

2013

Factors affecting basal and post-exercise prolactin secretion in horses

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FACTORS AFFECTING BASAL AND POST-EXERCISE PROLACTIN
SECRETION IN HORSES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in
the School of Animal Sciences

by
Lisa C. DiGiovanni
B.S., Syracuse University, 2011
May 2013

ACKNOWLEDGEMENTS

I wish to express my appreciation and gratitude to my major professor, Dr. Donald L. Thompson, Jr., for his remarkable patience, guidance, and support throughout my graduate career at LSU. I would also like to extend my thanks and appreciation to Dr. Cathleen C. Williams and Dr. Christine B. Navarre for being on my committee and for their teaching and support throughout my research and time at LSU. To Mr. Franklin “Randy” Wright, I am extremely grateful for his help and maintenance of the horses and horse farm.

I would like to extend a genuine thank you to all the graduate students who helped make this all possible. Pamela B. Mitcham, thank you for your instruction and guidance at the farm and in the lab, it was greatly appreciated. Caitlin Hebert, thank you for your assistance in the lab and at the farm and for teaching me radioimmunoassay techniques. Jeanne Lestelle, thank you for all your help at the farm, it was extremely appreciated. Nicole Arana Valencia, I am extremely grateful for all your help with early mornings at the farm and all the time and effort you have donated. Erin Oberhaus, thank you for all your help at the farm and for your wonderful advice.

To my family and friends who have been here to support me through my journey here at LSU, thank you so very much. This all would not be possible without all the love and support in my life that I have. I would also like to extend the greatest appreciation to my mother, Jacquelyn DiGiovanni; you have been there through the good and the bad and a single phone call to you always made everything better.

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ABSTRACT

There has been thorough documentation to support the role of dopamine in the control of prolactin production and secretion in various mammalian species, including the horse. However, there is evidence that other factors are involved in prolactin secretion. Seven experiments were conducted to assess factors that potentially might affect prolactin secretion in the horse. The first two experiments were conducted (separately) to test whether arginine vasopressin (AVP) or vasoactive intestinal polypeptide (VIP) affected prolactin secretion. In each experiment, AVP or VIP was administered intravenously and blood samples were collected to determine the effect on prolactin secretion. Neither peptide produced any alteration in plasma prolactin concentrations compared to simultaneous saline-injected controls ($P > 0.1$). Five subsequent experiments were conducted to assess the effects of various drugs on prolactin secretion in response to acute exercise. Pre-exercise treatments included dexamethasone (a glucocorticoid analog, administered 15 h before exercise), naloxone (an opioid antagonist, administered 2 min before exercise), cabergoline (a dopaminergic agonist, administered 15 h before exercise), flunixin meglumine (a prostaglandin inhibitor, administered 15 min before exercise), and sulpiride (a dopamine antagonist that causes the release of prolactin, administered 1.5 h before exercise). In all experiments, exercise induced an immediate increase ($P < 0.05$) in plasma prolactin concentrations in control horses. Pretreatment with dexamethasone, naloxone, or flunixin meglumine did not alter ($P > 0.1$) plasma prolactin concentrations relative to saline-treated controls. Pretreatment with cabergoline completely obliterated ($P < 0.01$) the exercise induced rise in prolactin concentrations. Pretreatment with sulpiride caused an immediate increase ($P < 0.001$) in prolactin concentrations relative to controls, but resulted in no change in prolactin response to exercise 90 min later relative

to controls. It is concluded that the only drug that had a significant effect on prolactin secretion was the dopaminergic agonist cabergoline. Direct administration of AVP or VIP, or perturbations of the adrenal cortical axis, the opioid system, or the prostaglandin system, had no effect on prolactin secretion as has been reported previously for other species.

INTRODUCTION

Prolactin is referred to as the “hormone of maternity” due to its regulation of mammary growth and development and its lactogenic properties (Hadley and Levine, 2007). Other known functions in the body include hair growth, reproduction, and follicular activity (Hadley and Levine, 2007). Lactotropes (prolactin secreting cells) are found in the pars distalis of the adenohypophysis and have been shown to be the prolactin secreting cells (Gregory et al., 2000).

A consistent rise in plasma prolactin concentrations has been shown to be detrimental in numerous animals. Increased levels of prolactin (hyperprolactinemia) have been shown to cause infertility in human males and females (Serri et al., 2003). In females, high prolactin levels can also cause galactorrhea (abnormal lactation), oligomenorrhea (infrequent menstruation) and even amenorrhea (absence of menstruation), while in men it causes hypogonadism, (little to no production of hormones in the sex glands; Serri et al., 2003). Hyperprolactinemia can be caused by pathologies, such as a pituitary tumor, or diseases, such as hypothyroidism (Serri et al., 2003).

Dopamine has been shown to be a potent prolactin secretion inhibitor. In the presence of dopamine, prolactin secretion is minimal, whereas when dopamine is absent, prolactin secretion rates are high (Moore, 1987). It has been shown that prolactin has an auto-regulatory feedback onto tuberoinfundibular dopamine neurons (Moore, 1987). Increased concentrations of prolactin due to a lack of stimulation of dopamine receptors located on lactotropes causes an auto-regulatory feedback loop to the tuberoinfundibular dopamine neurons, which are then activated to produce more dopamine, resulting in reduced prolactin secretion (Moore, 1987).

The regulation of prolactin secretion in horses seems to be the same as the tonic dopaminergic inhibition described for most mammalian species. Pioneer work by Johnson and Becker (1987) showed that administration of the dopamine antagonist, sulpiride, stimulated

prolactin secretion in mares, and that administration of the dopaminergic agonist, bromocriptine, reduced resting prolactin concentrations. These effects of agonists and antagonists have been reported by many authors since then (Donadeu and Thompson, 2002; Colborn et al., 1991a; Redmond et al., 1994; Thomson et al., 1996).

In addition to the dopaminergic effects on prolactin secretion, several other neuropeptides and hormones have been implicated in the control of prolactin secretion in other species. Moreover, various factors, particularly exercise and other forms of sympathetic nervous system stimulation, have been shown to stimulate prolactin secretion in horses (Thompson et al., 1988; Rabb et al., 1989; Colborn et al., 1991b). What is not known is whether the exercise-induced prolactin response is due to an immediate reduction in dopamine input to the adenohypophysis, or whether some intermediate neurotransmitter, peptide, or hormone is released and directly affects the lactotropes.

The experiments described herein were conducted to assess potential intermediates in the control of prolactin secretion. The hormones tested, or the systems chosen for perturbation, were selected based on their known effects in other, usually smaller, species. The first two experiments were conducted to examine how two brain peptides, arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP), affect resting prolactin levels in the horse. These two peptides were chosen due to previous research showing stimulated prolactin secretion in various species after administration (Frawley and Neill, 1981; DePaolo et al., 1986).

Subsequent experiments were conducted to assess how perturbation of various systems (specifically the adrenal cortical, opioid, and prostaglandin systems) might alter the prolactin response to exercise. It was assumed that a significant perturbation of the response would indicate an involvement of that system in the normal prolactin response to exercise.

CHAPTER I REVIEW OF LITERATURE

Pituitary Gland

The horse pituitary consists of two lobes: the adenohypophysis and the neurohypophysis (Ginther, 1992). The adenohypophysis is also called the anterior lobe and it is primarily composed of glandular tissue (Hadley and Levine, 2007). The adenohypophysis consists of the pars distalis and the pars tuberalis (Hadley and Levine, 2007). The pars distalis has been shown to contain the cells that produce prolactin, the lactotropes (Gregory et al., 2000). In a study done by Gregory et al. (2000), seasonally anestrous mares in November and sexually active mares in November had lactotropes that were only found in the pars distalis. This was also detected in six of seven mares that were sexually active in July (Gregory et al., 2000). The one mare that this did not pertain to had lactotropes in the pars distalis as well as in the pars tuberalis (Gregory et al., 2000). In this one mare, there were more isolated lactotropes that were identified within the pars distalis compared to the pars tuberalis (Gregory et al., 2000). This indicates that during the peak-breeding season some individuals may be able to express prolactin-secreting cells selectively within the pars tuberalis (Gregory et al., 2000).

Prolactin

Prolactin has been referred to as the “hormone of maternity” for its regulation of mammary growth and development and for its lactogenic properties (Hadley and Levine, 2007). Prolactin is also known for many other functions, depending on species. Stricker and Greuter (1929; summarized in Turner, 1977) reported that giving rabbits an extract of the anterior pituitary gland stimulates milk secretion. However, when mammary ducts were injected with anterior pituitary gland extract, only the alveoli attached to the treated ducts produced milk. These results showed that hormones in addition to prolactin act together with prolactin when

controlling mammary gland development. In addition to its involvement with mammary growth and development in mares (Redmond et al., 1994; Cross et al., 1995), prolactin has been reported to have other roles in the horse, including hair coat shedding in spring (Thompson et al., 1997) and induction of follicular activity and ovulation in seasonally anovulatory mares (Nequin et al., 1993, Thompson et al., 1997; Kelley et al., 2006; Mitcham et al., 2010). In most mammals, prolactin is a single-chain protein made up of 197 to 199 amino acids (Hadley and Levine, 2007). In 1988, Lehrman et al. reported that equine prolactin had 199 amino acid residues and had a 93% homology with porcine prolactin.

Factors Affecting Prolactin Secretion in the Horse

Several factors have been identified that cause prolactin release from the pituitary, including season (Johnson, 1986), feeding behaviors (Nadal et al., 1997), exercise and other forms of stress (Colborn et al., 1991b; Sticker et al., 1995; Thompson et al., 1994), consumption of endophyte-infected tall fescue grass (Cross et al., 1995; McCann et al., 1992), dopaminergic antagonists (Moore, 1987), thyrotropin releasing hormone (TRH; Johnson, 1986), and prostaglandin- $F_{2\alpha}$ (PGF $_{2\alpha}$; Thompson et al., 2013). From various experiments, plasma prolactin concentrations in the horse have been shown to follow a cyclic pattern throughout the year. That is, concentrations are high in the summer, start to decrease at the end of August, and reach their nadir in the months of November to February (Johnson, 1986; Fitzgerald et al., 2000). It has been reported that seasonal changes in concentrations of prolactin are directly correlated to both photoperiod and temperature (Johnson, 1986). This same study also noted that an increase of prolactin concentrations in the spring paralleled the loss of the winter hair coat, and decreasing concentrations of prolactin in the fall paralleled with the acquisition of the winter hair coat (Johnson, 1986).

According to Depew et al. (1994) and Nadal et al. (1997), consumption of a meal results in an increase in prolactin concentrations approximately 4 to 6 h after onset of feeding. However, the prolactin increases after meal consumption did not vary when several types of feedstuffs were fed (pelleted, complete grain mixture; alfalfa cubes; or crushed corn; Nadal et al., 1997).

Prolactin concentrations increase in horses that are stressed or exercised. As little as 5 minutes of exercise is enough to increase prolactin concentrations in stallions, geldings, and mares (Colborn et al., 1991b; Thompson et al., 1994). An increase in prolactin concentrations can be seen in as quickly as 10 minutes after onset of exercise, and prolactin can continue to be elevated through 30 minutes (Colborn et al., 1991b). Other forms of stress or physical activity also result in a surge in plasma prolactin concentrations, including twitching and mounting a mare, with or without ejaculation (Thompson et al., 1988; Rabb et al., 1989).

Pregnant mares that consume endophyte-infected tall fescue in the last three months of their pregnancy display a decrease in serum prolactin (Cross et al., 1995). Along with the decrease in prolactin concentrations, they also display increased gestation lengths, agalactia, foal and mare mortality, tough and thickened placentas, weak and dysmature foals, increased sweating during warm weather, reduced progesterone concentrations, and an increase in serum estradiol-17 β concentrations (Cross et al., 1995). Antidopaminergic drugs have been shown to reverse the effects of endophyte-infected tall fescue ingestion (Cross et al., 1995). Domperidone is a dopamine receptor antagonist that blocks both the normal dopaminergic input to the lactotropes as well as the ergot alkaloid that causes fescue toxicity. Domperidone is currently available commercially for treatment of pregnant mares grazing endophyte-infected tall fescue as Equidone^(R), distributed by Dechra Veterinary Products in Overland Park, Kansas.

Thyrotropin releasing hormone is a naturally occurring hypothalamic tripeptide that is the main regulator of thyroid stimulating hormone production and secretion (Hadley and Levine, 2007). It has also been shown to cause a release of prolactin after intravenous injection in most species tested (Hadley and Levine, 2007). Johnson (1986) was the first to show that TRH administration stimulated immediate prolactin secretion in horses. The prolactin response was dose-related between 50 and 500 µg of TRH (Johnson, 1986). At lower doses (0.4, 2 or 10 µg of TRH), there was no difference between doses but there was still a significant increase in serum prolactin concentrations (Thompson et al., 1992). Thyrotropin releasing hormone has been shown to act directly on lactotropes, acting through a specific TRH receptor, to stimulate prolactin (Hadley and Levine, 2007).

Prostaglandin- $F_{2\alpha}$ has been shown to drastically increase the rate of prolactin synthesis in rat pituitary cells (Gautvik et al., 1976). It is thought that $PGF_{2\alpha}$ may inhibit the release of prolactin-inhibiting factor (dopamine) causing the increase in prolactin concentrations (Ojeda et al., 1979). The stimulatory effect of $PGF_{2\alpha}$ on prolactin secretion has also been demonstrated in horses (Thompson et al., 2013).

Other Factors Affecting Prolactin Secretion

Dexamethasone has been shown to decrease prolactin concentrations in the rat (Rossier et al., 1980). Naloxone is an opioid antagonist, and binds to the opioid receptors normally found in the brain and prevents the binding of the endogenous opioid peptides (Rossier et al., 1980). Prolactin release is inhibited by endogenous opioids in horses (Aurich et al., 1996). In a study done by Aurich et al. (1995), naloxone was shown to increase prolactin secretion in stallions in the months of May and August and almost significantly increase prolactin concentrations in December. In contrast, naloxone has been shown to suppress prolactin secretion, and in the

presence of stress, naloxone partially suppressed the secretion of prolactin but did not abolish it, as did dexamethasone (Rossier et al., 1980). A high dose of naloxone (10 mg/kg) caused a significant decrease in prolactin concentrations, both basal and stress-induced, whereas a lower dose of (0.2 mg/kg) did not (Rossier et al., 1980).

AVP Effects on Prolactin

There is conflicting evidence in the literature concerning AVP and prolactin secretion. In a study done by DePaolo et al. (1986), administration of AVP into the third ventricle of the brain of rats suppressed prolactin secretion, which was hypothesized to be via a dopaminergic effect. However, Mai and Pan (1990) reported that intravenous administration of AVP stimulated prolactin secretion in ovariectomized, estrogenized female rats. A study by Funabashi et al. (1999) showed that administration of an AVP receptor antagonist caused a decrease in prolactin secretion in proestrous rats, which supports the hypothesis that prolactin increases in the presence of AVP. Also, Kjaer et al. (1991) reported that intravenous infusion of AVP stimulated prolactin secretion in rats in a dose dependent manner, and that administration of an antiserum against AVP, or an AVP antagonist, both inhibited the increase in prolactin secretion induced by the intracerebroventricular infusion of histamine. In healthy human males, Erfurth et al. (1996) found that intravenous AVP infusion caused consistent increases in plasma concentrations of prolactin and adrenocorticotrophic hormone (ACTH). Moreover, Alexander et al. (1991) reported that exercise of racehorses produced an immediate increase in both ACTH and AVP in pituitary venous blood; AVP concentrations fell after exercise, whereas ACTH concentrations remained elevated for an extended period of time.

VIP Effects on Prolactin

A flurry of research in the late 1970's and early 1980's indicated that VIP was likely a physiologic releasing factor for prolactin in the rat (Kato et al., 1978; Samson et al., 1980; Enjalbert et al., 1980; Abe et al., 1985). However, this has not necessarily held true for other species (Falsetti et al., 1988; Mezey et al., 1985; Sawangjaroen et al., 1994). One study showed that VIP may contribute to the regulation of prolactin from the anterior pituitary due to the fact that lactation causes an increase in VIP (Mezey et al., 1985). It has been shown in rhesus monkey pituitary tissue that VIP will stimulate prolactin secretion in the absence and presence of dopamine (Frawley and Neill, 1981). There is evidence in rat pituitary cells that prolactin secretion is regulated in an autocrine fashion by VIP produced in the hypothalamus (Nagy et al., 1988). In a study where VIP did not increase prolactin secretion, VIP was infused into the carotid artery of the ewe over a 10-minute period (Sawangjaroen et al., 1994). While VIP is found within the external zone of the median eminence in sheep, there is a study that shows VIP is not found in the hypophyseal portal blood of the sheep like it is in other species (Sawangjaroen et al., 1997).

Oxytocin and the Suckling Stimulus

Suckling has been shown to elicit an immediate release of pituitary prolactin (Benson et al., 1956; Fuchs et al., 1984; Grosvenor et al., 1986; Hadley and Levine, 2007). Suckling also results in a rapid release of oxytocin from the neurohypophysis (Samson et al., 1986), which travels to the adenohypophysis causing prolactin to be released (Benson et al., 1956). It has also been shown that injections of oxytocin cause an increase in prolactin concentrations in the rat (Egil et al., 2006), by acting directly on the lactotropes. In contrast, Koprowski and Tucker (1971) reported that administration of oxytocin to lactating dairy cows did not alter serum

prolactin concentrations. In horses, Roser et al. (1989) reported that pregnant mares at term induced to deliver with oxytocin had higher plasma prolactin concentrations in the first stages of labor than mares that delivered spontaneously. However, there was no immediate prolactin response to the injected oxytocin. Similarly, administration of 100 units of oxytocin intramuscularly to stallions and geldings did not elicit a prolactin response (D. L. Thompson, Jr., *unpublished data*).

Rationale for Present Experiments

The following experiments were designed to provide additional information on the mechanism(s) responsible for prolactin release in horses, particularly in response to stress and other forms of sympathetic nervous stimulation. The stress response in horses, epitomized by a brief exercise bout, includes immediate increases in plasma concentrations of prolactin (Thompson et al., 1988; Rabb et al., 1989; Colborn et al., 1991b), growth hormone (Thompson et al., 1994), adrenocorticotropin (Alexander et al., 1991; Nagata et al., 1999), cortisol (Thompson et al., 1988; Nagata et al., 1999), epinephrine (Thornton, 1985; Snow et al., 1992; Nagata et al., 1999), and AVP (Alexander et al., 1991). In addition, other neuropeptides and hormones have been reported to be involved with prolactin secretion, both in the resting state and in response to various stressful stimuli. Thus, the first two experiments described herein tested whether two peptides, AVP and VIP, known to affect prolactin secretion in other species, would alter resting plasma prolactin concentrations in the horse. The subsequent experiments used the paradigm of exercise-induced prolactin secretion to test whether the adrenal cortical, opioid, or prostaglandin systems in the horse mediate prolactin release in response to stress. Note that one apparently obvious candidate, epinephrine, was not tested here, because recent research indicated that epinephrine administration does not stimulate prolactin secretion in geldings (Thompson et al.,

2013). Oxytocin, another potential candidate, has also been tested previously in horses and found not to affect prolactin secretion (Roser et al., 1989; D. L. Thompson, Jr., *unpublished data*).

CHAPTER II

RESPONSES TO POTENTIAL PROLACTIN SECRETAGOGUES IN HORSES: ARGININE VASOPRESSIN AND VASOACTIVE INTESTINAL POLYPEPTIDE

Introduction

Although dopaminergic control of prolactin secretion has been well defined in various species, other brain peptides and hormones have been identified that cause an immediate prolactin release. Mai and Pan (1990) reported that intravenous administration of AVP stimulated prolactin secretion in ovariectomized, estrogenized female rats. A study by Funabashi et al. (1999) showed that administration of an AVP receptor antagonist caused a decrease in prolactin secretion in proestrous rats, which supports the hypothesis that prolactin increases in the presence of AVP. Also, Kjaer et al. (1991) reported that intravenous infusion of AVP stimulated prolactin secretion in rats in a dose dependent manner, and that administration of an antiserum against AVP, or an AVP antagonist, both inhibited the increase in prolactin secretion induced by the intracerebroventricular infusion of histamine. In healthy human males, Erfurth et al. (1996) found that intravenous AVP infusion caused consistent increases in plasma concentrations of prolactin and adrenocorticotrophic hormone (ACTH). However, in a study reported by DePaolo et al. (1986), administration of AVP into the third ventricle of the brain of rats suppressed prolactin secretion.

Similarly, VIP has been shown to increase prolactin secretion in some species and have no effect on prolactin secretion in other species. In rhesus monkeys, VIP stimulated prolactin secretion (Frawley and Neill, 1981). Dopamine appears to have no effect on prolactin levels in VIP-treated rhesus monkeys (Frawley and Neill, 1981). This shows that VIP is a compelling stimulator of prolactin secretion in rhesus monkeys that is able to override the dopamine control on prolactin (Frawley and Neill, 1981). Numerous studies have shown that VIP causes the

release of prolactin in rats (Kato et al., 1978; Samson et al., 1980; Enjalbert et al., 1980; Abe et al., 1985). However, this has not necessarily held true for other species (Falsetti et al., 1988; Mezey et al., 1985; Sawangjaroen et al., 1994). It has been reported that VIP antibodies or antagonists suppress prolactin secretion in rat pituitary cells (Nagy et al., 1988), indicating that VIP acts directly on lactotropes to cause prolactin secretion in rats.

Due to the species variation in prolactin responses to AVP and VIP, it cannot be assumed that either would be a secretagogue for prolactin in horses. Given that stimulating prolactin has direct application in seasonally anovulatory mares (Kelley et al., 2006; Mitcham et al., 2010), the present experiments were conducted to determine whether AVP or VIP administration would stimulate prolactin release in horses.

Materials and Methods

Experiment 2.1. AVP administration to mares. Eleven light horse mares between the ages of 6 and 21 yr, weighing between 385 and 615 kg, and with a body condition scores between 6 and 8 (Henneke et al., 1983), were used. They were long term residents of the LSU AgCenter Horse Unit in Baton Rouge, Louisiana. They were maintained on pasture consisting primarily of Alicia bermudagrass and winter ryegrass. The experiment was conducted in mid-May of 2012.

All mares were brought in from pasture the night before treatments and kept in a dry lot with water available on an ad libitum basis. The following morning, the mares were quietly walked into an outdoor chute and tethered either in the chute or on the fence alongside the chute. At that time, each mare was fitted with an indwelling, 14-gauge catheter in the left jugular vein that was held in place with cyanoacrylate glue.

The AVP (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile 0.155 M saline such that all treatment injections were 5.0 mL. Doses prepared were 0 (control), 0.05, 0.1 and 0.2 mg in 5 mL. Two initial blood samples were drawn from each mare at 10 min apart, and then treatment was administered through the jugular catheter. Three mares each received the 0, 0.5, and 0.1 mg dose; two mares received the 0.2 mg dose. Post-treatment blood samples were collected at 5, 10, 20, 30, 40, and 60 minutes relative to treatment injection. All blood samples were immediately placed into sample tubes containing sodium heparin as an anticoagulant and were centrifuged at 1200 x g for 10 min. Plasma was stored at -15°C until they were assayed for prolactin. Prolactin was measured in all samples with a double-antibody radioimmunoassay previously validated for horse tissues (Colborn et al., 1991a).

Prolactin concentrations were analyzed by repeated measures analysis of variance (ANOVA) by the GLM procedure of SAS (SAS Instit., Cary, NC). The ANOVA tested the effect of treatment with the mare-within-treatment term, and tested the effect of time of sampling and its interaction with treatment with residual error.

Experiment 2.2. VIP administration to geldings. Twelve light horse geldings between the ages of 10 and 21 yr, weighing between 442 and 640 kg, and with body condition scores between 6 and 8, were used. They were long term residents of the LSU AgCenter Horse Unit in Baton Rouge, Louisiana, and were housed and maintained in the same pastures as the mares in Experiment 2.1. The experiment was conducted in late May of 2012.

The geldings were prepared starting the night before as described for the mares in Experiment 2.1. On the day of treatment, the geldings were tethered alongside the fence and chute and jugular blood samples were collected at -10, 0, 10, 20, 30, 40, 60, 90, and 120 minutes relative to treatment injections. In this case, blood was drawn via jugular venipuncture at each

time interval via 21-gauge needles attached to 7-mL evacuated blood tubes containing sodium heparin as the anticoagulant. Treatments were sterile saline (5 mL; controls, n = 6) and 0.25 (n = 4) or 0.5 (n = 2) mg of VIP (Anaspec, Inc., Fremont, CA) dissolved in sterile saline (5 mL volume). Blood samples were handled, stored, and assayed for prolactin as described for Experiment 2.1. Statistical analysis of the prolactin concentrations was the same as described for Experiment 2.1.

Results

Experiment 2.1. Mean prolactin concentrations for mares receiving 0, 0.25, and 0.5 mg of VIP are presented in Figure 2.1. There was no effect of AVP dose in the ANOVA ($P = 0.99$). There was an effect of time of sampling ($P = 0.0007$), but no interaction of dose and time of sampling ($P = 0.39$).

Experiment 2.2. Prolactin concentrations for geldings receiving 0, 0.25, or 0.5 mg of VIP are presented in Figure 2.2. Data for individual geldings at each dose are presented, as well as the means for each dose, to illustrate the variation among horses within the dose groups. Individual geldings had spurious, large increases in prolactin concentrations not typical of the groups. Although there was an effect of time of sampling ($P = 0.0012$) in the ANOVA, there was no effect of dose of VIP ($P = 0.38$) and no interaction with time of sampling ($P = 0.49$).

Discussion

Administration of AVP or VIP at the doses used had no effect on prolactin secretion other than that seen in control horses, even though there were effects of time on prolactin concentrations in both experiments. Responses to known secretagogues of prolactin (e.g., sulpiride, TRH, or $\text{PGF}_{2\alpha}$; Johnson, 1986; Johnson and Becker, 1987; Thompson et al., 2013) in horses occur typically within 5 to 10 min of administration. The time effects of

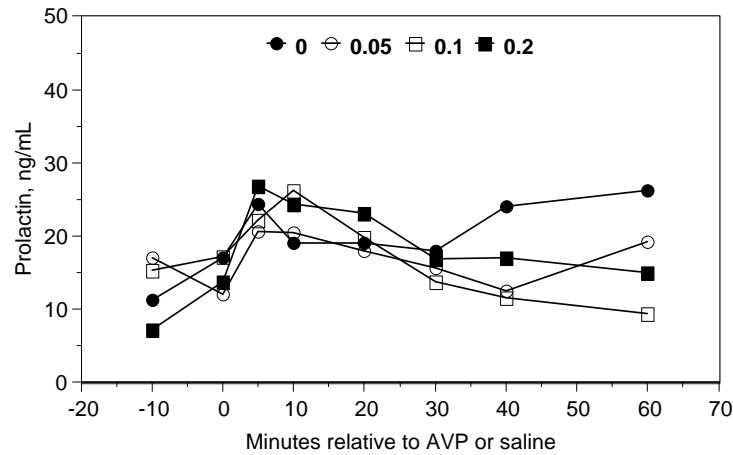


Figure 2.1. Mean plasma concentrations of prolactin in horses administered arginine vasopressin (AVP) at time 0 at 0, 0.05, 0.1, or 0.2 mg in Experiment 2.1. There was an effect of time of sampling ($P < 0.0007$) in the ANOVA, but no effect ($P > 0.1$) of dose or interaction of dose and time. The pooled SEM was 3.5 ng/mL.

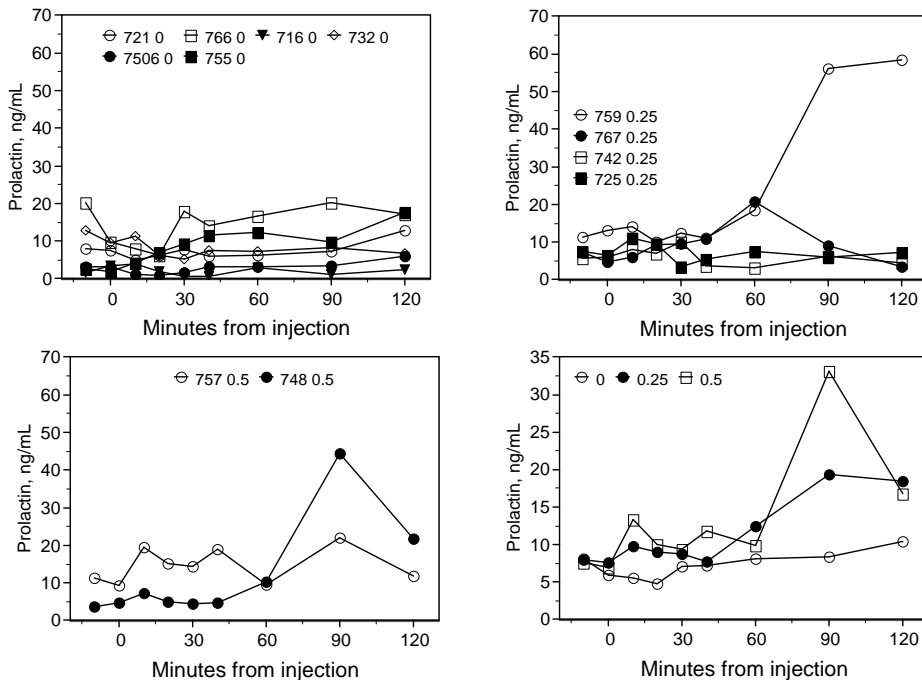


Figure 2.2. Plasma concentrations of prolactin in horses administered vasoactive intestinal polypeptide (VIP) at time 0 at 0, 0.25, or 0.5 mg in Experiment 2.2. There was an effect of time of sampling ($P < 0.0012$) in the ANOVA, but no effect ($P > 0.1$) of dose or interaction of dose and time. Data for the individual horses are shown to illustrate the variation in prolactin concentrations. The pooled SEM was 4.1 ng/mL.

prolactin concentrations observed in both experiments were unexpected; normally horses receiving 5 mL of saline intravenously have no immediate change in prolactin concentrations. However, spontaneous surges in prolactin concentrations have been reported (Roser et al., 1987; Thompson et al., 1992), and likely account for the variations in Experiment 2.2. Whether the time variations in Experiment 2.1, which seemed more coordinated, were physiologic, is unknown.

Route of administration of these two peptides has varied in previous experiments. DePaolo et al. (1986) administered AVP into the third ventricle of the brain of rats resulting in a suppression of prolactin secretion, which was hypothesized to be via a dopaminergic effect. However, most stimulatory effects of AVP on prolactin secretion have been after intravenous injection (Mai and Pan, 1990; Kjaer et al., 1991; Erfurth et al., 1996). Dose of peptide is another factor that must be considered, especially in the face of negative results on prolactin secretion. Synthetic AVP has a biological activity of approximately 600 IU/mg of peptide (Stedman's Medical Dictionary, 2006). A dose of AVP used in humans of 0.0143 IU/kg BW infused over a 1.5-min period elevated plasma ACTH and cortisol concentrations within 15 min (Rye et al., 1997). Based on that dose, the highest dose used in the present experiment (0.2 mg in a horse of approximately 500 kg, or 0.218 IU/kg BW) was approximately 15 times that dose used in humans. Doses of 0.05 to 0.4 IU/min administered to adult horses are recommended for increasing blood pressure in animals experiencing septic shock that do not respond to fluid replacement and norepinephrine therapy (Divers, 2008). It has been reported that 0.4 and 0.8 mg of AVP administered to normal horses of about 500 kg caused undesirable side-effects indicative of elevated blood pressure and peripheral vasoconstriction (D. L. Thompson, Jr., *unpublished*

data). Thus, it is unlikely that the lack of prolactin response in this experiment was due to insufficient dose of AVP.

There is no mention of effects of VIP in horses in the literature, thus the only possible comparison of dose is restricted to its administration in other species. Infusion of VIP into the uterine artery of sheep at 1 to 30 $\mu\text{g}/\text{min}$ resulted in whole-body vasodilatation that was evident in both the infused and the contralateral sides of the uterine vasculature (Clark et al., 1982). In newborn sheep, VIP was a powerful pulmonary vasodilator with a threshold of 0.3 $\mu\text{g}/\text{kg BW}$ (Kulik et al., 1984). Sawangjaroen and Curlewis (1994) infused VIP (1.8 nmol/min, or 6 $\mu\text{g}/\text{min}$) into the carotid artery of sheep over a 10 min period and observed an increase in heart rate to almost three-fold the resting value; in contrast, prolactin secretion was not affected. The highest dose of VIP administered in the present experiment was 0.5 mg, which would be approximately 1 $\mu\text{g}/\text{kg BW}$ for a horse of average body condition. Thus, this dose is similar to those causing significant biological effects in sheep. Unfortunately, possible effects of VIP on blood pressure were not monitored in the present experiment to confirm its biological activity at the doses administered.

In conclusion, these results of AVP and VIP administration to horses do not support the hypothesis that either peptide is involved with prolactin secretion in mares or geldings. Further study with higher doses of VIP may be warranted, but would unlikely result in responses different than those presented herein.

CHAPTER III

EFFECT OF PRETREATMENT WITH DEXAMETHASONE, NALOXONE, CABERGOLINE, FLUNIXIN MEGGLUMINE, OR SULPIRIDE ON THE PROLACTIN RESPONSE TO EXERCISE IN HORSES

Introduction

It has been shown that various forms of stress in horses cause prolactin secretion to increase (Rabb et al., 1989; Colborn et al., 1991b; Thompson et al., 1994). Plasma prolactin concentrations can continue to be increased as long as 30 min from acute exercise for 5 minutes in stallions (Colborn et al., 1991b). The prolactin response to exercise can occur with as little as 1 min of trotting in mares (D. L. Thompson, Jr., *unpublished data*).

The secretagogues of prolactin that are known to act via specific hormonal or neurotransmitter receptors on pituitary lactotrobes are dopamine antagonists, which bind to dopamine receptors and block the inhibitory action of dopamine, and TRH, which binds to specific TRH receptors on the lactotrobes and directly stimulates prolactin secretion (Hadley and Levine, 2007). When horses are stressed, it is not known how prolactin secretion is mediated. The two most commonly hypothesized models are 1) the stress causes an immediate reduction in dopaminergic input to the pituitary via the hypothalamic-pituitary portal system, and 2) the stress causes the release of an unknown stimulatory factor, such as TRH, that acts directly on the lactotrobes. The following experiments were designed to test several drugs known to perturb the adrenal axis (dexamethasone), the opioid system (naloxone), the dopaminergic system (cabergoline), the prostaglandin synthetic pathway (flunixin meglumine), and possibly a short-loop feedback system of prolactin on its own secretion (sulpiride).

Dexamethasone is a glucocorticoid analog that mimics the action of cortisol in the body. Cortisol is released as part of the sympathetic nervous system response to stress (Rabb et al., 1989; Colborn et al., 1991b). Pretreatment with dexamethasone 1 day before exercise should

preclude ACTH from being secreted in response to exercise, due to its negative feedback on the hypothalamic-pituitary axis (Hadley and Levine, 2007).

It has also been proposed in the horse that opioid agonists inhibit the secretion of prolactin (Aurich et al., 1996). Naloxone, which is an opioid antagonist, has been shown to increase prolactin secretion in stallions, in the absence of exercise or stress (Aurich et al., 1995). In contrast, naloxone has been shown to decrease prolactin secretion in the presence of stress in rats (Rossier et al., 1980). The study done by Rossier et al. (1980) is contradictory to the study done by Aurich et al. (1996) unless there is an unknown relationship between naloxone and stress.

Given that dopamine is a known physiologic regulator of prolactin secretion (Hadley and Levine, 2007), the action of its analogs should be inhibitory to prolactin release after stress. In fact, Thomson et al. (1996) treated stallions with bromocriptine before sexual stimulation and seminal collection, and reported that it precluded the prolactin increase normally seen after collection (Rabb et al., 1989; Colborn et al., 1991b). Cabergoline is a powerful dopamine D2 receptor agonist (Seeman, 2007) that can suppress prolactin secretion for at least 10 days in horses (Hebert, 2012).

Banamine is a trade name for flunixin meglumine, a non-steroidal anti-inflammatory drug that has been shown to inhibit prostaglandin synthesis in horses in general (Semrad et al., 1985) and PGF-2 α production and secretion specifically in mares (Daels et al., 1991). Prostaglandin-F2 α administration has been reported to cause immediate prolactin release in horses (Thompson et al., 2013), and Ginther et al. (2012) reported that inhibition of PGF_{2 α} production with flunixin meglumine in mares resulted in a reduced prolactin secretion during the luteolytic phase. Similar results were reported for heifers by Pugliesi et al. (2012). Pretreatment with flunixin meglumine

before exercise should provide evidence as to whether $\text{PGF}_{2\alpha}$ is involved with the prolactin response to that exercise.

Finally, sulpiride causes immediate prolactin release in horses due to its antagonism of dopamine receptors on the lactotrobes. There have been reports that prolactin can act via short-loop feedback, either at the hypothalamus or directly on the pituitary (autoregulation) to inhibit further prolactin release (Hadley and Levine, 2007). Thus, sulpiride pretreatment was assessed as a factor that might perturb exercise-induced prolactin release in the last experiment.

Materials and Methods

General procedures. Horses in the following experiments were long-term residents of the LSU AgCenter Horse Unit in Baton Rouge, Louisiana, and were routinely housed on pasture. For each exercise challenge, they were pretreated either the evening before exercise (dexamethasone, cabergoline) or the morning of exercise (naloxone, flunixin meglumine, and sulpiride). In all experiments, the horses were kept in a dry lot overnight and quietly walked into an outdoor chute in the morning. A halter was placed on each horse, which was then removed from the chute and tethered to the fence near the chute, or kept in the chute untethered.

When all horses were in place, blood sampling for the first horse began. The order of horses was randomized, with the exception that treated and control horses were exercised alternately. Blood sampling in each case involved a pre-exercise sample followed by a sample at 5, 10, and 20 min after the onset of exercise. Exercise was for 2 min in a round pen at a trot or canter; horses were encouraged by voice commands only. Blood was drawn by jugular venipuncture with 21-gauge needles into evacuated 7-mL tubes containing sodium heparin (143 units). Blood samples were subsequently placed at 4°C and centrifuged at 1200 x g for 15 min; plasma was harvested and stored at -15°C.

Plasma concentrations of prolactin were determined by radioimmunoassay as described by Colborn et al. (1991a). Limit of sensitivity and intra- and interassay coefficients of variation averaged 0.2 ng/mL and 7 and 12%, respectively.

Data from each experiment was analyzed by ANOVA that took into account the repetitive nature of the data (split-plot; Steel et al., 1997). Main effects of sex (where appropriate), treatment, and sampling time were assessed, as well as all two- and three-way interactions. Differences between treatment and control means for each time period were assessed by the least significant difference test (Steel et al., 1997).

Experiment 3.1. Dexamethasone pretreatment. Six light horse mares and six light horse geldings between the ages of 7 and 23 years, weighing between 410 and 615 kg, with body condition scores between 5 and 8 were used. The experiment was conducted during June of 2012. Three mares and three geldings were administered dexamethasone (Sigma Chem. Co., St. Louis, MO; 40 µg/kg BW) in oil intramuscularly on the evening before the exercise challenge; three mares and three geldings received similar injections of oil and served as controls.

Experiment 3.2. Naloxone pretreatment. Six light horse mares and six light horse geldings between the ages of 14 and 21 years, weighing between 442 and 640 kg, with body condition scores between 6 and 8 were used. The experiment was conducted during June of 2012. Three mares and three geldings were administered naloxone (Sigma; 0.25 mg/kg BW) in saline intravenously 2 min before the exercise challenge; three mares and three geldings received similar injections of saline and served as controls.

Experiment 3.3. Cabergoline pretreatment. Six light horse mares and six light horse geldings between the ages of 7 and 23 years, weighing between 430 and 615 kg, with body condition scores between 6 and 8 were used. The experiment was conducted during July of

2012. Three mares and three geldings were administered cabergoline (1 mg) in long-acting vehicle intramuscularly the evening before the exercise challenge; three mares and three geldings received similar injections of saline and served as controls.

Experiment 3.4. Flunixin meglumine pretreatment. Ten light horse geldings between the ages of 7 and 20, weighing between 442 and 540 kg, with body condition scores between 6 and 7 were used. The experiment was conducted in October of 2012. Five geldings were administered flunixin meglumine (Schering-Plough Animal Health, Kenilworth, NJ; 1.5 mg/kg BW) solution intravenously and five control geldings received a similar injection of saline. Blood samples were collected immediately before flunixin meglumine or saline injection, and then again 15 min later, which was immediately before exercise. Additional blood samples were collected at 5, 10, 15, 20 and 30 minutes after onset of exercise.

Experiment 3.5. Sulpiride pretreatment. Ten light horse geldings between the ages of 7 and 21, weighing between 410 and 570 kg, with body condition scores between 5 and 7 were used. The experiment was conducted in October of 2012. Five geldings were administered sulpiride (racemic mixture; Sigma; 0.01 mg/kg BW) in saline intravenously and five control geldings received a similar injection of saline. Blood samples were drawn immediately before injections and again at 5, 10, 20, 30, 60, and 89 minutes after injection. Horses were then exercised starting at 90 min post-injection. Blood samples were collected at 5, 10, 15, 20 and 30 minutes after onset of exercise.

Due to the large prolactin response in sulpiride-treated geldings relative to the normal response to exercise, data from Experiment 3.5 were analyzed in two separate ANOVA: one with all data included, and one with only the 90-min samples and subsequent data included. This

allowed for better testing the effects of pretreatment with sulpiride (resulting in a large variation) on the exercise-induced prolactin concentrations (with smaller variations).

Results

Experiment 3.1. There was an effect of time ($P < 0.0001$) in the ANOVA, reflecting the significant rise in prolactin concentrations after exercise in the two groups of horses (Figure 3.1). There was no effect ($P > 0.1$) of sex, dexamethasone treatment, or sex by treatment interaction, nor any interaction with time and the other factors in the analysis.

Experiment 3.2. There was an effect of time ($P < 0.0001$) in the ANOVA, again reflecting the significant rise in prolactin concentrations after exercise in the two groups of horses (Figure 3.2). However, there was no effect ($P > 0.1$) of sex, naloxone treatment, or sex by treatment interaction, nor any interaction with time and the other factors in the analysis.

Experiment 3.3. There was an effect of time ($P < 0.001$) as well as a treatment by time interaction ($P < 0.001$) in the ANOVA, but no effect of sex or any interaction of other factors with sex (Figure 3.3). Control horses displayed the expected rise in prolactin concentrations after exercise, but horses treated with cabergoline had low and unchanging concentrations throughout the blood sampling period. Prolactin concentrations differed ($P < 0.01$) between groups for all time periods.

Experiment 3.4. There was an effect of time ($P < 0.0001$) in the ANOVA, reflecting the significant rise in prolactin concentrations after exercise in the two groups of horses (Figure 3.4). There was a trend ($P = 0.074$) towards higher prolactin concentrations in the treated group relative to controls; however, the difference was present from the first (pre-flunixin meglumine injection) sample and remained at a consistent level throughout the blood sampling period. There was no treatment by time interaction ($P > 0.1$).

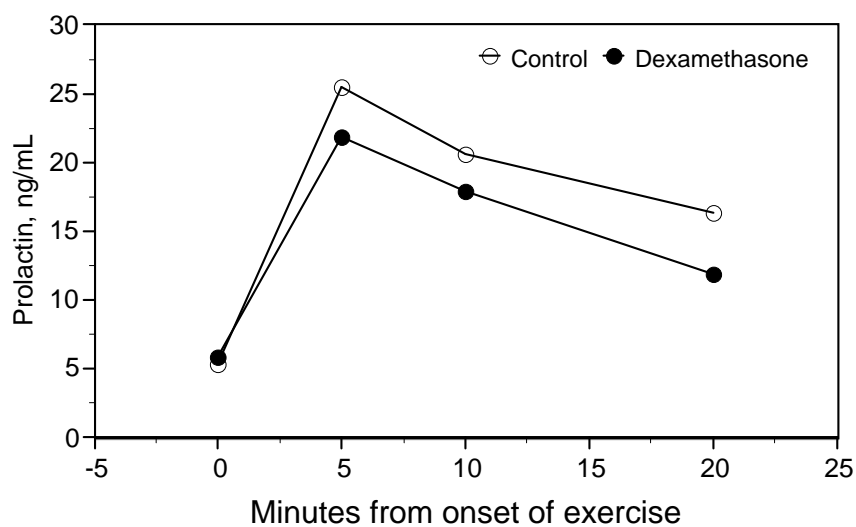


Figure 3.1. Mean plasma concentrations of prolactin in horses administered dexamethasone the evening before exercise for 2 min in Experiment 3.1. There was an effect of time of sampling ($P < 0.0001$) in the ANOVA, but no effect ($P > 0.1$) of treatment or interaction of treatment and time. The pooled SEM was 3.7 ng/mL.

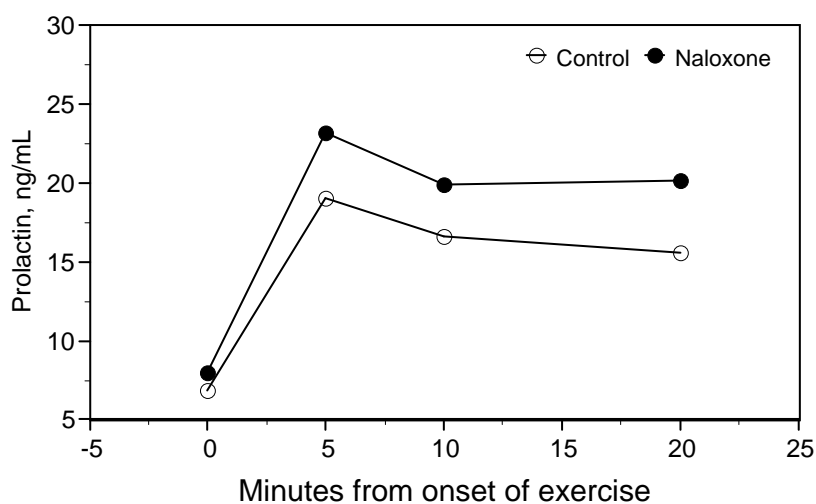


Figure 3.2. Mean plasma concentrations of prolactin in horses administered naloxone 2 min before exercise for 2 min in Experiment 3.2. There was an effect of time of sampling ($P < 0.0001$) in the ANOVA, but no effect ($P > 0.1$) of treatment or interaction of treatment and time. The pooled SEM was 2.2 ng/mL.

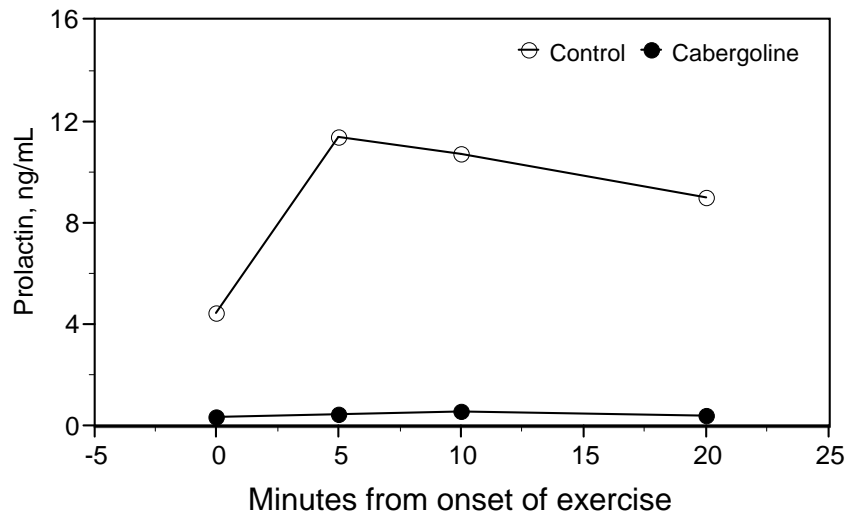


Figure 3.3. Mean plasma concentrations of prolactin in horses administered cabergoline the evening before exercise for 2 min in Experiment 3.3. There was an interaction of treatment with time of sampling ($P < 0.0001$) in the ANOVA. Prolactin concentrations of control horses differed ($P < 0.01$) from those of treated horses for all time periods. The pooled SEM was 0.6 ng/mL.

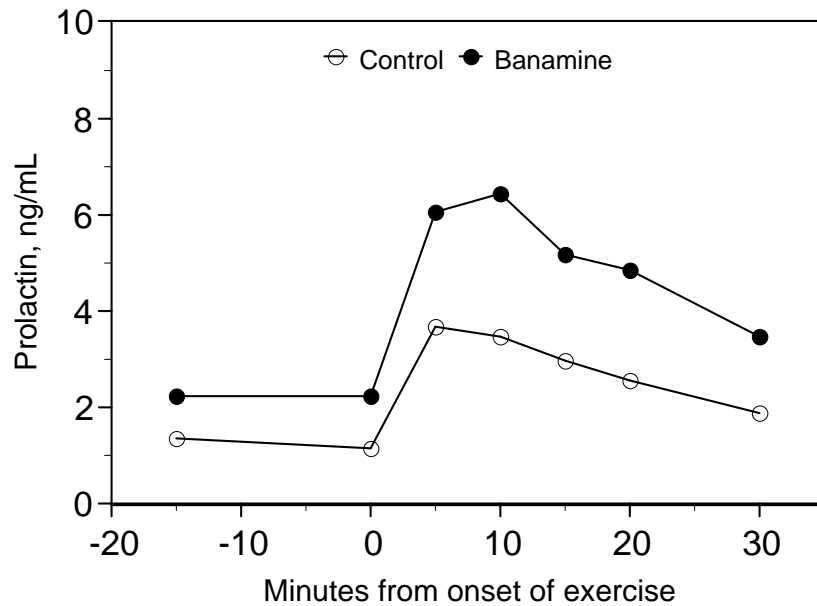


Figure 3.4. Mean plasma concentrations of prolactin in geldings administered flunixin meglumine (Banamine) 15 min before exercise for 2 min in Experiment 3.4. There was an effect of time of sampling ($P < 0.0001$) in the ANOVA, and a trend ($P = 0.074$) for lower concentrations in control geldings. There was no interaction of treatment and time ($P > 0.1$). The pooled SEM was 0.7 ng/mL.

Experiment 3.5. After sulpiride injection, prolactin concentrations increased ($P < 0.001$) to a peak of >16 ng/mL in treated geldings and then gradually decreased to concentrations similar to controls by 89 min (Figure 3.5); concentrations in control geldings were relatively low and unchanged during the same time, as reflected by the treatment by time interaction ($P < 0.001$) in the first ANOVA (all data). Analysis of the 90-min and subsequent data separately indicated no effect of treatment, time, or treatment by time interaction ($P > 0.1$).

Discussion

Each treatment in the five experiments contained herein was designed to perturb a system in the body that could potentially be involved in prolactin secretion. With the known effects of hypothalamic dopamine input to the pituitary (Hadley and Levine, 2007), it was assumed that cabergoline treatment would likely abolish the prolactin response to exercise. However, if a prolactin releasing factor existed in horses as has been reported for other species (Watanobe et al., 2000; Curlewis et al., 2002), it could possibly stimulate prolactin directly at the lactotrope and override the suppressive effects of the dopamine agonist. Given that no response was observed after exercise in horses receiving cabergoline, it is concluded that either the cabergoline suppression was too great, or that no alternate stimulatory factor is involved.

In general, the prolactin responses to exercise were consistently observed in control mares and geldings in all five experiments. These data also confirm that the magnitude of the prolactin response to exercise and other forms of stress is not as great as is observed after treatment with sulpiride (Clavier et al., 2012). As seen in Experiment 3.5, the prolactin response after sulpiride treatment was almost four times as great as that observed in control geldings after exercise.

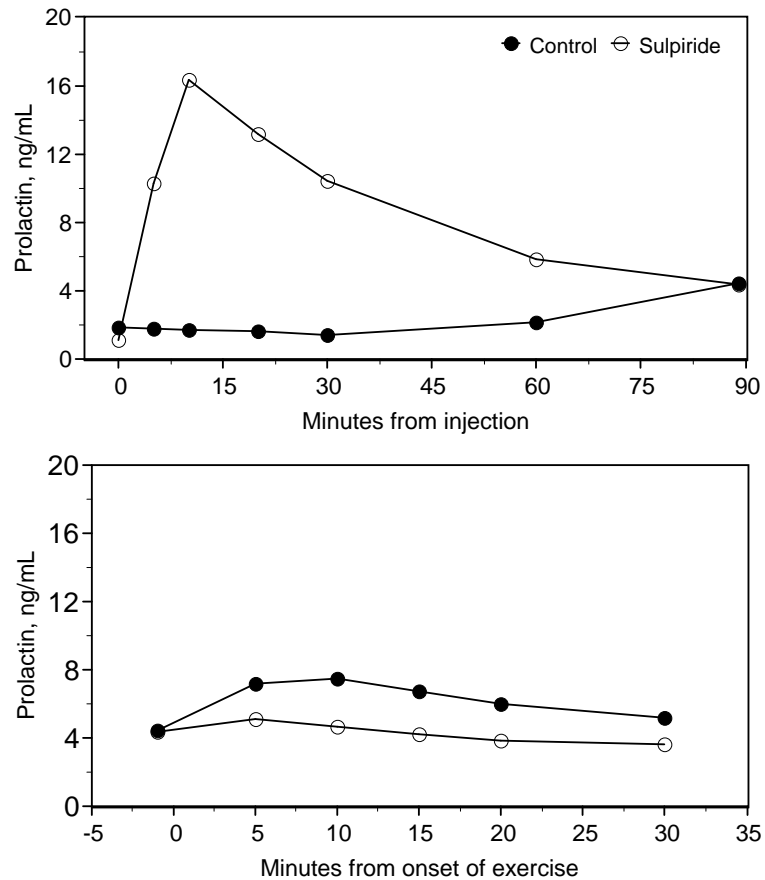


Figure 3.5. Mean plasma concentrations of prolactin in control geldings and geldings administered sulpiride 90 min before exercise for 2 min in Experiment 3.5. Sulpiride stimulated ($P < 0.0001$) prolactin concentrations (top panel), but had no effect ($P > 0.1$) on the exercise-induced prolactin response (bottom panel). Pooled SEM was 1.4 ng/mL for the sulpiride effect, and 1.1 ng/mL for the exercise effect.

Dexamethasone treatment was designed to feedback on the hypothalamic-pituitary axis to suppress corticotropin releasing hormone and ACTH secretion, which are known to respond to exercise in horses (Alexander et al., 1991). Blocking these two hormones should abolish, or diminish, the prolactin response to exercise, if in fact they were involved in that response. The very similar prolactin responses to exercise in the treated and control horses indicate that neither hormone is apparently involved in the prolactin response to exercise. Dexamethasone treatment

of rats actually decreases prolactin secretion (Rossier et al., 1980), however no decrease was observed in the horses in this experiment.

Aurich et al. (1995a) reported that naloxone administration to stallions increased prolactin concentrations during May and August, and had a tendency to do so in December. Aurich et al. (1996) also reported that naloxone stimulated prolactin release in stallions, but not in luteal phase mares or geldings. In contrast, Aurich et al. (2002) reported no change in prolactin concentrations in stallions treated with naloxone. Aurich et al. (1995b) reported that prolactin secretion was induced by naloxone treatment in ovariectomized pony mares that had been pretreated with estradiol benzoate. However, Aurich et al. (1997) reported that prolactin release was significantly increased by naloxone administration in ovariectomized pony mares after mares had been pretreated with only melatonin, but not when they received a combination of estradiol benzoate and melatonin. Thus, the literature is contradictory on the effects of opioid antagonists on prolactin secretion in horses. The results of Experiment 3.2 indicate that naloxone treatment of mares and geldings had no direct effect on prolactin secretion in the first 15 min after administration, and subsequently no effect on the exercise-induced prolactin response.

Flunixin meglumine is a potent inhibitor of prostaglandin synthesis in horses (Semrad et al., 1985) and has been used in doses similar to that used herein to abolish $\text{PGF}_{2\alpha}$ production and release in mares (Daels et al., 1991; Ginther et al., 2012). Although intravenous administration of $\text{PGF}_{2\alpha}$ was shown to stimulate prolactin secretion in mares (Thompson et al., 2013), flunixin meglumine administration at a dose used in previous studies had no effect on exercise-induced prolactin secretion in the geldings in Experiment 3.4. It is concluded that the prolactin response to exercise is likely not mediated by prostaglandin(s) in the horse.

Sulpiride was first reported to increase prolactin concentrations in horses by Johnson and Becker (1987). Since then, multiple studies have shown the stimulatory effects of sulpiride on prolactin secretion in horses (Colborn et al., 1991a; Thomson et al., 1996; Donadeu and Thompson, 2002; Kelley et al., 2006). Stimulating prolactin secretion with sulpiride before exercise tested the possibility that a short-loop feedback, or perhaps an autoregulation, of prolactin secretion, is present in horses. This approach has the limitation that release of prolactin 90 min before exercise may only deplete the releasable stores of prolactin rather than feeding back in a negative manner. Regardless, there was no significant effect of pretreatment with sulpiride on the exercise-induced release of prolactin 90 min later. A better approach to testing whether a negative short-loop or autoregulation of prolactin exists would be to inject prolactin exogenously. The main reason that approach has not been attempted is that no source of large amounts of equine prolactin are available, and use of a similar hormone, recombinant porcine prolactin, caused antibody production in pony mares (Thompson et al., 1997).

SUMMARY AND CONCLUSIONS

Seven experiments were conducted to investigate factors that affect pituitary prolactin secretion in the horse in the resting state and in response to exercise. The first two experiments explored how two brain peptides affect resting prolactin levels. The subsequent five experiments were conducted to examine the effects of perturbation of various systems within the body on the prolactin response to the stress of exercise.

In the first experiment, various doses of AVP (0, 0.05, 0.1 and 0.2 mg in 5 ml solution) in saline administered intravenously to determine their effect on prolactin secretion. Although there was a time effect on prolactin concentrations, there was no alteration relative to the 0 dose response. It was concluded that AVP likely is not involved in prolactin secretion in a calm horse.

Various doses of VIP (0, 0.25 and 0.5 mg) were administered in the second experiment and it was determined that neither dose of VIP altered prolactin concentrations compared to the 0 dose. It was concluded that VIP also does not affect the lactotrope directly and is not a likely regulator of prolactin secretion in the horse.

The third experiment tested dexamethasone, a glucocorticoid agonist that feeds back negatively on corticotropin releasing hormone and ACTH, as a pretreatment to acute exercise. Prolactin responded to 2 min of exercise as expected, but pretreatment with dexamethasone did not perturb that response.

In the fourth experiment, the opioid antagonist naloxone was administered intravenously as a pre-exercise treatment. Naloxone had no effect on the prolactin response to exercise, thus endogenous opioid peptides are not likely involved with exercise-induced prolactin secretion in horses.

Cabergoline, a long-acting dopamine agonist, was administered in the fifth experiment. The morning after administration, prolactin concentrations were suppressed and remained low throughout exercise and the post-exercise blood sampling period. Control horses displayed the normal exercise-induced prolactin response. It was determined that cabergoline abolished the prolactin response even in the presence of exercise. It was concluded that cabergoline is a potent inhibitor of prolactin secretion, and likely would override any stimulatory effect of exercise (such as a releasing hormone) even if it existed.

The sixth experiment was conducted using flunixin meglumine, a nonsteroidal anti-inflammatory drug that prevents prostaglandin production and secretion, as a pretreatment to exercise. The exercise-induced prolactin response was not altered by flunixin meglumine treatment, indicating that prolactin response to exercise is unlikely mediated by prostaglandins.

The final experiment tested the effect of sulpiride treatment, which normally stimulates prolactin secretion directly at the lactotrope level by competing with dopamine for its receptors. As expected, pretreatment with sulpiride stimulated prolactin secretion, but it had no effect on the exercise-induced prolactin response 90 min later.

It is concluded that the only drug that had a significant effect on prolactin secretion was the dopaminergic agonist cabergoline. Direct administration of AVP or VIP, or perturbations of the adrenal cortical axis, the opioid system, or the prostaglandin system, had no effect on prolactin secretion as has been reported previously for other species.

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