cells to adhere to the bottom of the culture plate before treatments were administered. This allowed the removal of media from the

culture wells without removing the cells and several treatments could be conducted on the same population of cells. The maximum release of LH occurred on d 4 of incubation and decreased steadily until d 21, when the experiment was terminated.

Dispersed cell cultures are generally used in short-term studies, but they have also been used in studies that continue longer. Rodgers-Neame (1984) cultured human pituitary cells for 20 to 30 d. With 14 d of continuous LHRH treatments, LH increased five-fold during the first 4 d of treatments and then declined steadily for the remainder of the trial. When cell cultures were allowed a 7-d recovery period after LHRH treatment, LH concentrations returned to base levels and remained there until the second treatment period on 4 d which yielded a three-fold increase in LH.

Dispersed cell cultures tend to respond better to LHRH when they are allowed an incubation period without treatment. Chao (1987) observed that when bovine anterior pituitary cells were pre-incubated for less than 12 h, subsequent treatment had little effect on the total LH content. Cells that had been pre-incubated for 18 or 24 h had 81% and 234% increases in LH content, respectively, when compared with cells that had little or no pre-incubation. The maximum amount of LH release occurred in cells that had been pre-incubated for 24 h. This maximum response was achieved by treating cells with a 32 nM concentration of GnRH for 2 h. GnRH did not elicit a significant LH response after 48 h of incubation, even at higher concentration.

Culture of Whole Glands

The use of whole anterior pituitary glands is a less commonly used technique to study secretion of pituitary hormones (Smith and Follett, 1972; Martin and Klein, 1976). It does however, yield reliable information because cellular integrity is maintained for at least 4 d in a static culture system (Martin and Klein, 1976). Similar to dispersed cells, whole glands release large amounts of LH during the first 24 h of incubation in the absence of LHRH. Maximum LH stimulation was observed when glands were exposed to 10^{-6} M LHRH, and unlike dispersed cells, the response to LHRH increased with time in culture (Martin and Klein, 1976). Smith and Follett (1972) found that the amount of LH released into the media decreased to baseline concentrations within 45 min after the beginning of superfusion of quail pituitaries. Stimulation of pituitary explants, from quail, with hypothalamic extracts at a dose equivalent to .5 to 1.0 quail hypothalamus, elicited a response that was 2- to 10-fold greater than basal release during a period of 6 to 11 h. This response occurred rapidly after the infusion of the extract, and the response declined as the extract diffused out of the chamber containing the pituitary.

DISPERSED CELL CULTURE SYSTEMS TO EVALUATE CONTROL OF LH SECRETION

Rodent Model

The majority of the information that is available on dispersed anterior pituitary cell cultures is with rats. Rats have been used to investigate the effects of LHRH on release of LH in vitro (Drouin and Labrie, 1976; Nakano et al., 1976;

Reel et al., 1978; Loughlin et al., 1981; Grotjan and Leveque, 1984). Others have used rat glands to perfect enzymatic dissociation of anterior pituitary cells (Portanova et al., 1970; Vale et al., 1972; Hopkins and Farquhar, 1973).

Reel (1978) used dispersed anterior pituitary cells from rats to determine if the differences in responsiveness of the pituitary to LHRH throughout the estrous cycle of the rat was maintained when the cells were dissociated. It was observed that there was no difference in LH release when cells were obtained on different days of the estrous cycle. However, cell response to treatment was greater during 3 to 4 d in culture when compared with d 1 and d 2. It is possible that the collagenase used to dissociate cells may cause temporary damage to the cells that is overcome within a few days in culture.

The effect of LHRH or other secretory compounds on LH secretion has been evaluated using cell cultures of rat anterior pituitaries. It has been consistently observed that LHRH is more effective at stimulating LH release than cAMP, cGMP (Grotjan and Leveque, 1984), elevated K^+ (Grotjan and Leveque, 1984; Nakano et al., 1976) or dibutyryl cyclic AMP (Nakano et al., 1976). Drouin and Labrie (1976) determined the inhibitory effects of androgens, such as testosterone and 3α-androstanediol, on LHRH stimulated LH release in vitro. When anterior pituitary cells were incubated for 72 h with 10^{-10} M LHRH in combination with 10^{-8} M testosterone or 10^{-8} M 3α-androstanediol, release of LH was 15 to 20% less than when cells were incubated with only LHRH.

Ovine Model

McIntosh and McIntosh (1983) used dispersed sheep anterior pituitary cells to determine the effect of pulsatile treatment with 1 ng/mL GnRH on LH release. Pulse frequency and duration, but not amplitude, influenced LH response. Intervals of between GnRH pulses that were less than 16 min caused a decrease in stimulation, and desensitization to GnRH occurred more quickly. The leading edge of a GnRH pulse caused a greater release of LH than did the continuous administration of GnRH over a pulse period. Greater concentrations of GnRH did not cause desensitization, but shortening the intervals between GnRH pulses resulted in release of less LH.

Porcine Model

Enzymatic dissociation of anterior pituitary cells has also been performed with porcine tissue. Walker and Hopkins (1978) applied this procedure to determine if porcine anterior pituitary cells in culture would response to LHRH treatment and if the release of LH in response to LHRH treatment is biphasic. They found that the maximal response to LHRH was at a concentration of 10^{-8} M after a 2-h incubation period. Barb et al (1990) also found that 4 h incubations with 10^{-9}, 10^{-8} and 10^{-7} M GnRH resulted in a 140%, 210% and 250% increases in LH release, respectively. However, after 24 h of incubation, pituitary cells no long responded with a dose response, but GnRH treated cells still had greater concentrations of LH in medium than did the controls. Similar to rodent cells,

porcine cells also were unresponsiveness to GnRH treatment during the first days of incubation (Walker and Hopkins, 1978).

Bovine Model

Secretion of LH in response to LHRH treatment by dispersed bovine anterior pituitary cells has been studied by several investigators (Padmanabhan et al., 1978a; Padmanabhan and Convey, 1978b; Padmanabhan et al., 1981; Baratta et al., 1994). Cells have been maintained in static culture for approximately 6 d (Panmanabhan et al., 1978a; Padmanabhan and Convey, 1978b; Baratta et al., 1994) or in a perifusion culture system for up to 5 d (Padmanabhan et al., 1981; Hassan and Merkel, 1994). The number of cells in static cultures ranged from $5x10^5$ cells/well (Padmanabhan and Convey, 1978a; Padmanabhan et al., 1978b) to $1.0\text{-}1.5x10^5$ cells/well (Baratta et al., 1994), and $5x10^6$ cell/tube when using superfusion techniques (Padmanabhan et al., 1981).

Padmanabhan and Convey (1978a) found that bovine pituitary cells, dispersed with collagenase, responded poorly to LHRH treatments early in culture, but the response improved with increased culture time. This recovery of response with increased incubation time is consistent with the responses in rodents (Reel et al., 1978) and swine (Walker and Hopkins, 1978). Release of LH increased linearly with dose of LHRH, and cells had a consistent maximum response to 100ng/mL of LHRH after 24 h of incubation. Incubation of cells with estradiol in combination with LHRH for more than 3 h resulted in increased LH released when compared with controls (Padmanabhan and Convey, 1978b).

Baratta (1994) found similar results. Release of LH by cells had begun to decline after 24 h in culture. However, LH release was significantly increase after cells were incubated with estradiol for 15 h. When cells were incubated with LHRH in combination with estradiol, there was an initial inhibitory effect on LH due to estradiol, then a stimulatory response followed by another inhibitory phase after 48 h of co-incubation.

SUMMARY OF CULTURE SYSTEMS

A summary of several pituitary culture systems is represented in Table 1. This table allows comparisons to be made among species. It also allows comparison of culture systems along with hormone dosages used and results of each study. References are included as a source to where detailed information about each experiment can be found.

SUMMARY

Calf crops can be reduced by increased postpartum intervals of cows, due to inadequate nutrient intake of the cow during gestation and early lactation. The links between nutrient intake, body composition and pituitary function are not known, however, they clearly exist. Several signals, most likely acting in combination with one another, are known to be involved in the relationship among food intake, leptin, pituitary hormones and consequently reproduction.

However, it is not know in what particular sequence these signals occur and how species differences could be involved.

It has been determined that leptin controls food intake and body composition. Treatment with leptin also restores reproduction in sterile ob/ob and advances the onset of puberty in normal mice. Leptin receptor mRNA is located in the anterior pituitary and hypothalamus of sheep and fasting reduces the expression of these receptors. In cattle and other species, these areas of the brain also control LH synthesis and release through GnRH. Therefore, it is logical to conclude that there is a possible link between serum leptin levels and LH release.

Due to the difficulty of injecting leptin, or any other compound, directly into the bovine brain, an in vitro approach was chosen. Several other investigators have demonstrated that dispersed rat, swine and bovine cells show a dose dependent response to GnRH and LHRH treatments in vitro.

Taking these factors into consideration, our objectives were to determine if GnRH and leptin have a direct effect on bovine pituitary cells to influence the release of LH.

CHAPTER III

EFFECT OF GNRH AND LEPTIN ON LUTEINIZING HORMONE RELEASE FROM DISPERSED BOVINE ANTERIOR PITUITARY CELLS IN VITRO

ABSTRACT

Enzymatically dispersed bovine anterior pituitary cells were used to determine the effects of GnRH, leptin and serum on release of luteinizing hormone in vitro. Steer pituitaries were obtained immediately after slaughter. Connective tissue was removed from anterior pituitaries and slices of tissue were exposed to collagenase to disperse cells. Dulbecco's modified Eagle medium with 1% newborn calf serum, 2% glutamine, and antibiotics was used for culture of cells. Cells (approximately 100,000/well) were plated in 24-well culture plates and incubated at 38.5° C with 95% air and 5% CO_2. After 72 h of incubation, medium was removed and cells were washed three times with medium and cells were treated with 0, 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M GnRH (Experiment 1). Cells were incubated for 1 h with GnRH and medium was removed and frozen (Exposure 1). Cells were then incubated for 4 h with the same concentration of GnRH (Exposure 2). After 4 h, medium was removed and frozen and medium without GnRH was added. After 20 h, medium was removed and frozen and medium containing the same concentrations of GnRH as the previous day was added. After 4 h medium was removed and frozen. In Experiment 2, cells were prepared

and incubated as described for Experiment 1. Cells were treated with 0, 10^{-11}, 10^{-10} or 10^{-9} M concentrations of recombinant mouse leptin, with and without 10^{-7} M GnRH. After 4 h incubation, medium was removed and frozen (Exposure 1) and medium without leptin was added. After 20 h, medium was removed and frozen for analysis (Post-leptin) and medium containing the same leptin concentrations used the previous day was added for 4 h, then medium was removed and frozen (Exposure 2). Concentrations of LH were quantified in all media.

Concentrations of LH increased after 1 h of incubation with 10^{-9} and 10^{-8} M GnRH ($P < .01$). Other concentrations of GnRH did not alter release of LH during the incubation periods. During experiment 2, GnRH at 10^{-8} M did not influence LH release. Leptin at 10^{-9} and 10^{-10} M suppressed ($P < .003$) LH release by 42% and 35% respectively, in rep 1 when compared with control cells during Exposure 1. However, leptin did not alter LH release during rep 2. Leptin (10^{-10}, 10^{-9} and 10^{-8} M) suppressed LH release by 32% in a second trial ($P < .005$). During the 20-h post-leptin incubation, LH release was 46% greater ($P < .0002$), in reps 1 and 2, and 29% greater ($P < .02$) in a second trial, by cells previously treated with leptin when compared with control cells. When cells were exposed to leptin for 4 h on the second day of treatment (Exposure 2), LH release was decreased by 41% (reps 1 and 2) compared with control cells ($P < .0001$). In trial 2, leptin at 10^{-8} M decreased LH release by 19% on d 2 when compared with other concentrations of leptin ($P < .09$). We conclude, that with this culture system, GnRH causes release of LH from bovine pituitary cells in vitro. The decrease in LH release during exposure to leptin followed by increased LH release after

leptin treatment was removed, indicates that leptin acts directly on bovine pituitary cells to alter the release of LH.

INTRODUCTION

The hypothalamus secretes gonadotropin-releasing hormone (GnRH) which controls synthesis and secretion of luteinizing hormone (LH) by the pituitary (Schally et al., 1971, McCann, 1977). Secretion of LH in the bovine occurs in pulses (Rahe et al., 1980) and pulses of LH are associated with pulses of GnRH (Gazal et al., 1998). The bovine pituitary requires stimulation by the hypothalamus for LH synthesis and release (Anderson et al., 1981). Exogenous GnRH results in secretion of LH in vivo and in vitro (Zolman et al., 1973; Hall et al., 1988). Repeated injections of a low dose of GnRH to postpartum (Jagger et al., 1987) and nutritionally induced anovulatory cows, causes secretion of LH that is similar to secretion during the preovulatory period of cows (Bishop, 1995; Vizcarra et al., 1997).

Analogues of GnRH are effective stimulators of LH release in several species. Serum LH in cattle increases rapidly following GnRH treatment (Zolman et al., 1973; Kaltenbach et al., 1974). Injection of dairy cows with synthetic LHRH resulted in increased release of LH and two injections at a 1.5 h interval resulted in greater LH release than did a single injection (Foster, 1978).

Body fat stores regulate reproduction in cattle (Richards et al., 1989; Selk et al., 1988; Vizcarra, et al., 1998). Leptin has been proposed to be a metabolic

signal that links the amount of body fat to reproduction. Leptin is a protein produced by adipose tissue and is responsible in some species for the regulation of food intake and body composition, and may have a role in reproduction (Dyer et al., 1997; Carro et al., 1997; Ahima et al., 1997). The leptin gene is expressed in the hypothalamus and adipocytes of rats (Morash et al., 1999) and receptors for leptin are localized in the paraventricular and arcuate nuclei of the hypothalamus (McCann et al., 1998; Yu et al., 1997).

Fasting reduces the expression of the leptin gene in fat cells and decreases serum concentrations of leptin in prepubertal heifers (Amstalden et al., 2000). Fasted heifers had reduced pulsatile secretion of LH and changes in LH pulses were related to changes in GnRH secretion by the hypothalamus. Leptin may be a signal by which body fat and/or nutrient intake regulates LH secretion and reproduction.

The objectives of these experiments were to determine if GnRH, leptin and serum have direct effects on bovine pituitary cells to influence release of LH.

MATERIALS AND METHODS

Dispersion of Cells

Pituitary glands were removed from steers immediately after slaughter, placed on ice in 50-mL polystyrene conical tubes (Corning) containing 25 mL of Hank's Balanced Salt Solution (HBSS; Gibco BRL, Grand Island, NY) at 4°, and transported to the laboratory. Steers were of beef breeding and weighed

approximately 550 kg. Pituitaries were processed under sterile conditions in a laminar flow hood. Instruments were washed with 70% ethanol and air-dried in the hood.

Glands were dissected from connective tissue and the posterior lobe was discarded. The anterior portion of the pituitary was washed with HBSS to remove blood cells. Anterior pituitaries from 2 to 3 steers were sliced into 1mm slices with a Stadie-Riggs Microtome and slices were pooled. Then slices were cut into smaller pieces (approximately 2 X 2 mm) with a scalpel blade. The pieces of pituitary tissue were exposed to a collagenase solution, which consisted of .075 g of lypholized type I collagenase (Gibco BRL, Grand Island, NY) in 25 mL of HBSS, for 45 min on a rocker plate in an incubator at 38.5°C. Then the tissue and collagenase solution was triturated 20 times with a sterile 10-mL pipette to loosen cells clinging to connective tissue. Solution was then filtered through a 25 mm wire mesh syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) into a 50-mL conical tube. The filtrate was centrifuged at 2000 x g for 5 min at 22°C. The supernatant was discarded and the pellet of cells was resuspended in 5 mL of HBSS and transferred into a 12-mL conical tube (Corning). The 50-mL tube was washed with an additional 2-mL of HBSS and this was also added to the 12-mL conical tube. The suspension of cells was recentrifuged for 5 min at 2000 x g. The cell washing procedure was repeated twice. The pellet was suspended in a Dulbecco's Modified Eagle Medium solution (DMEM; Gibco BRL, Grand Island, NY) which contained 1% newborn calf serum (Gibco BRL, Grand Island, NY), 2% L-glutamine-200mM (Gibco BRL)

and .001% Antibiotic Antimycotic (Gibco BRL). Dulbecco's Modified Eagle Medium plus additives will be designated as DMEM throughout the manuscript, except when stated that serum was not added. The resuspended pellet was centrifuged, the supernatant discarded, and the cells were resuspended in DMEM. DMEM was also used as the culture medium.

Pituitary cells were stained for counting by combining 400 uL of the cell suspension, in DMEM solution, with 100 uL of 0.4% trypan blue dye (Sigma, St. Louis, MO). Viable cells were counted on a hemacytometer at 430 x. the remainder of the cells were diluted in DMEM and plated in Falcon polystrene 24-well tissue culture plates (Becton Dickinson and Company, Lincoln Park, NJ) at a concentration of 100,000 viable cells per well. Cells were incubated for 72 h in a humidified incubator with 95% air and 5% CO_2 at 38.5°C to allow cells to adhere to the culture plate before treatment. These conditions were used for all incubations.

Experiment 1: Effect of GnRH on LH Release

Experiment 1A. After the 72-h incubation period, medium was removed from the wells. Wells were washed three times with 500 uL of DMEM and GnRH treatments were added. A well was assigned to a treatment for all incubation periods. A total volume of 1 mL of DMEM per well was maintained throughout the incubation period. Medium with 0, 10^{-9}, 10^{-8}, 10^{-7}or 10^{-6} M concentrations of GnRH (Sigma) was added to appropriate wells.

During the first treatment period (d1), DMEM with the designated concentration of GnRH was added to the wells and cells were incubated for 1 h, then medium was removed and stored (-20°C) for LH analysis (Exposure 1). Then cells were washed twice with 500 uL of DMEM and treatments were repeated. After 4 h of incubation (Exposure 2), medium was removed and stored (-20°C), cells were washed twice with 500 uL of DMEM, and 1 mL of DMEM without GnRH was add to each well for a 20-h incubation (Post-treatment). On d 2, medium was removed and frozen for analysis and wells were washed twice with 500 ul of DMEM solution. A second series of 1-h and 4-h incubations with the same concentrations of GnRH were conducted as described for d 1. At completion of the 4-h incubation, medium was removed and frozen, and the trial was terminated. Luteinizing hormone was quantified in all medium. Four replications of the experiment were conducted with cells from 2 to 4 steers per replication and 1 to 4 wells per treatment.

Experiment 1B. Effect of GnRH on LH Release in the Absence of Serum in Medium. An experiment was conducted to determine the effect of serum on the response of pituitary cells to GnRH. Dulbecco's Modified Eagle Medium plus additives was used for incubations, except for the treatment without serum. Incubation times for this experiment were similar to Experiment 1A. Concentrations of GnRH used were 0, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}or 10^{-6} M, with and without 1% new born calf serum. Each of two replications included cells from two steers, with 3 to 6 wells per treatment combination.

Experiment 2: Effect of Leptin on LH Release

Experiment 2A: After the 72-h pre-treatment incubation period, medium was removed and wells were washed three times with 500 ul of DMEM and leptin treatments were added. A 4 X 2 factorial design was used with 1 mL of medium containing 0 (Control), 10^{-11}, 10^{-10} or 10^{-9} M leptin (NHPP-NIDDK) added to appropriate wells with and without a final concentration of 10^{-7} M GnRH.

After a 4 h incubation, medium was remove and frozen (-20°C) for LH analysis (Exposure 1). Then wells were washed twice with 500 uL of DMEM and 1 mL of DMEM without leptin was added to each well and cells were incubated for 20 h (Post-treatment). Then medium was removed and frozen (-20°C), wells were washed twice, and the same leptin treatments were added to each well. After 4 h incubation (Exposure 2), medium was removed and frozen. Wells were washed twice and 1 mL of DMEM without leptin was added. After 20-h incubation, medium was removed and frozen. Luteinizing hormone was quantified in all medium. Each of two replications included cells from 2 or 3 steers, with 3 or 4 wells per treatment combination.

Experiment 2B. This experiment was similar to Experiment 2A, except an additional concentration of leptin was evaluated (10^{-8} M) and 1-h incubations were added. A 5 X 2 factorial design was used and medium (1 mL) with 0 (Control), 10^{-11}, 10^{-10}, 10^{-9} or 10^{-8} M leptin was added to appropriate wells with and without 10^{-7} M GnRH. One replication was conducted, including cells

from 4 steers, with 2 to 5 wells per treatment combination. Luteinizing hormone was quantified in all medium.

After the 72-h pre-treatment, cells were triple washed and 1 mL of medium with the designated concentration of leptin was added and cells were incubated (d 1). After 1 h, medium was removed and frozen for LH analysis (Exposure 1). Then wells were washed twice with 500 uL of DMEM and leptin treatments were repeated. After 4 hr, medium was removed and frozen for later analysis (Exposure 2). Wells were washed twice with 500 uL of DMEM and 1 mL of DMEM without leptin and GnRH was added to each well and cells were incubated for 20 h (Post-treatment). Then medium was removed and frozen, and wells were washed twice with DMEM. On d 2, cells were exposed to the same treatments as on d 1 for 1 h (Exposure 3) and 4-h (Exposure 4) incubations were conducted. After 4 h, medium was removed and frozen for later analysis. Wells were washed twice with DMEM, 1 mL of DMEM without leptin and GnRH was added to each well and cells were incubated. After 20 h, medium was removed and frozen.

Experiment 2C: Effect of leptin on the LH release in the absence of serum in medium. The effect of serum in DMEM on the response of pituitary cells to leptin was evaluated using the procedure described to evaluate the effect of leptin. A 2 X 2 X 5 factorial design was used. Wells received medium with or without new born calf serum, GnRH (10^{-7} M) or no GnRH, and leptin

concentrations of 0, 10^{-10}, 10^{-11}, 10^{-12} or 10^{-13} M. Two replicates included cells from 3 steers per replicate, with 1 to 2 wells per treatment combination.

After the 72-h pre-treatment period, medium was removed and wells were washed three times with serum free DMEM and then exposed to leptin in DMEM (1 mL) with or without serum, and with or without GnRH. Wells were assigned to a treatment for all incubation periods. After 1 h of incubation, medium was removed and frozen for analysis (Exposure 1). Then wells were washed twice with 500 uL of serum free DMEM, and treatments were repeated for 4 h (Exposure 2). After 4 h, medium was recovered and frozen, wells were washed twice with serum free DMEM, and 1 mL of either DMEM plus serum or DMEM without serum, without leptin and GnRH, was added to each well. After 20 h, medium was removed and frozen, wells were washed twice with DMEM without serum, and cells were exposed for 4 h to the same leptin, GnRH, and serum treatments as the previous day. After 4 h, medium was recovered and frozen for later analysis (Exposure 3). Wells were washed twice with 500 uL of serum free DMEM and either serum-free DMEM (1 mL) or 1 mL of DMEM plus serum (same serum treatment as the previous incubation) was added to the wells and incubated for 20 h. Then medium was removed and frozen and the trial was terminated. Luteinizing hormone was quantified in all medium.

STATISTICAL ANALYSIS

Treatment effects and their interactions were assessed using the GLM procedure of SAS. Each experiment was replicated two, three or four times with

a different pool of cells from 2 to 4 steers for each pool. For each incubation period, main effects were treatments (GnRH, leptin, serum), replication and the interactions. When mean concentrations of LH among replicates differed by more than 2-fold, concentrations of LH were expressed as a % of the control wells before analysis. Concentrations of LH are expressed as Least Squares Mean ng per mL of medium. Differences among treatments were determined using Fisher's protected lsd procedure.

RESULTS

Experiment 1A

There was a rep X treatment effect on concentrations of LH in medium after 1 h of exposure to GnRH (Exposure 1). This effect was due to a difference in the magnitude of LH release between replicates, not a difference in direction of change in the concentrations. Thus, the main effect of concentration of GnRH on LH is presented in Figure 1. Concentrations of GnRH at 10^{-9} and 10^{-8} M increased ($P < .04$) LH release into medium following the first 1-h incubation when compared with control cells. Gonadotropin releasing hormone tended to increase LH ($P < .12$) in medium during the ensuing 4-h incubation period (Exposure 2; Figure 2). During the second 4-h incubation (d 2), greater concentrations of GnRH (10^{-8}, 10^{-7} and 10^{-6} M) resulted in decreased concentrations of LH in medium ($P < .001$; Figure 3).

Experiment 1B

The addition of serum to medium during the first 1-h incubation period resulted in a 37% increase ($P < .0001$). However, treatment with GnRH did not alter LH release ($P > .10$; Exposure 1; Figure 4). Addition of serum to medium increased concentration of LH released into medium by 25% ($P < .0006$) during the first 4-h incubation period (Exposure 2) compared with wells that received no serum. Gonadotropin releasing hormone (10^{-7} and 10^{-8} M) also increased LH concentrations after 4 h of incubation ($P < .05$; Figure 5). During the 20-h incubation period without GnRH, concentrations of LH were increased by 62% ($P < .0001$) in wells that received serum when compared with wells without serum. Wells that had previously received GnRH (10^{-6} and 10^{-8} M) had decreased LH concentrations ($P < .03$) during the 20-h incubation period (Figure 6). Serum increased LH concentrations by 20% ($P < .01$) on d 2 after 1 h of incubation when compared with wells that did not receive serum, but GnRH did not alter ($P > .10$) LH concentrations (Figure 7). Addition of serum or GnRH did not alter ($P > .20$) concentrations of LH after 4 h incubation on d 2 (Figure 8).

Experiment 2

Experiment 2A: Treatment with GnRH did not alter ($P > .10$) concentrations of LH in medium when cells were treated with leptin. There was a leptin X rep effect on concentrations of LH in medium after 4 h of incubation. During rep 1, leptin at 10^{-9} and 10^{-10} M suppressed concentrations of LH ($P < .003$) in medium by 42% and 35% respectively, compared with control cells

(Figure 9; Exposure 1). Leptin did not influence LH release in rep 2. During the first 20-h post-leptin treatment period, cells treated with 10^{-10} M leptin during exposure 1 had a 46% increase in LH concentration ($P < .002$) in medium when compared with control cells (Figure 10). There was a rep x leptin effect on concentrations of LH in medium after the second 4-h period (Exposure 2). This effect was due to a difference in magnitude of LH release between replicates, not a difference in direction of change in concentrations. The main effect of concentration of leptin on LH within replicate is presented in Figure 11. Leptin resulted in an average decrease of 41% in LH concentrations ($P < .0001$) when compared with the control cells.

Experiment 2B: This experiment was similar to Ex. 2A, with the exception of the addition of 10^{-8} M leptin and 1-h incubations. Treatment with GnRH did not alter LH release. After cells were incubated with leptin for 1 h (Exposure 1), leptin (10^{-8}, 10^{-9} and 10^{-10} M) decreased LH concentrations in medium by an average of 32% ($P < .005$; Figure 12), but leptin did not influence ($P > .20$) LH in medium during the ensuing 4-h incubation (Exposure 2; Figure 13). Cells previously treated with leptin (1-h and 4-h) had increased LH concentrations (29%; $P < .02$) in medium during the 20-h incubation period without leptin (Figure 14). Leptin at a concentration of 10^{-8} M resulted in a 25% decrease in LH release ($P < .09$) during the 1-h incubation on d 2 (Exposure 3; Figure 15). During the second 4-h incubation with leptin and during the 20-h incubation without leptin, leptin did not alter ($P > .10$) LH concentrations in medium (Figures 16 and 17).

Experiment 2C: The addition of serum to medium resulted in almost a 2.5-fold increase in LH concentration during both the first 1-h incubation period ($P < .0001$; Table 2) and also during the ensuing 4-h incubation period ($P < .0001$; Table 3) when compared with cells incubated without serum. Leptin and GnRH did not alter LH concentrations during the first 1-h incubation. However, there was a serum x leptin effect, associated with an increase in LH concentrations in medium during the first 4-h incubation period when cells were exposed to leptin and serum ($P < .0001$; Table 3). Cells previously treated with serum had increased (80%; $P < .001$) LH concentrations in medium during the 20-h incubation period (Table 4). Treatment with leptin and/or GnRH did not alter LH concentrations during this time. Serum, leptin and/or GnRH did not alter LH concentrations during the 1 or 4-h incubations on d 2 (Tables 5 and 6). Cells previously treated with leptin on d 2 had increased ($P < .10$; 21%) Treatment with leptin and GnRH failed to alter LH concentrations during the 20-h incubation period (Table 7).

Discussion

Treatment with GnRH for 1 h resulted in an increase in LH released into medium. This was consistent with other studies in which GnRH stimulated LH release in vitro (Chao et al., 1987; Pandmanabhan and Convey et al., 1978; Baratta et al., 1994). In our model, 10^{-9} and 10^{-8} M GnRH, were the only concentrations that induced a significant increase in LH released into medium.

Incubation with GnRH for 4 h, after the 1-h exposure on the first day of treatment, did not result in a significant increase in LH concentrations in medium. After 4 h of incubation with GnRH (10^{-8}, 10^{-7} and 10^{-6} M) on d 2, concentrations of LH were decreased in medium. Barb et al. (1990) found a significant linear response to 10^{-9}, 10^{-8} and 10^{-7} M GnRH after 4-h incubation with porcine anterior pituitary cells. The reason porcine cells responded linearly to increased GnRH at longer incubations, when bovine cells did not, could be due to greater doses of GnRH at longer incubation times causing negative feedback and/or down regulation to occur on bovine pituitary cells. The lack of LH response on d 2 could also be attributed to the fact that the cells had been in culture for 5 d at this point and possibly had depleted their LH stores.

Infusion of nutritionally anestrous cows with GnRH, increased LH concentrations when given in pulses within a period from 0.1 to 10 min (Bishop et al., 1993; Vizcarra et al., 1999). However, when GnRH was infused at a constant amplitude and frequency to anestrous cows (Vizcarra et al., 1999) or, to OVX sheep (Handelsan et al., 1988), LH secretion decreased. Sheep pituitary cells in vitro responded to an increase in GnRH pulse duration with a decrease in LH concentrations (McIntosh and McIntosh, 1983). This suggests that the pituitary of sheep and cattle are sensitive to pulses of GnRH. By giving large doses of GnRH in a static culture system, pituitary cells could become unresponsive to GnRH because of down regulation or internalization of receptors for GnRH.

The addition of serum to medium with GnRH (Experiment 1B) resulted in a 37% increase in LH release after the first 1-h incubation period and a 25%

increase during the ensuing 4-h incubation. Addition of GnRH (10^{-8} and 10^{-7} M) to medium during the 4-h incubation also increased LH concentrations. Cells exposed to serum continued to have greater concentrations of LH in medium during the 20-h incubation and 1-h incubation on d 2. However, GnRH treatments on d 1, decreased LH concentrations in medium. During the 4-h incubation (d 2), neither serum nor GnRH altered LH concentrations in medium. By d2 (5 d after the initiation of culture), medium in serum free wells was cloudy, indicating that the cells were detaching from the bottom of the plate. Serum in vitro provides nutrients, as well as binding factors which help the cells attach to the plate (Barnes and Sato, 1980). Perhaps our cells were detaching due to lack of serum in the medium.

Hayashi and Sato (1976) found that the addition of serum to culture medium is critical to primary rat pituitary cell cultures. When cells were cultured in serum free medium, growth of cells was severely retarded or did not occur. This lack of growth was not overcome when pituitary hormones were added to the medium, but when T_3, thyrotropin-releasing hormone and transferrin were added to the serum free medium, cell growth was comparable to cells grown in medium containing serum. Serum may provide certain hormones and growth factors needed by cells in vitro.

Leptin suppressed the release of LH from pituitary cells during incubations for 1 or 4 h. This is in agreement with reduced LH release from rat hemipituitaries incubated with leptin (Gonzalez et al., 2000). Leptin at 10^{-9} and 10^{-10} M significantly decreased LH concentrations in medium after 4 h of

incubation. However, during the 20-h incubations without leptin, LH concentrations increased in wells that had been previously exposed to leptin when compared with wells that had not been exposed to leptin. During the second 4-h incubation, all concentrations of leptin decreased LH concentrations. In vivo, leptin had a stimulatory effect on serum LH concentrations. Nagatani et al. (2000) injected castrated males sheep with recombinant leptin. During the fed period, LH concentrations between control and leptin treated groups did not differ. During fasting, leptin treated sheep had higher levels of LH than did control sheep. However, Blache et al. (2000) found that acute increases in feed intake results in increased concentrations leptin in plasma and cerebrospinal fluid. This increase in leptin was related to an increase in GnRH release, which in turn, cause LH to increase. This suggests that leptin may have a role in sheep by linking food availability and reproduction through LH secretion. However, the rational behind why leptin is stimulatory in vivo and causes suppression of LH in vitro is not clear. Perhaps the concentrations of leptin we selected were nonphysiological, or the concentrations could have caused down regulation to occur. Concentrations of leptin used were similar to those used with other species in vitro, but continuous exposure in vitro for 1 or 4 h gives a different response than acute in vivo exposure.

Treatment with GnRH did not alter LH concentrations during this experiment to evaluate the effect of leptin on LH release. In vivo, constant infusion of exogenous GnRH causes the depression of LH release and numbers of GnRH receptors (Lamming and McLeod, 1988; Ronayne et al., 1993; Vizcarra

et al, 1997; Vizcarra et al., 1999). The lack of a GnRH response during this experiment could be caused by down regulation of GnRH receptors. The concentration of GnRH (10^{-7} M) used during Experiment 2 was determined from the results of Experiment 1. In an effort to choose the concentration of GnRH, which would elicit the greatest LH response, we may have inadvertently selected a concentration that was too great and caused down regulation of the cells.

When a lesser concentration of leptin (10^{-11} M) and a shorter incubation (1 h) were used, results were similar to the previous experiment. Incubation with leptin decreased LH concentrations after the first 1 h of incubation, but not during the following 4-h incubation. Similar to the previous experiment (Experiment 2A), cells previously treated with leptin had greater concentrations of LH in medium during the 20-h incubation without leptin. On d 2, leptin (10^{-8} M) decreased LH release after the 1-h incubation, but leptin, at any concentration, did not alter LH concentrations during the second 4 and 20-h periods. The lack of response during these two incubations could be caused by several factors. Cells had been in culture for 5 d at this point, may have had depleted LH stores or had become less functional or leptin could have down regulated LH release to a point the cells could not recover on the second day of treatment.

Addition of serum to medium with leptin resulted in an approximately 2.5-fold increase in LH concentrations on d 1 of incubation. Leptin increased LH concentrations in medium during the first 4-h incubation period. Cells previously treated with leptin had greater LH concentrations during the 20-h incubation period after exposure to leptin on d 1 and d2, when compared with cells that

were not previously exposed to leptin. This is in agreement with results from Experiments 2A and 2B. Treatment with serum, leptin and/or GnRH did not alter LH concentrations on d 2. This could be do to death of cells or that leptin, or a combination of leptin and GnRH, down regulated the cells to a point at which they could not recover this late in culture.

IMPLICATIONS

The decrease in LH release during exposure to leptin followed by increased LH release after leptin was removed indicates that leptin has a direct effect on the pituitary to modulate LH release, therefore, regulating reproduction in cattle. Leptin may also have effects on the brain or hypothalamus to regulate LH release. This study clearly shows that leptin modulates LH release at level of the pituitary.

Treatment with GnRH stimulated LH release. The decrease in LH release after 4 h of treatments could have been caused by down regulations of the cells by persistent treatment with GnRH. In vivo, GnRH is released in a pulsatile manner, which was not duplicated during treatments in these experiments. These results support the concept that constant exposure of bovine pituitary cells to GnRH, decreases secretion of LH. Pulsatile treatment of cattle with GnRH is necessary to increase LH secretion.

Leptin influences secretion of LH by bovine pituitary cells and may be a signal by which nutrition and fat deposition influence reproduction. Additional

research is necessary to evaluate the effects of duration of exposure and concentration of leptin on bovine LH secretion in vitro and in vivo.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Treatment of bovine anterior pituitary cells in vitro with GnRH, for 1 h, stimulates LH release. Incubation for an additional 4 h tended to increase LH release and exposure to GnRH for 4 h the next day decreased LH release. Decreased LH release on d 2 of treatment could be due to down regulation of pituitary cells by GnRH and/or a decrease in viability of cells.

The addition of serum to media resulted in a dramatic increase in LH release until d 2 of treatments. By this point, cells had been in culture for 5 d and viability could be decreased. Cells that received serum-free media were also detaching from the bottom of the culture plate by d 3 of treatment.

During incubation with leptin, LH release was suppressed. However, after leptin treatments were removed, cells previously exposed to leptin had greater concentrations of LH in media, than cells that had not been exposed to leptin. In vivo, leptin is stimulatory. The explanation for the leptin suppressive effect in vitro remains uncertain, but perhaps the concentrations of leptin we used in our experiments were nonphysiological for LH release.

We conclude that GnRH stimulates and leptin inhibits the release of LH from bovine anterior pituitary cells in vitro. These results indicate that leptin has a direct effect on the bovine pituitary to regulate LH release.

Table 1. Influence of GnRH/LHRH on LH Release from Pituitaries or Dispersed Pituitary Cells in Perifusion or Static Culture.

Animal	Culture Type	Culture System	Hormone	Dose	Results	Reference
Rats	Whole gland	Static	Synthetic LHRH	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M	Increased	Martin et al., 1976
Rats	Whole gland	Perifusion	Synthetic GnRH or HE	10 ug/ml or 1 HEq/mL	Increased	Kao et al., 1977
Human	Dispersed	Static	LHRH	5 ng/mL	Increased	Rodgers-Neame et al., 1984
Quail	Whole gland	Perifusion	LHRF		Increased	Smith and Follett, 1972
Pig	Dispersed	Static	LHRH	10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M	Increased	Walker and Hopkins, 1978
Rats	Dispersed	Perifusion	LHRH or HE	50 ng/mL or 1 HEq	Increased	Loughlin et al., 1981
Rats	Dispersed	Static	Synthetic LHRH	10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M	Increased	Drouin and Labrie, 1976
Rats	Dispersed	Static	Synthetic LHRH	10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M	Increased	Nakano et al., 1976
Rats	Dispersed	Static	LHRH	10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M	Increased	Grotjan and Leveque, 1984
Rats	Dispersed	Static	LHRH	10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M	Increased	Reel et al., 1978
Rats	Dispersed	Static	Synthetic LRF	10^{-10}, 10^{-9}, 10^{-8} M	Increased	Vale et al., 1972
Sheep	Dispersed	Perifusion	GnRH	.001, .01, .1, 1, 10, 100 ng/mL	Increased	McIntosh and McIntosh, 1983
Cattle	Dispersed	Static	GnRH	.01, .1, 1, 10 ng/mL	Increased	Chao et al., 1987
Cattle	Dispersed	Perifusion	LHRH	.1 ng/mL	Increased	Padmanabhan et al., 1981
Cattle	Dispersed	Static	LHRH	.1, 1, 10, 100 ng/mL	Increased	Padmanabhan and Convey, 1978
Cattle	Dispersed	Static	LHRH	.1, 1, 10 ng/mL	Increased	Padmanabhan et al., 1978
Cattle	Dispersed	Static	LHRH	.1, 1, 10, 100 nM	Increased	Baratta et al., 1994

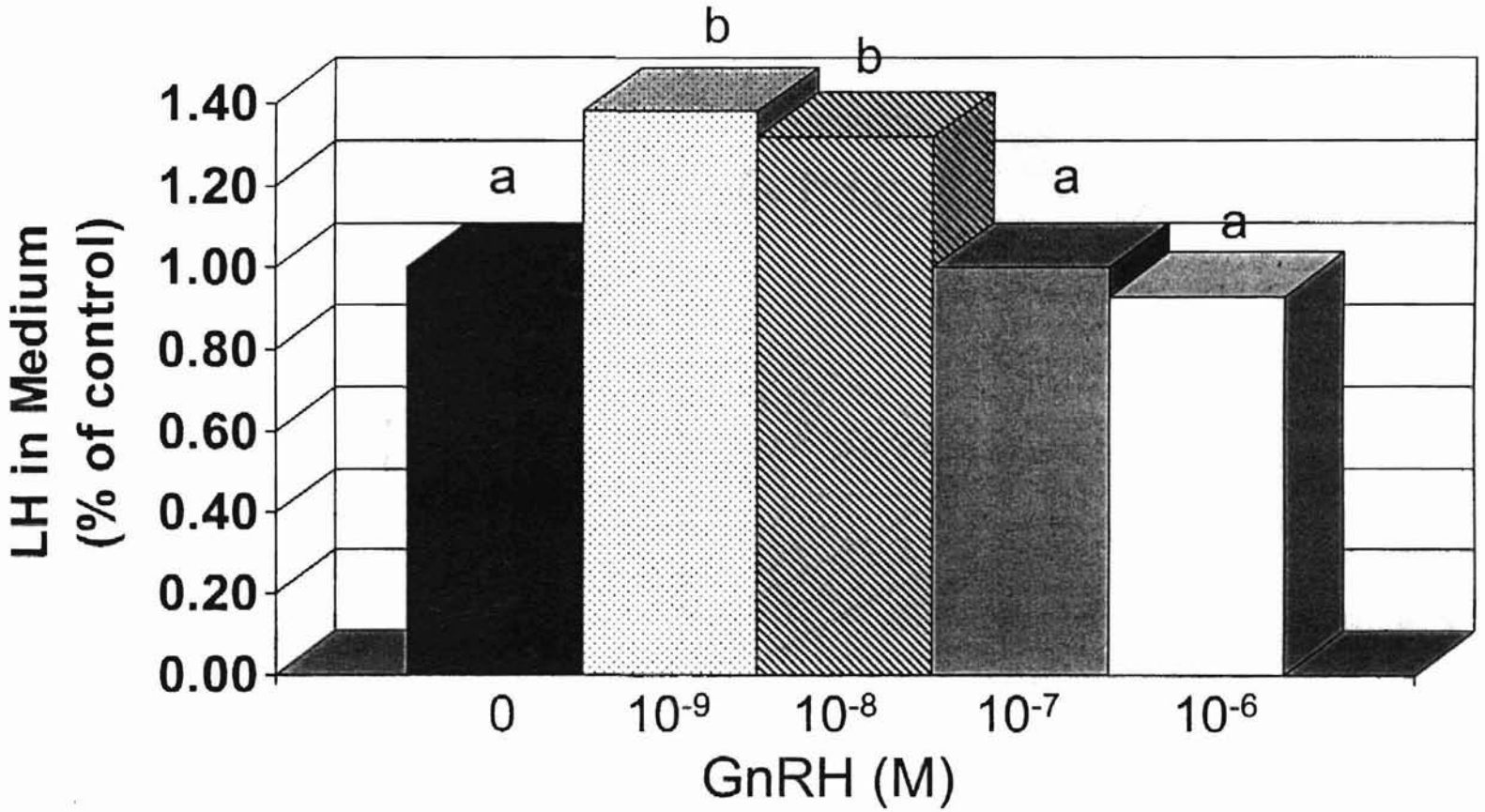

Figure 1. Experiment 1A. Concentration of LH in medium from pituitary cells after the first 1-h incubation with GnRH. Four reps with cells from 2 to 4 steers with 1 to 4 wells/treatment/rep (a, b $P < .04$; MSE=.14).

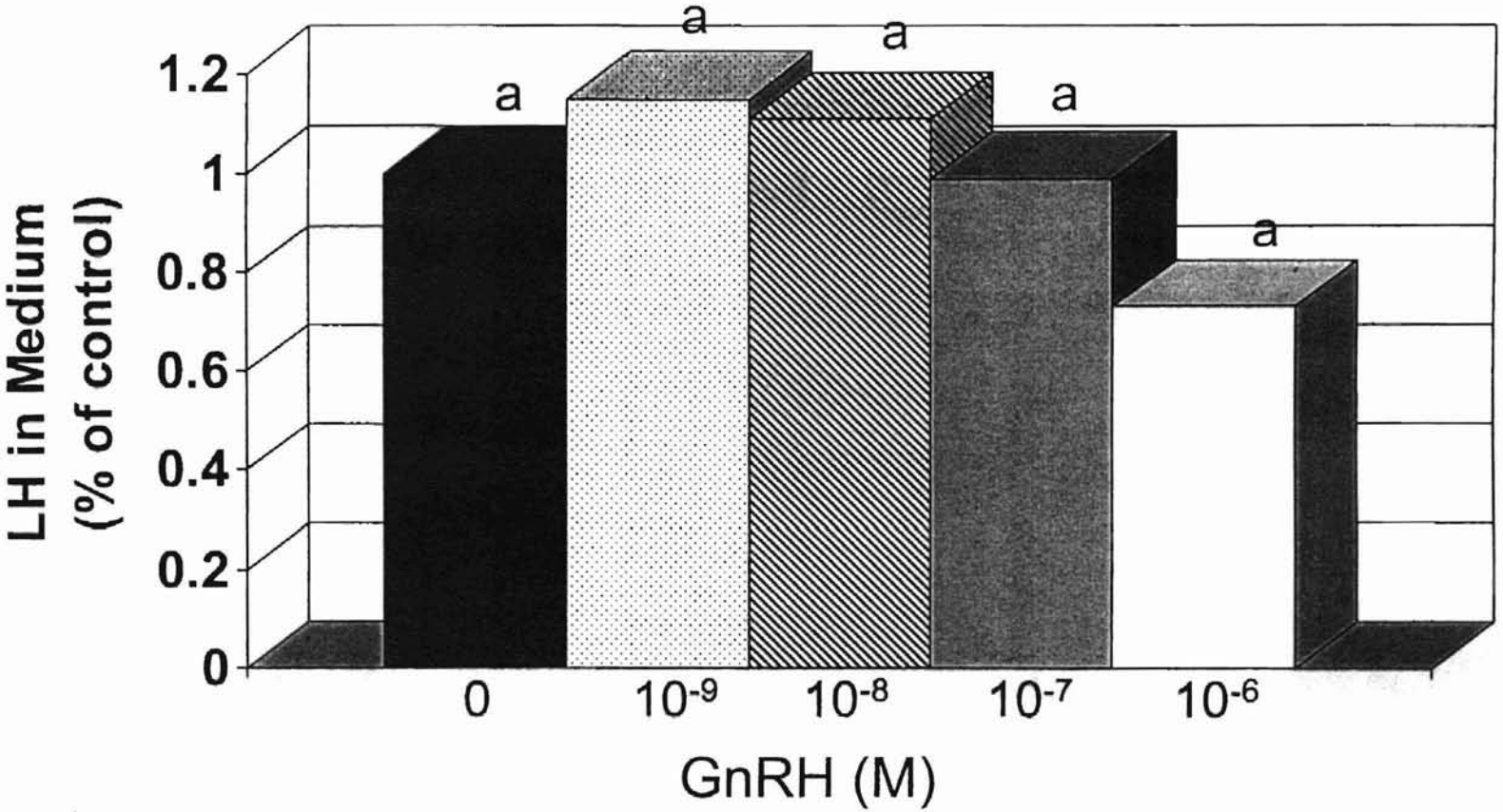

Figure 2. Experiment 1A. Concentration of LH in medium from pituitary cells after the first 4-h incubation with GnRH. Four reps with cells from 2 to 4 steers with 1 to 4 wells/treatment/rep (a, b $P < .12$; MSE=.10).

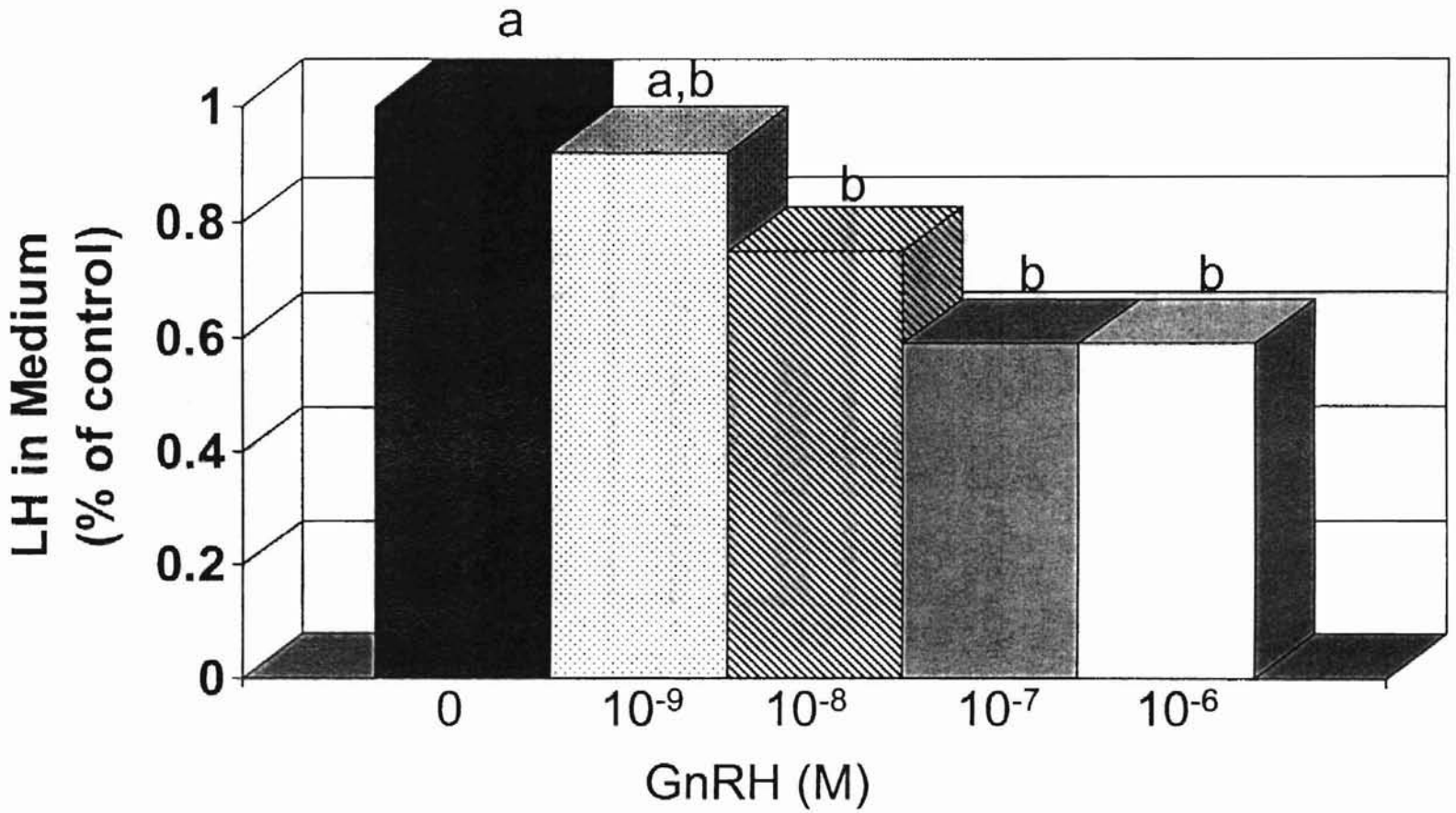

Figure 3. Experiment 1A. Concentration of LH in medium from pituitary cells after the second 4-h incubation with GnRH. Four reps with cells from 2 to 4 steers with 1 to 4 wells/treatment/rep (a, b $P < .0001$; MSE=.03).

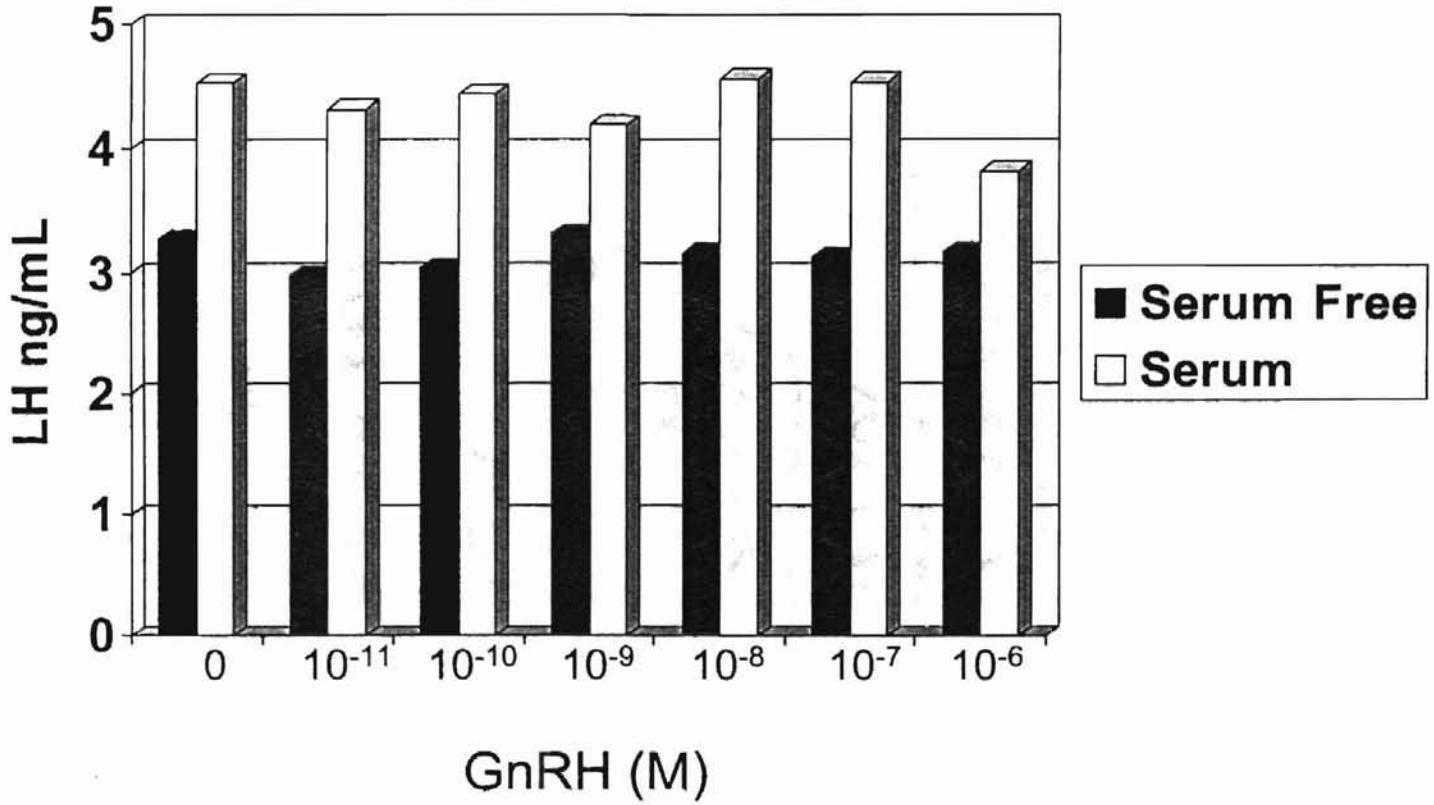

Figure 4. Experiment 1B. Concentration of LH in medium from pituitary cells after the first 1-h incubation with and without serum ($P < .0001$) and GnRH ($P > .10$). Two reps with cells from 4 steers with 3 to 6 wells/treatment/rep (MSE=.520).

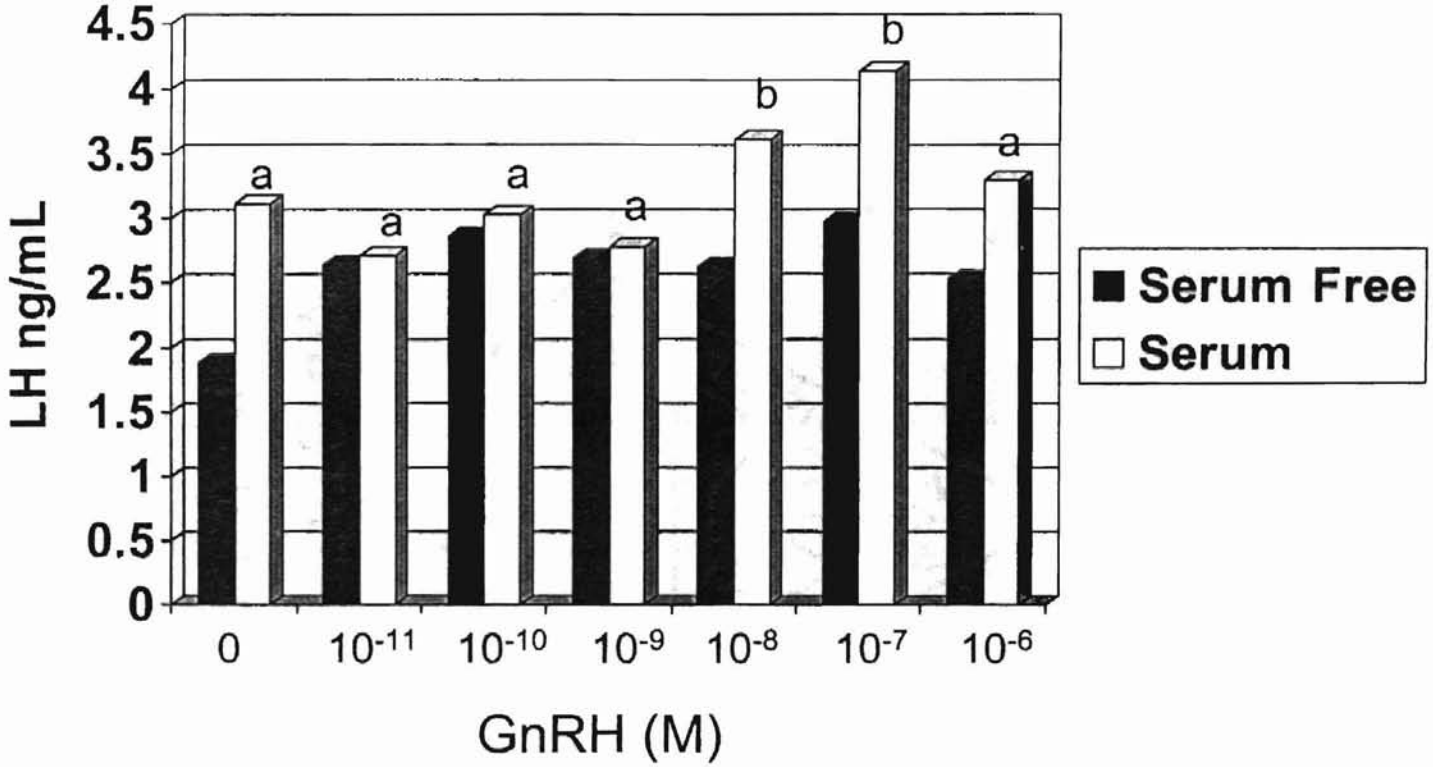

Figure 5. Experiment 1B. Concentration of LH in medium from pituitary cells after the first 4-h incubation with and without serum ($P < .0006$) and GnRH (a, b $P < .05$). Two reps with cells from 4 steers with 2 to 6 wells/treatment/rep (MSE=.418).

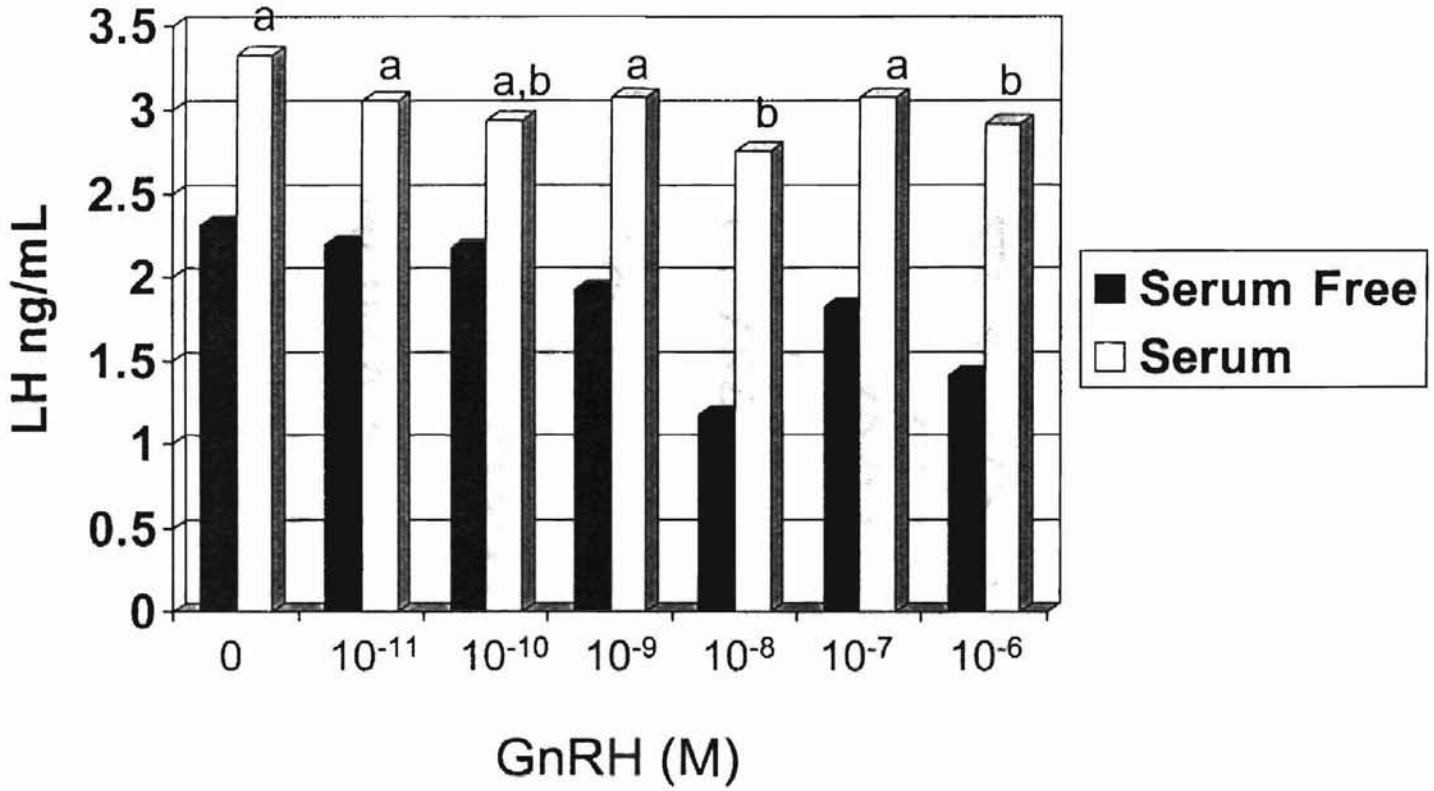

Figure 6. Experiment 1B. Concentration of LH in medium from pituitary cells during 20-h incubation with and without serum ($P < .0001$) and without GnRH (a, b $P < .03$). Two reps with cells from 4 steers with 3 to 6 wells/treatment/rep (MSE=.225).

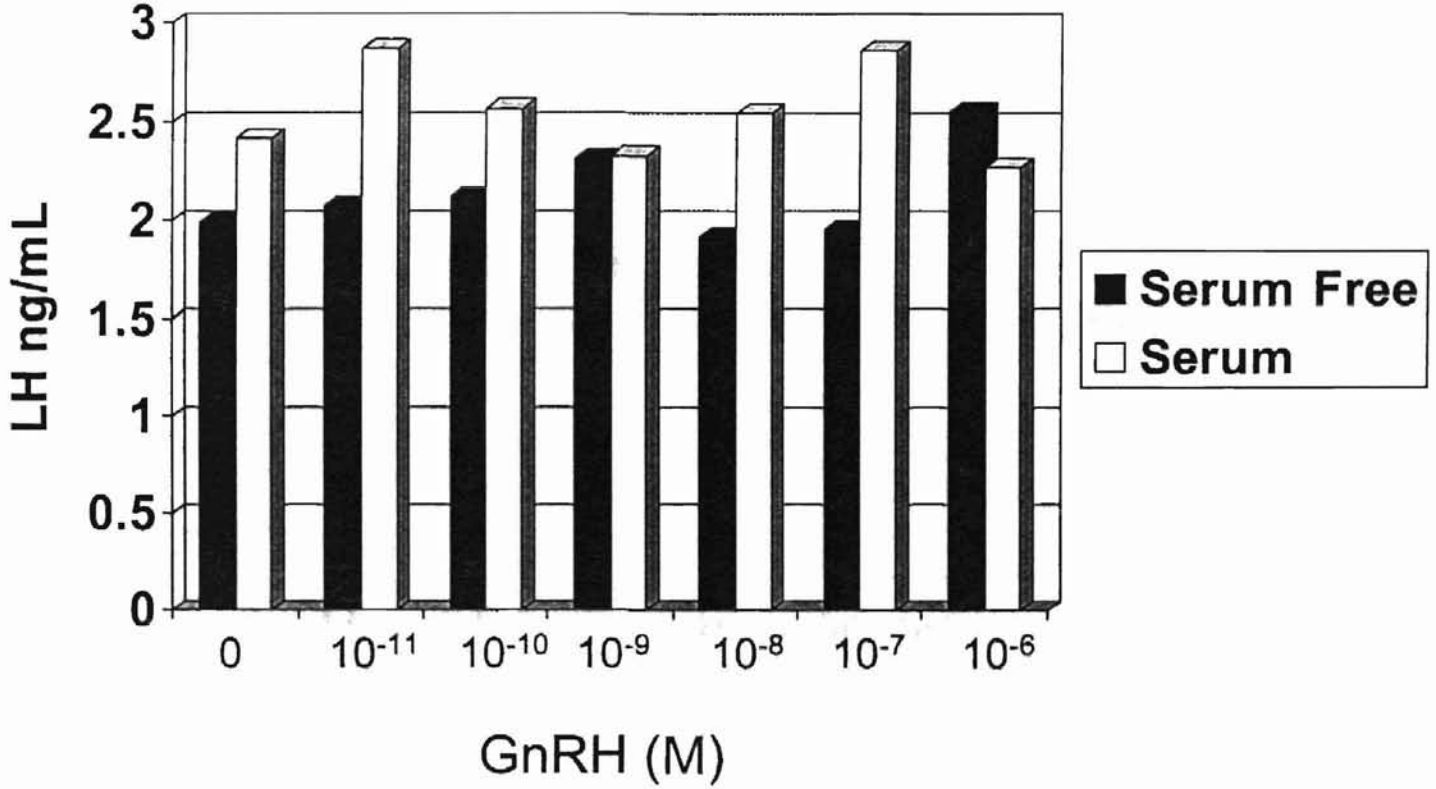

Figure 7. Experiment 1B. Concentration of LH in medium from pituitary cells after the second 1-h with and without serum ($P < .01$) and exposed to GnRH ($P > .10$). Two reps with cells from 4 steers with 2 to 6 wells/treatment/rep (MSE=.333).

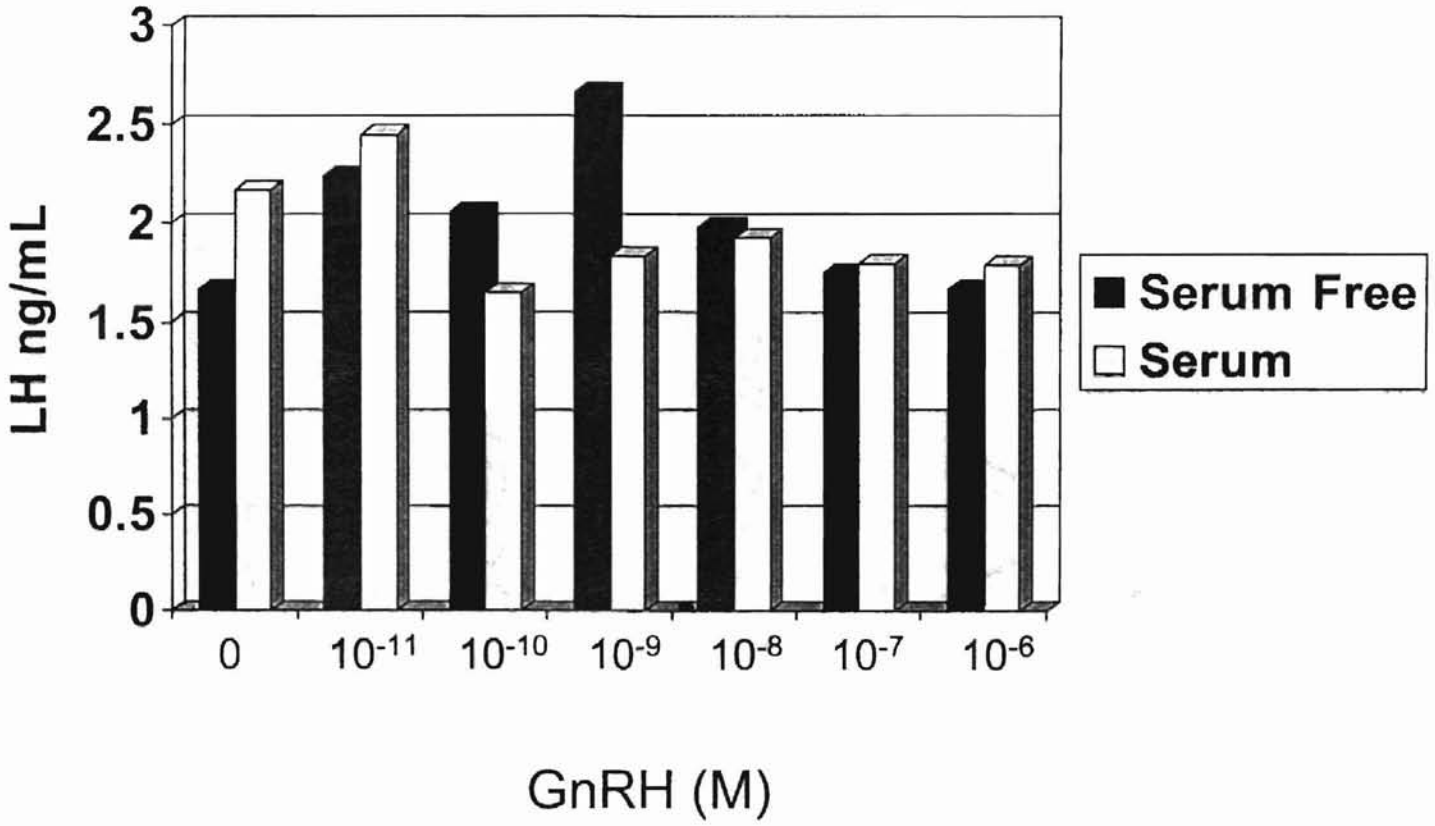

Figure 8. Experiment 1B. Concentration of LH in medium from pituitary cells after the second 4-h incubation with and without serum ($P > .10$) and GnRH ($P > .20$). Two reps with cells from 4 steers with 3 to 6 wells/treatment/rep (MSE=.831).

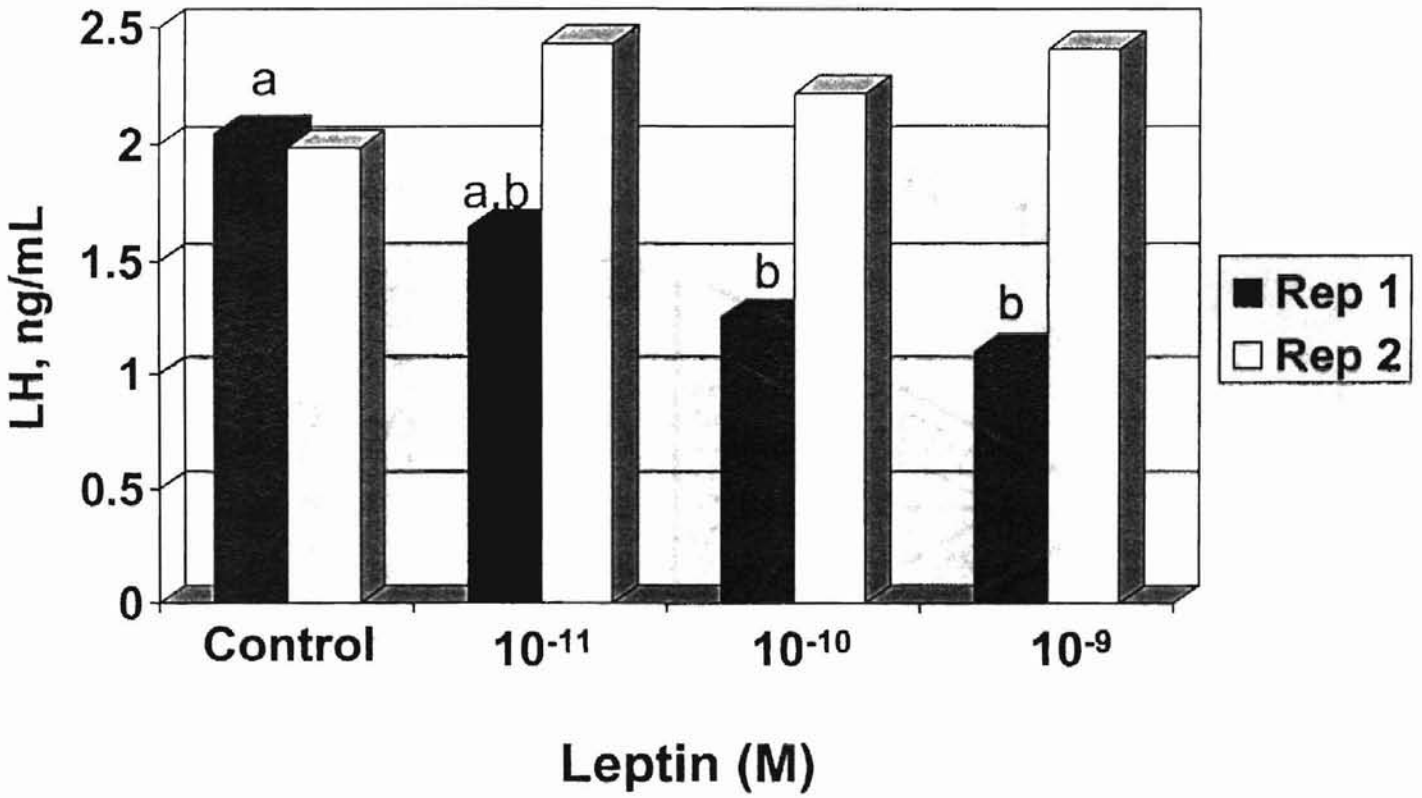

Figure 9. Experiment 2A. Concentration of LH in medium from pituitary cells after the first 4 h incubation with leptin (Trt x Rep). Cells were from 2 to 3 steers/rep with 3 to 4 wells/treatment (a, b $P < .003$; MSE=.191).

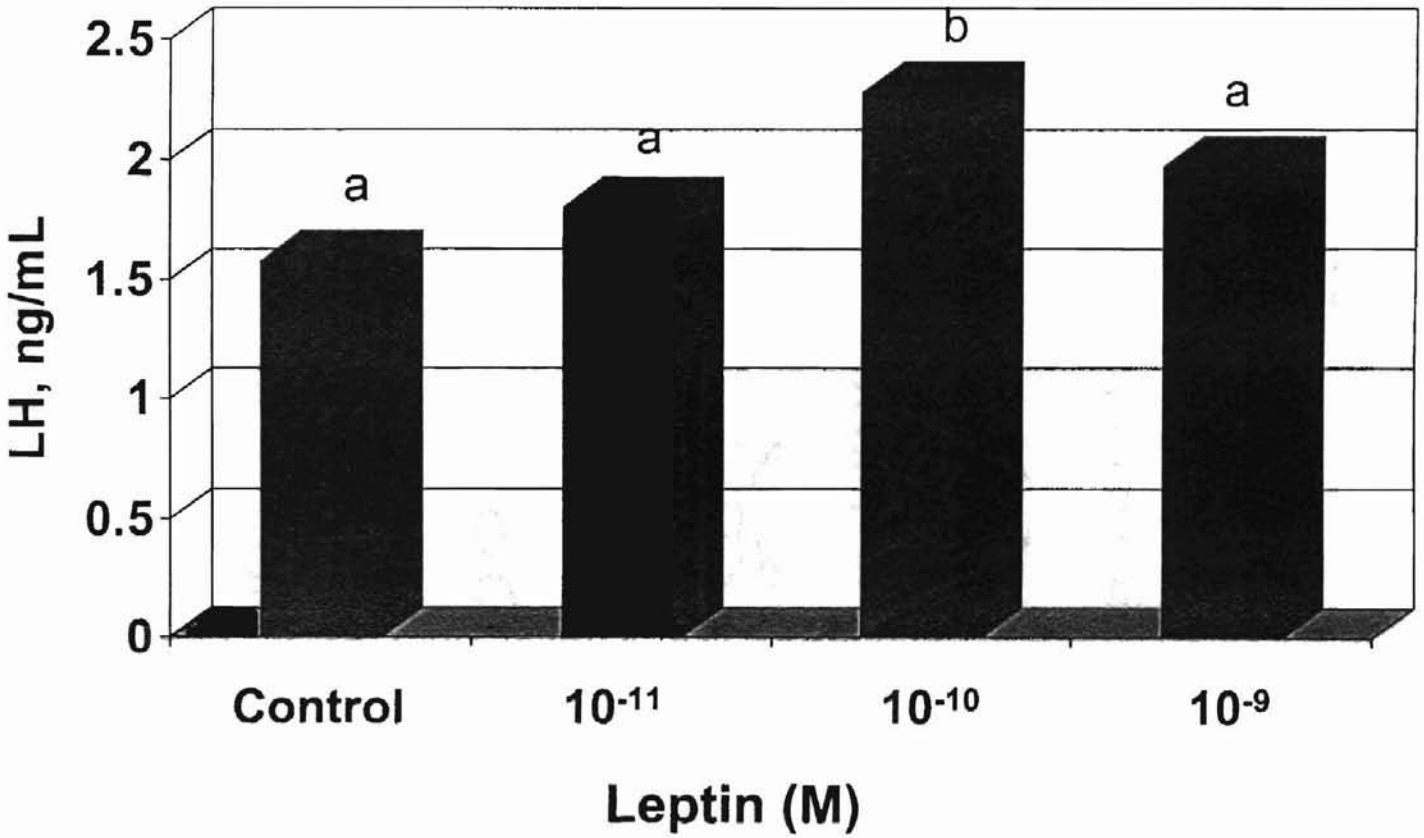

Figure 10. Experiment 2A. Concentrations of LH in medium from pituitary cells after 20 h exposure to control medium after 4 h incubation with leptin (a, b $P < .002$; MSE =.179).

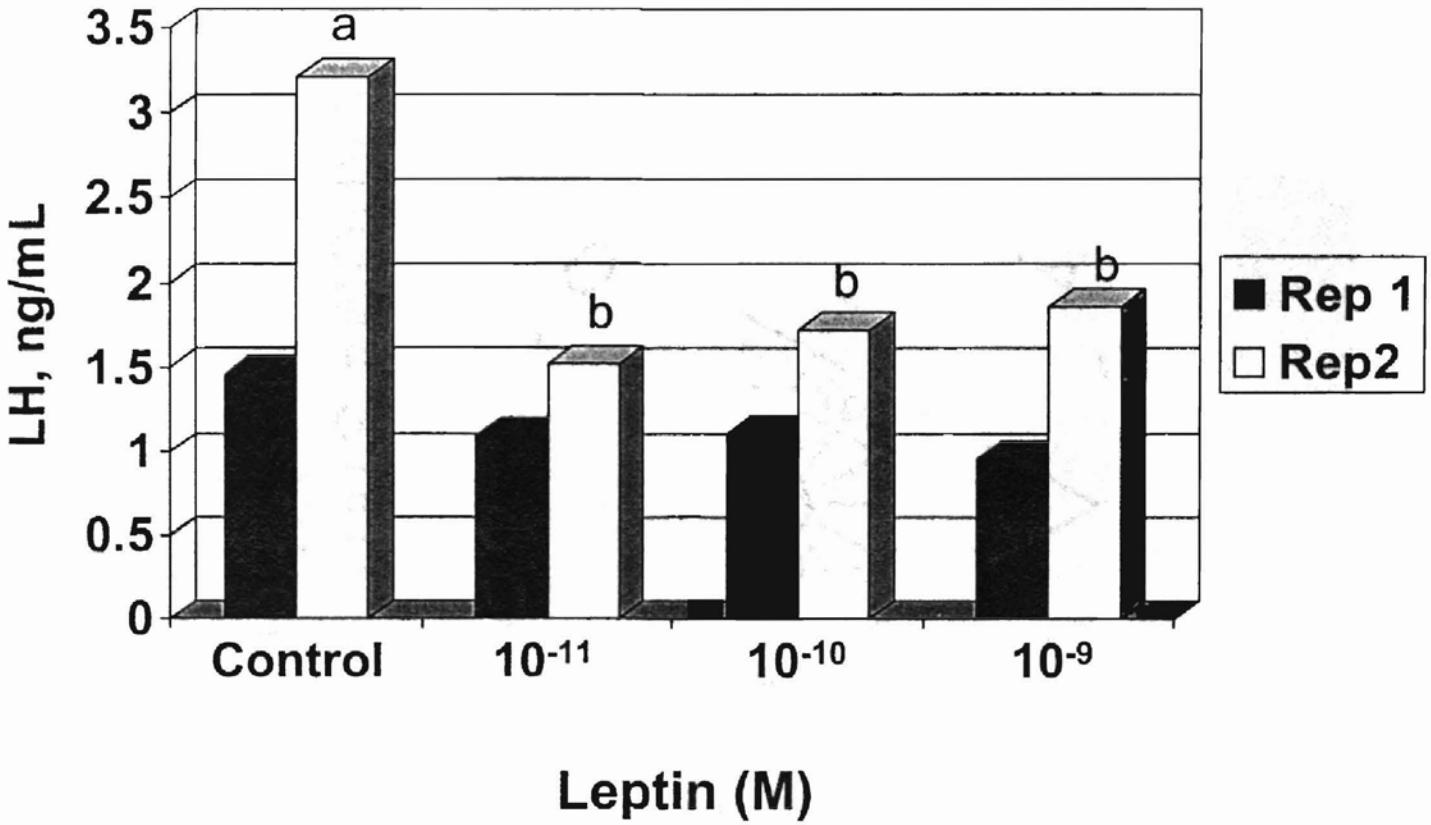

Figure 11. Experiment 2A. Concentrations of LH in medium from pituitary cells after the second 4 h incubation with leptin (a, b $P < .001$; MSE =.106).

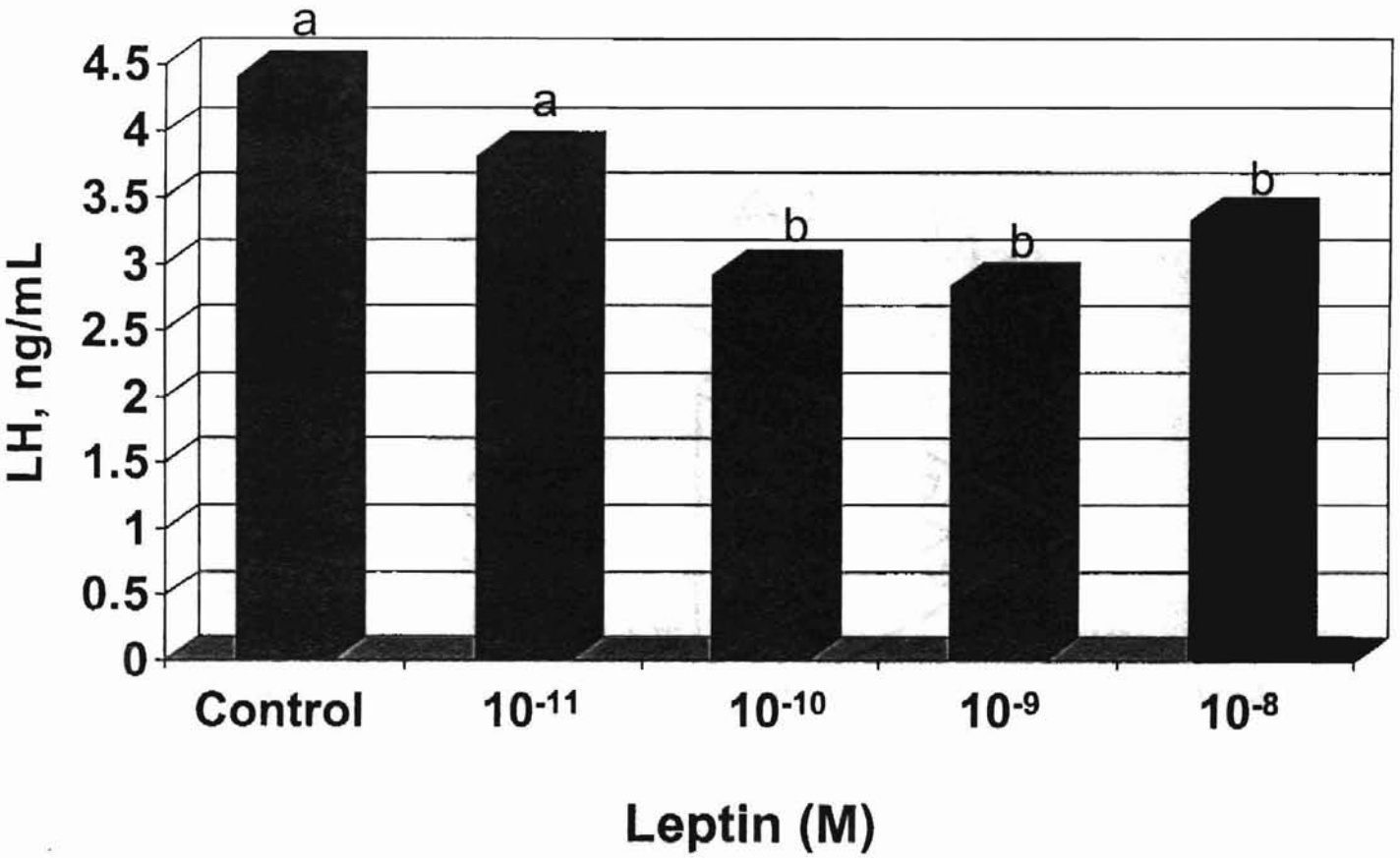

Figure 12. Experiment 2B. Concentrations of LH from pituitary cells after the first 1 h incubation with leptin (a, b $P < .005$; MSE =.636). Cells were from 4 steers with 2 to 5 wells/treatment/rep.

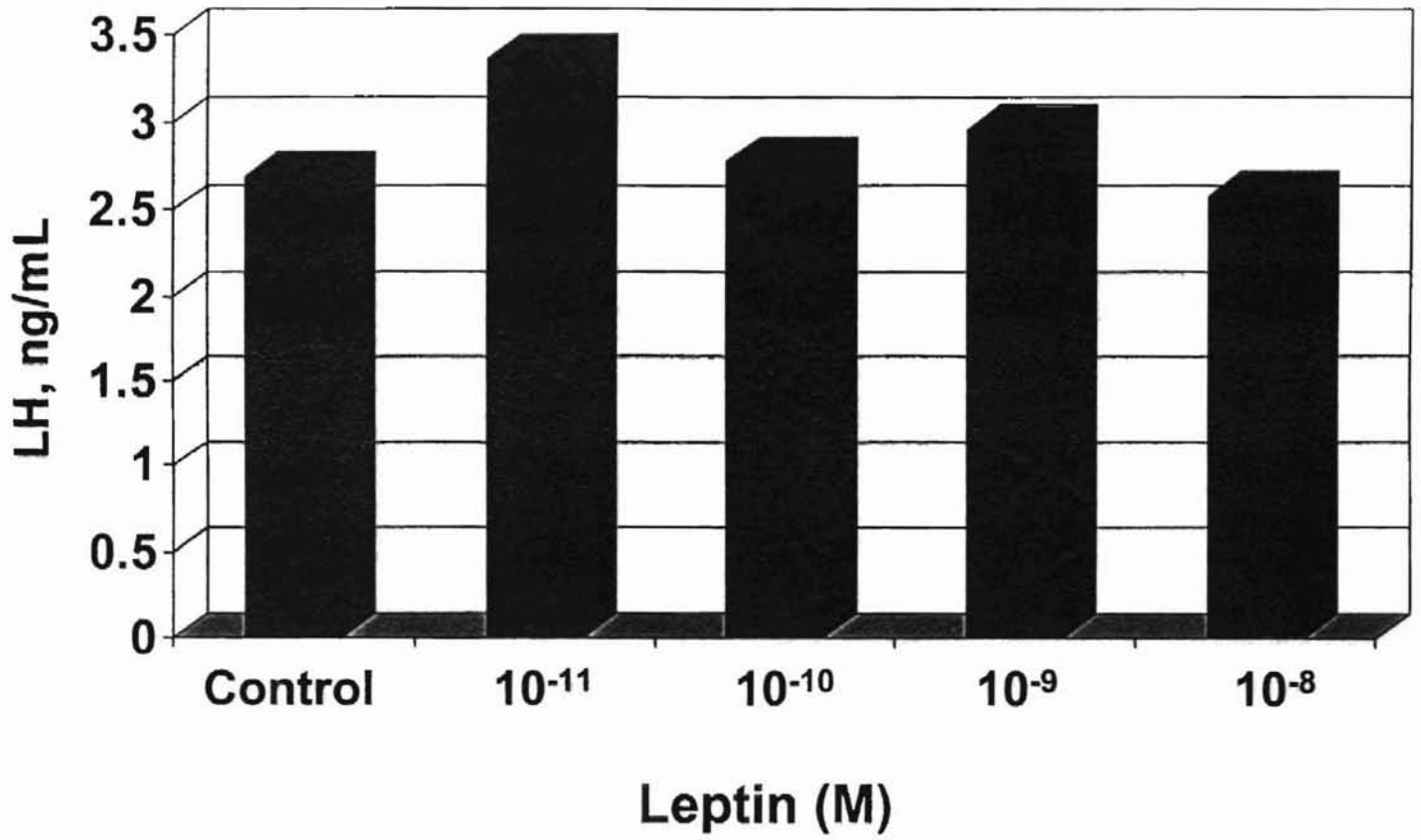

Figure 13. Experiment 2B. Concentrations of LH in medium from pituitary cells after the first 4 h incubation with leptin ($P > .20$; MSE =1.19).

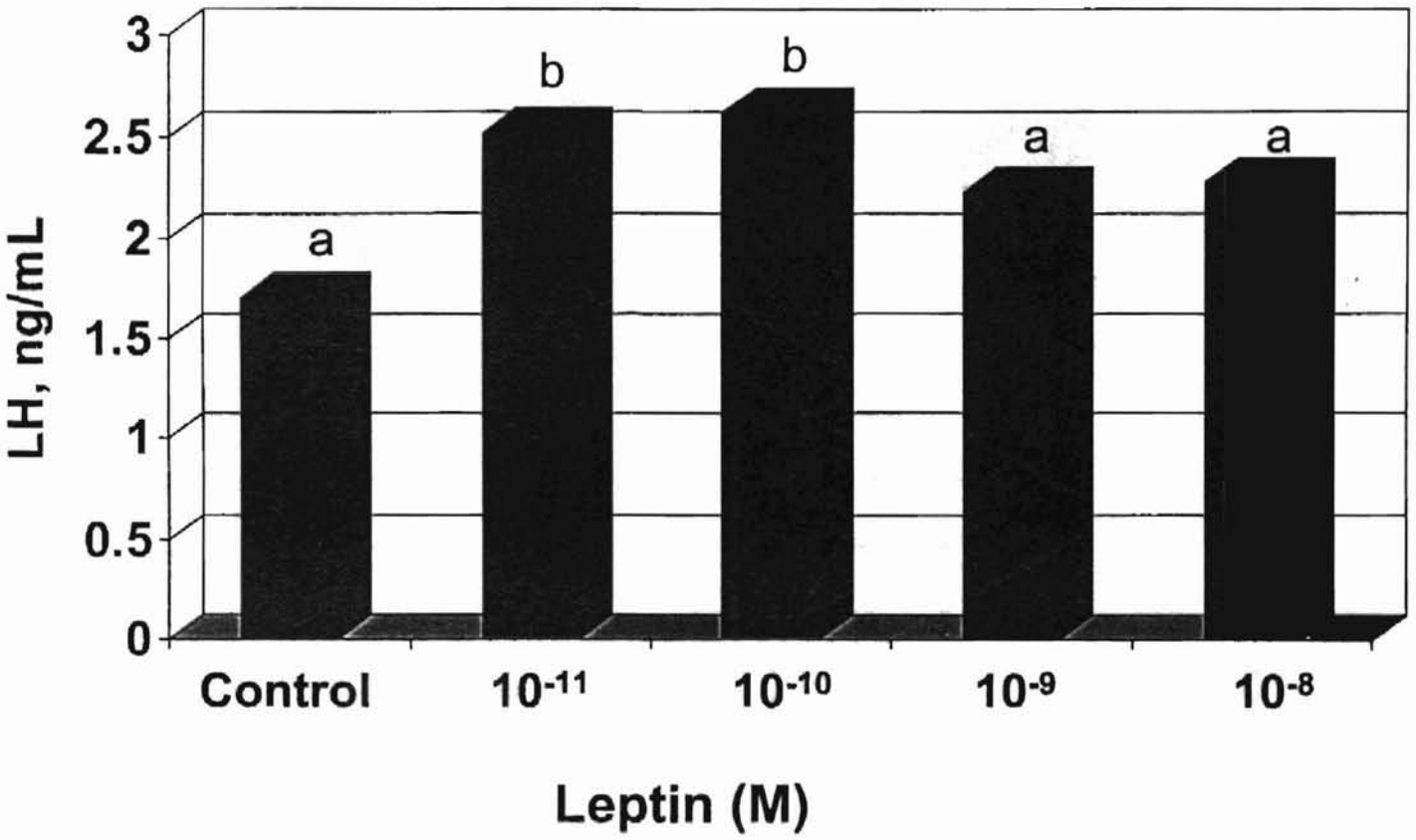

Figure 14. Experiment 2B. Concentrations of LH in medium from pituitary cells after 20 h exposure to control medium after cells were incubated with leptin (a, b $P < .02$; MSE =.147).

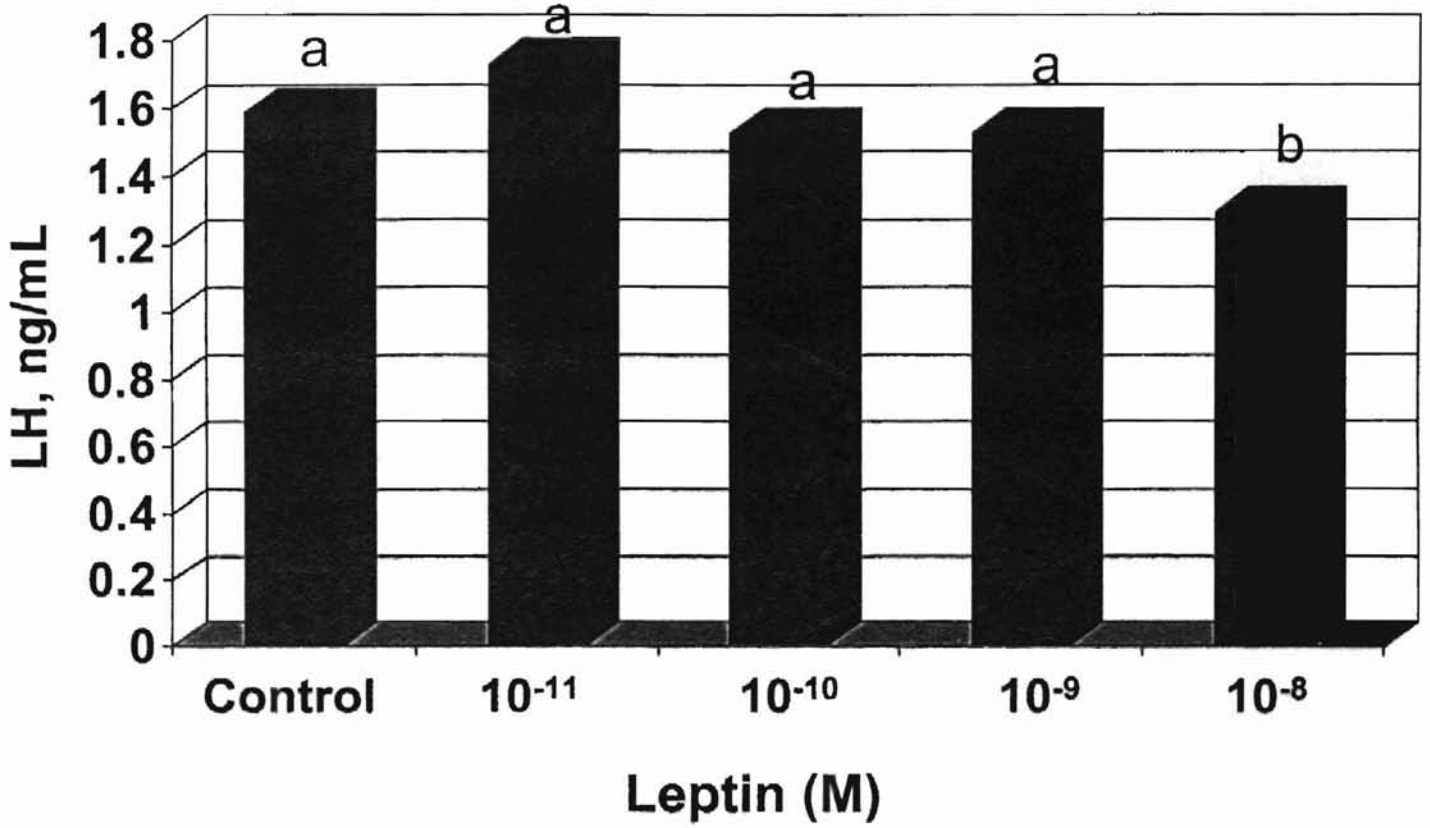

Figure 15. Experiment 2B. Concentrations of LH in medium from pituitary cells after second 1 h incubation with leptin (a, b $P < .007$; MSE =.075).

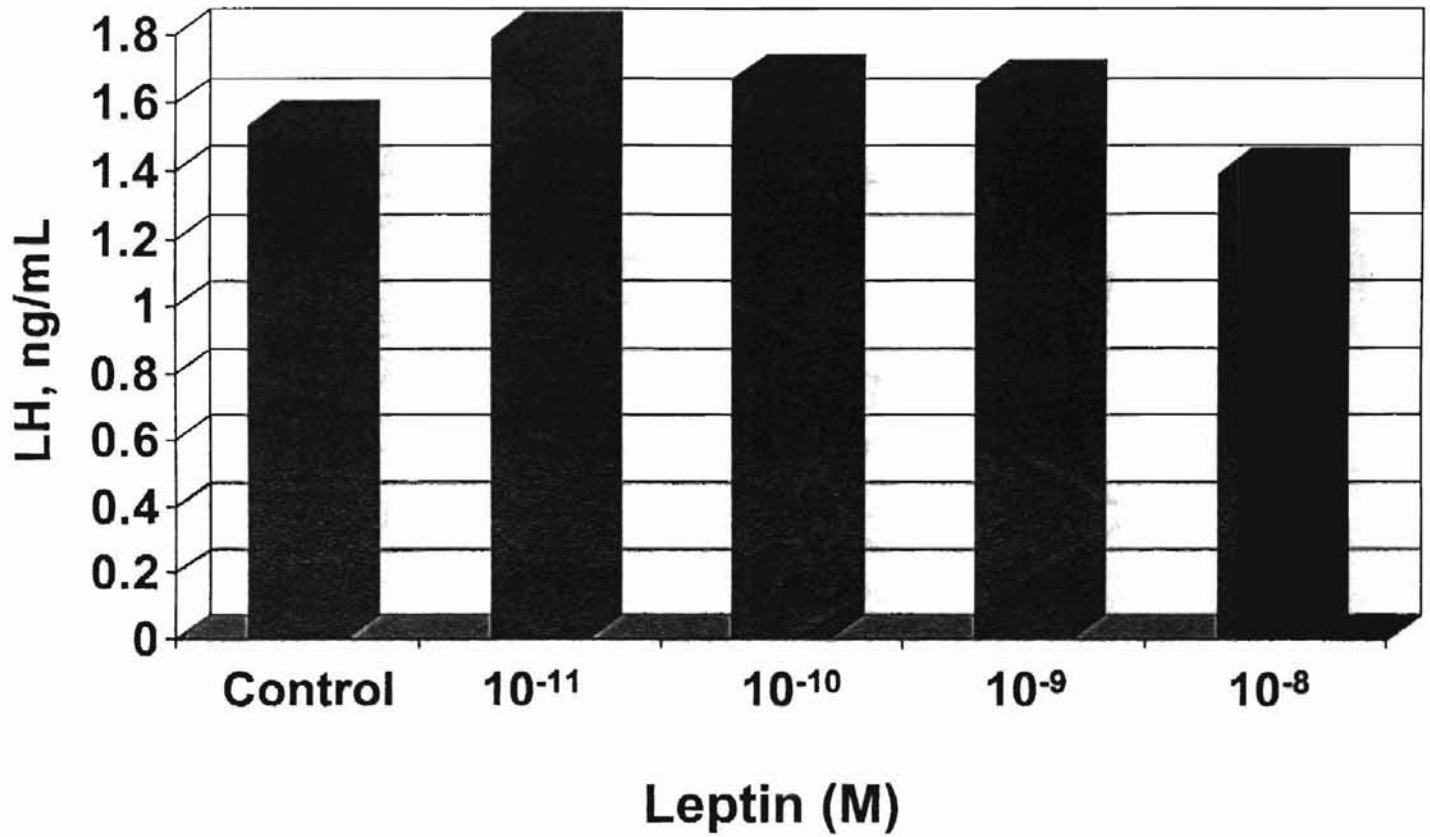

Figure 16. Experiment 2B. Concentrations of LH in medium from pituitary cells after the second 4 h incubation with leptin ($P > .10$; MSE =.064).

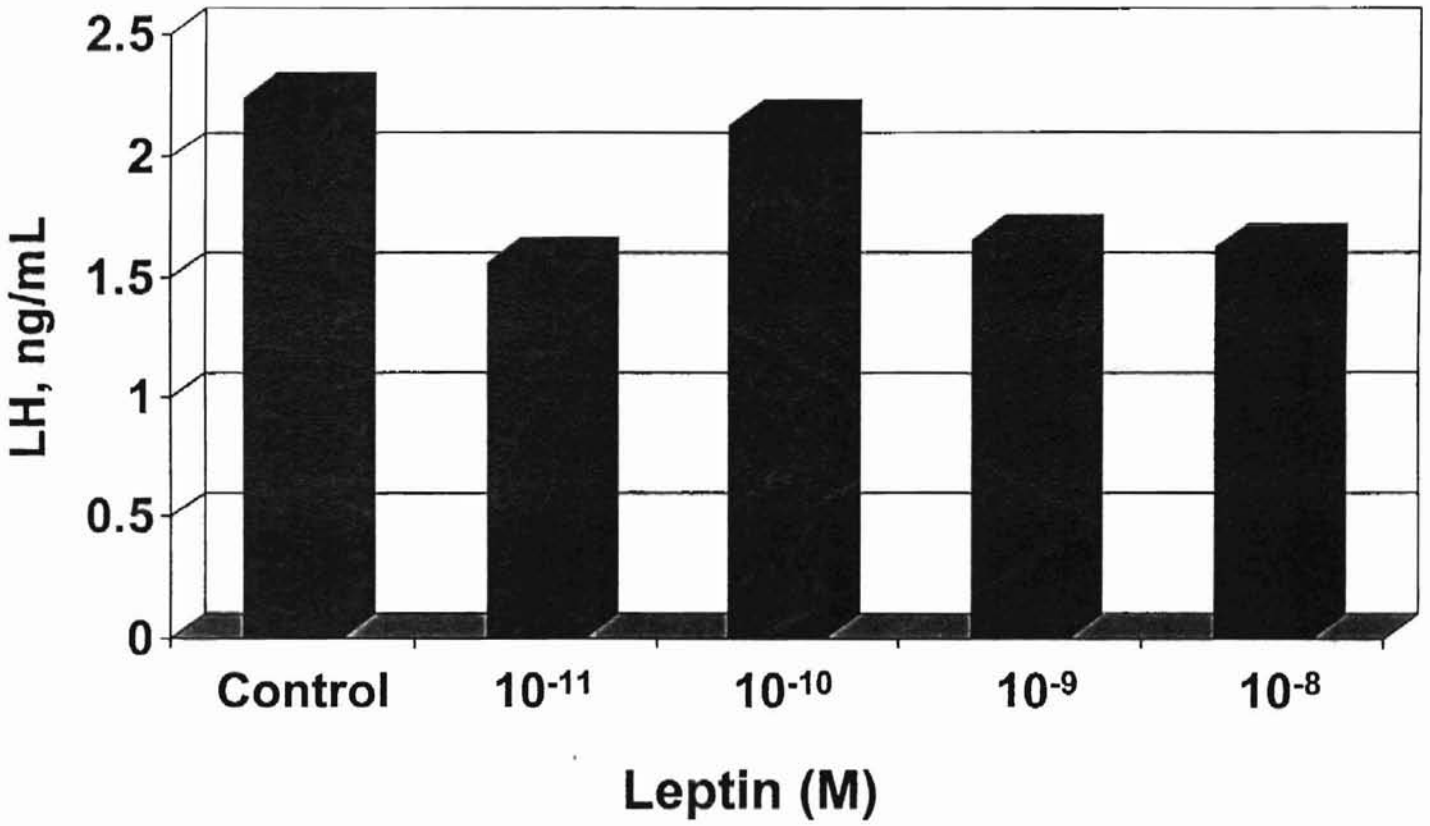

Figure 17. Experiment 2B. Concentrations of LH in medium from pituitary cells after 20 h exposure to control medium after incubation with leptin (P > .10; MSE =.195).

Table 2. Concentrations of LH in medium of pituitary cells after the first 1-h incubation with and without serum (P < .0001), leptin and GnRH (MSE = 1.48; two reps with cells from 3 steers with 2 to 4 observations per treatment combination).

		Leptin (M)				
		0	10^{-11}	10^{-10}	10^{-9}	10^{-8}
Serum	GnRH	5.41	3.52	3.18	3.56	4.83
	No GnRH	1.94	3.71	3.44	3.72	3.48
No Serum	GnRH	0.98	1.16	1.49	1.56	1.52
	No GnRH	1.52	1.52	1.83	1.73	1.20

Table 3. Concentrations of LH in medium of pituitary cells after the first 4-h incubation with and without serum (P < .0001), serum x leptin (a, b P < .0001) and GnRH (MSE = .04; 1 to 2 observations per treatment combination).

		Leptin (M)				
		0	10^{-11}	10^{-10}	10^{-9}	10^{-8}
Serum	GnRH	1.79^{a}	3.32^{b}	2.06^{b}	3.02^{b}	2.47^{b}
	No GnRH	1.76	3.29	2.32	2.84	3.10
No Serum	GnRH	1.54	1.54	1.48	1.13	1.07
	No GnRH	1.25	1.44	1.40	1.13	1.18

Table 4. Concentrations of LH in medium of pituitary cells after 20 h exposure to control medium after 4-h incubation with serum ($P < .001$), leptin and GnRH (MSE = .69; 2 to 4 observations per treatment combination).

		Leptin (M)				
		0	10^{-11}	10^{-10}	10^{-9}	10^{-8}
Serum	GnRH	1.43	1.88	1.07	2.62	1.71
	No GnRH	1.13	1.70	1.54	1.67	NE
No Serum	GnRH	0.79	0.95	0.79	0.89	0.96
	No GnRH	0.86	0.84	1.05	1.08	1.22

NE = Non-estimatable

Table 5. Concentrations of LH in medium of pituitary cells after second 1-h incubation with and without serum, leptin and GnRH (MSE = .43; 1 to 4 observations per treatment combination).

		Leptin (M)				
		0	10^{-11}	10^{-10}	10^{-9}	10^{-8}
Serum	GnRH	0.72	1.34	0.93	0.83	0.70
	No GnRH	1.41	1.46	0.89	0.79	0.35
No Serum	GnRH	0.78	0.70	0.76	1.07	0.94
	No GnRH	0.86	0.79	0.92	1.02	0.97

Table 6. Concentrations of LH in medium of pituitary cells after second 4-h incubation with and without serum, leptin and GnRH (MSE = 0.291; 1 to 4 observations per treatment combination).

		Leptin (M)				
		0	10^{-11}	10^{-10}	10^{-9}	10^{-8}
Serum	GnRH	1.12	1.31	0.74	0.92	0.83
	No GnRH	1.04	1.33	0.90	0.99	1.51
No Serum	GnRH	0.87	1.12	0.65	0.75	1.25
	No GnRH	0.74	0.96	0.84	0.86	1.08

Table 7. Concentrations of LH in medium of pituitary cells after 20 h exposure to control medium after 4-h incubation with serum ($P < .10$), leptin and GnRH (MSE = .22; 2 to 4 observations per treatment combination).

		Leptin (M)				
		0	10^{-11}	10^{-10}	10^{-9}	10^{-8}
Serum	GnRH	1.11	1.23	1.25	1.44	1.15
	No GnRH	0.98	1.22	1.06	1.37	1.05
No Serum	GnRH	0.87	0.97	0.94	0.92	0.69
	No GnRH	0.88	0.98	1.19	1.00	1.29

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