MODULATION OF THE ACTH RESPONSE TO STRESS BY IL-6, NITRIC OXIDE, DIET AND EXERCISE

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by
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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

MODULATION OF THE ACTH RESPONSE TO STRESS
BY IL-6, NITRIC OXIDE, DIET AND EXERCISE

Presented by Ryan Jankord

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance

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Professor James Turk

DEDICATIONS

I greatly appreciate those who made this possible and made this worthwhile. I would like to dedicate this thesis to…

My Father
My family, Rekha and Hailey
My parents, Steve and Dolly
My mentor, Dr. Laughlin
Teachers past and present that have encouraged and enlightened me

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The work completed in this thesis required a multidisciplinary approach and was made possible by the opportunities provided by my committee to complete work in their labs, discuss potential projects and assist in the interpretation of results. As a whole, I greatly appreciate the time that I was afforded to individually discuss many aspects of this thesis. Thank you for your help and I hope you are pleased by the work presented in this thesis. In addition, I wish to acknowledge the following technical contributions.

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ABSTRACT

The release of ACTH from the anterior pituitary is one step in the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis. The HPA axis is activated in response to stress and the secretion of ACTH is under the control of hypothalamic releasing factors. This thesis examined the role of several different factors in the modulation of the ACTH response to stress by testing the following hypotheses: 1) hypothalamic IL-6 is indirectly involved in stimulating the HPA response to stress by affecting the activity of corticotropin releasing hormone (CRH) neurons in the hypothalamus; 2) nitric oxide synthase (NOS) inhibition will result in an increase in ACTH response to stress and a decrease in blood flow to tissues of the HPA axis; and 3) high fat diet-induced changes in HPA activity could be attenuated with exercise training. The experimental design, results and discussion for each one of these hypotheses constitutes a single chapter in this thesis. The major findings include: 1) IL-6 is a novel hypothalamic factor found expressed in the external zone of the median eminence and released into the hypophyseal portal vessels in response to stress; 2) Compared to males, female pigs show a greater expression of IL-6 in the median eminence and a greater ACTH response to exercise and restraint stress; 3) Nitric oxide activates the HPA axis in response to restraint but limits the HPA response during maximal exercise; 4) Nitric oxide maintains basal cerebral blood flow; and 5) a high fat diet increases free fatty acids and attenuates the ACTH response to stress and exercise training reverses both of these high fat diet induced alterations. These data demonstrate that multiple factors influence the release of ACTH in response

to stress. Thus, the ability of the body to respond to stress through the activation of the HPA axis can be affected by IL-6, nitric oxide, diet and exercise.

PREFACE

Recorded here are the events from the exploratory journey I, Ryan Jankord, took under the direction and guidance of my dissertation committee. As many journeys from the department of Biomedical Sciences have begun, this journey set out to see how activity affects health. I began the trek to obtain a better view of how exercise affects markers of chronic inflammation. I made a decided stop along the way to take a closer look at the biology of neuro-immuno-endocrine interactions. This dissertation is sequenced to first introduce you to the area of study then share with you the discoveries of my work. To finish, I will attempt to place the results from these studies into context for how they relate to our current understanding of the areas explored and the possible implications these results may have for human health.

CHAPTER 1

INTRODUCTION

Inflammation and Health

The term inflammation conjures different images to different people. Celsus described the cardinal clinical signs of inflammation nearly 200 years ago as redness (*rubor*), heat (*calor*), swelling (*tumor*) and pain (*dolor*). Rudolph Virchow added dysfunction of the organs involved (*function laesa*) in 1871 (Scott et al. 2004a). Understanding of the physiological, cellular and molecular processes involved in inflammation has improved dramatically in recent years (Scott et al. 2004b). Acute inflammation is rapid and composed primarily of neutrophils that accumulate within minutes, while chronic inflammation requires a minimum of 24 to 48 hours and is composed predominantly or monomuclear cells, such as macrophages and lymphocytes (Gabay 2006). The acute response involves large increases (100-1000 fold) in various inflammatory markers like plasma IL-1β, TNF-α and IL-6 that can remain elevated for several days. After the acute response plasma IL-1β, TNF-α and IL-6 all return to their pre-infection basal levels (Gabay and Kushner 1999).

In comparison to an acute response the chronic inflammatory state refers to smaller changes (from approximately 1-2pg/ml to 3-4pg/ml for IL-6) that persist over longer periods, years to decades, of time (Bruunsgaard 2005). It is this chronic low-grade inflammatory state that is frequently present during the development of a multitude of diseases and disorders. In fact, the American Heart Association includes the proinflammatory state (defined as elevated C-Reactive Protein, CRP) as one of the risk factors/components of the metabolic syndrome (AHA 2006). Understanding the relation between this chronic low-

grade inflammatory state and health is what prompted the experiments in Chapter 2.

IL-6

The inflammatory marker on which the research in this thesis is focused is IL-6. Depending on the researcher, or clinician, you are talking with, IL-6 may be referred to as a pro- or anti-inflammatory cytokine. Both terms are appropriate for describing the biological activity of IL-6 as IL-6 possesses both pro- (increases acute phase proteins, like CRP) and anti- (turns off IL-1β and TNF-α production) inflammatory properties. Although short duration increases in plasma IL-6 have been suggested as beneficial (Pedersen and Febbraio 2005), no report has provided evidence to suggest that chronic maintenance of elevated plasma IL-6 can be beneficial for health. In fact, chronic maintenance of plasma IL-6 above basal levels is associated with a variety of diseases and disorders (Cohen et al. 1997; Conway et al. 2004; Ferrucci et al. 1999; Ikonomidis et al. 1999; Ridker et al. 2000).

IL-6 and Disease

The inflammatory state is one of the components of the metabolic syndrome. In addition, plasma IL-6 has been associated with, or implicated in, the development of the other components comprising the metabolic syndrome. IL-6 is associated with obesity (Bastard et al. 2002; Chan et al. 2002; Wernstedt et al. 2004), dyslipidemia (Chan et al. 2002), insulin resistance (Bastard et al. 2002; Pickup et al. 1997), prothrombotic state (Conway et al. 2004) and

hypertension (Fernandez-Real et al. 2001). However, we do not know if IL-6 precedes and causes these other risk factors, if IL-6 is an effect of these risk factors, or if the associations are nothing more than independent co-occurrences.

In addition to its association with disorders presenting peripheral dysfunctions, IL-6 is also associated with disorders of central nervous system function. In individuals with depression there is a dysregulation of the circadian rhythm of IL-6 (Alesci et al. 2005; O'Brien et al. 2004). There is also a dysregulation of IL-6 seen in individuals with sleep disorders (Spath-Schwalbe et al. 1998; Vgontzas and Chrousos 2002; Vgontzas et al. 2003). As was the case with the associations between IL-6 and components of the metabolic syndrome we do not know whether IL-6 is a cause, or result, of these central alterations.

We do not fully understand the source of plasma IL-6 or the site of action for plasma IL-6 to elicit biological effects. Thus, we do not fully understand the relationship between plasma IL-6 and disease development.

Stress and Disease

Stress is another term that stirs up different meanings in different minds. Stress, in the biological sense, is simply a disruption of homeostasis. The terms stress and stressors refer to threats to homeostasis. The stress response refers to the body's reaction to a threat to homeostasis. This reaction, or stress response, is how the body attempts to counter the threat to homeostasis and involves the activation of the HPA axis and the sympathetic nervous system (Miller and O'Callaghan 2002).

> "In response to stressors, the forces that threaten the constancy of an organism's internal milieu, the body mounts an adaptive or stress response to preserve its internal homeostasis (Campeau et al. 1998; Chrousos and Gold 1992; Johnson et al. 1992). The hypothalamic-pituitary-adrenal axis constitutes a major aspect of this stress response." (Kim and Rivier 2000)

The activation of the HPA axis results in the release of glucocorticoids from the adrenal gland. Although the sympathetic nervous system is also involved in the stress response, this thesis will focus on the activity of the HPA axis. Therefore, this section on stress and disease will examine disorders which occur alongside an alteration in the activity of the HPA axis. In this context, chronic stress refers to a constant overactivation of the HPA axis while acute stress refers to the rapid responsitivity of the HPA axis to a threat on homeostasis.

Prolonged hyperactivity of the HPA axis can result in Cushing's disease. Individuals with Cushing's disease present with obesity, fatigue, high blood

pressure and high blood glucose (NIDDK 2002). This is the result of the body being exposed to high levels of cortisol for an extended length of time. The constant hyperactivity and high cortisol seen in Cushing's disease is an extreme example of overactivity of the HPA axis. Overactivity of the HPA axis, even if it is below the extent of change seen in Cushing's disease, has been implicated in the development of peripheral dysfunctions like obesity, insulin resistance, and hypertension (Bjorntorp and Rosmond 2000).

The dysfunctions that result from overactivity of the HPA axis include most components of the metabolic syndrome (Chrousos 2000). Stress is thought of as an underlying cause of the dysregulation of HPA activity. It doesn't matter whether the threat to homeostasis is real or perceived as either can result in overactivity of the HPA axis. Thus, running a marathon or being in a situation that increases your sense of chaos in the world and results in anxious feelings are both examples of stress. If these stressors are acute and removed, the body will have an opportunity to adapt and become more adept at handling these threats to homeostasis in the future (Selye 1946). However, if you continually run without resting, or if you remain in a situation of perceived chaos, the body will not have an opportunity to appropriately adapt to the situation and dysfunction will result (Selye 1946). This is chronic stress.

IL-6 and Stress

IL-6 and Acute Stress

More than a decade ago it was reported that plasma IL-6 increases in response to acute psychological (LeMay et al. 1990; Zhou et al. 1993) and physical stressors (Zhou et al. 1993). Since that time studies have reported that plasma IL-6 increases in response to acute exercise (Papanicolaou et al. 1996) and the source of plasma IL-6 during exercise can be skeletal muscle (Ostrowski et al. 1998), not circulating monocytes (Starkie et al. 2001). It has also been reported that IL-6 is released from the brain during prolonged exercise in humans (Nybo et al. 2002) and that the cerebral release of IL-6 during exercise is reduced with hypoglycaemia (Nybo et al. 2003). Of key interest to human health is the report that the magnitude of increase in IL-6 in response to acute stress predicts future ambulatory blood pressure (Brydon and Steptoe 2005) and that the hypertensive response to acute stress is attenuated in IL-6 KO mice (Lee et al. 2004). Since plasma IL-6 increases in response to acute stress, the next section examines the interactions between IL-6 and the HPA axis.

IL-6 and the HPA axis

IL-6 has the ability to acutely activate each level of the HPA axis (Dunn 2004) and a dose-dependent increase in ACTH and cortisol is seen in response to IL-6 administration in man (Tsigos et al. 1997). IL-6 can stimulate CRH release from the hypothalamus (Navarra et al. 1991) and median eminence (Spinedi et

al. 1992), ACTH release from the anterior pituitary (Abraham and Minton 1997) and corticosteroid production in the adrenal cortex (Silverman et al. 2004).

IL-6 is also found to be expressed in the hypothalamus (Miyahara et al. 2000; Shizuya et al. 1998), co-localized with vasopressin in the paraventricular and supraoptic nuclei, the internal zone of the stalk median eminence (SME) and the posterior pituitary (Ghorbel et al. 2003; Gonzalez-Hernandez et al. 2006). The expression of IL-6 in the hypothalamus fluctuates with the light-dark cycle (Guan et al. 2005) and acutely changes in response to various stressors such as restraint (Miyahara et al. 2000; Shizuya et al. 1997), odorants (Komori et al. 2003) and dehydration (Ghorbel et al. 2003). With the discovery of IL-6 in the posterior pituitary (Ghorbel et al. 2003) it is possible that hypothalamic IL-6 contributes to the increase in plasma IL-6 during the acute stress response.

IL-6 and Chronic Stress

In addition to the response of IL-6 to acute stressors, we also know that conditions of chronic stress, such as providing care for a spouse with dementia, are associated with a chronic increase in plasma IL-6 (Kiecolt-Glaser et al. 2003). Kiecolt-Glaser et al., 2003 reported, “one core pathway behind the diverse health risks associated with caregiving and other chronic stressors: overproduction of IL-6, a key proinflammatory cytokine that appears to enhance morbidity and mortality among older adults (Papanicolaou et al. 1998).”

Connecting Stress, HPA activity, IL-6 and Disease

The common disease conditions that occur with overactivity of the HPA axis and increased plasma IL-6 include obesity, hypertension and insulin resistance. During acute stress there is an acute increase in HPA activity and an acute increase in plasma IL-6. IL-6 possesses the ability to affect the activity of the HPA axis and the final product of the HPA axis, cortisol, inhibits IL-6 production. The commonality and interrelations between IL-6 and HPA activity suggests that the co-occurrence of increased plasma IL-6 and HPA activity in response to stress is more than mere coincidence. In fact, the ability of IL-6 to affect HPA activity has been suggested as the mechanism where by IL-6 affects health and contributes to disease development (Elenkov et al. 2005; Yudkin et al. 2000).

Despite these suggestions, two important questions remain unanswered: 1) If IL-6 affects health through its action on HPA activity where is the site of action for IL-6? 2) What tissue is the source of IL-6 that affects HPA activity? The site of action for IL-6 could be at the level of the hypothalamus (Navarra et al. 1991), the median eminence (Spinedi et al. 1992), the pituitary (Abraham and Minton 1997) or the adrenal gland (Silverman et al. 2004). Most tissues are capable of producing IL-6 and some candidates that could produce IL-6 to affect HPA activity include immune (Ahluwalia et al. 2001), adipose (Bastard et al. 2002; Mohamed-Ali et al. 1997), muscle (Pedersen and Febbraio 2005), endothelial (Moreno et al. 2001) and neural cells (Shizuya et al. 1998).

If IL-6 and HPA activity are linked during chronic stress I propose that IL-6 is not derived from a peripheral source. This proposal is supported by evidence that circulating IL-6 only affects HPA activity when the concentration reaches acute inflammatory levels (Tsigos et al. 1997). Thus, the plasma IL-6 concentration required to affect HPA activity is approximately 40 times greater than plasma IL-6 concentrations seen during chronic stress. On the other hand, if IL-6 is acting in paracrine fashion in one of the tissues of the HPA axis then IL-6 could impact HPA activity despite a low circulating concentration.

If local tissue IL-6 and HPA activity are linked during chronic stress, I further propose the hypothalamus as the most likely source of IL-6 that affects HPA function. Evidence for this proposal is found in reports that hypothalamic IL-6 changes during stress conditions (Miyahara et al. 2000; Shizuya et al. 1998), hypothalamic tissue IL-6 correlates with circulating IL-6 levels (Guan et al. 2005) and that IL-6 is released from the brain during stress (Nybo et al. 2002). Hypothalamic tissue IL-6 levels could impact HPA activity by affecting the PVN neurons in the hypothalamus to release CRH (Navarra et al. 1991; Spinedi et al. 1992). Thus, in response to the questions presented about IL-6 in this section I propose that the hypothalamus is the source of increased circulating IL-6 during conditions of chronic stress and that hypothalamic IL-6 modulates HPA activity by stimulating the CRH neurons within the PVN in the hypothalamus.

HYPOTHESES

Hypothalamic IL-6 is indirectly involved in stimulating the HPA response to stress by affecting the activity of CRH neurons in the hypothalamus.

1. Cells expressing IL-6 will be juxtaposed to CRH neurons in the hypothalamus.

2. In response to acute stress IL-6 will be released and increase the activity of CRH neurons thereby increasing the HPA response to stress.

The next section, Chapter 2, describes the experiments conducted to address these hypotheses. Chapter 2 describes the novel expression pattern found for IL-6 in the hypothalamus and includes the follow-up experiments to elucidate the functional significance of this discovery. Chapters 3 and 4 describe additional experiments conducted to further examine other factors contributing to HPA activity. Chapter 3 addresses the role of nitric oxide in ACTH release and in the control of hypothalamic blood flow. Chapter 4 addresses the effects of diet and exercise on the HPA response to stress. In combination, these studies provide insight into the regulation of HPA activity by examining the modulation of the ACTH response to stress by IL-6, nitric oxide and diet and exercise. The last chapter, Chapter 5, will provide the final discussion and interpretation of results, and will expand upon the significance of the findings presented in this thesis.

CHAPTER 2

Sex Difference in Link between Inflammation and Stress.

ABSTRACT

Inflammation contributes to disease development and the neuro-immuno-endocrine interface is a potential site of action for inflammatory products like IL-6 to affect health. Plasma IL-6 increases in response to stress and the magnitude of increase predicts future ambulatory blood pressure [Brydon, L. & Steptoe, A. (2005) *J Hypertens* **23,** 1001-7]. Although plasma IL-6 can stimulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis, the precise role, if any, for IL-6 in the HPA response to non-immunological stressors is unclear. Interestingly, the sex difference in HPA response to restraint stress is eliminated in IL-6 knockout mice [Bethin, K. E., Vogt, S. K. & Muglia, L. J. (2000) *Proc Natl Acad Sci U S A* **97,** 9317-22]. Here we provide a novel mechanistic link between IL-6 and the HPA response to stress. We discovered IL-6 localized to the external zone of the stalk median eminence (SME) next to the hypophyseal portal vessels. In the SME, content of IL-6 decreased in response to stress along with an increase in phosphorylation of STAT3 in the anterior pituitary and a simultaneous increase in plasma concentrations of IL-6 and ACTH. Furthermore, we show that females concomitantly display greater SME content of IL-6 and greater HPA responsiveness to stress, thereby suggesting that IL-6 is an integral factor contributing to enhanced stress responsiveness in females. Our results provide a direct link between IL-6 and ACTH release and reveal a sex difference in this relationship.

INTRODUCTION

Chronic overproduction of IL-6 has been suggested as a core pathway contributing to the health risks associated with aging, inflammation, chronic stress and metabolic disease (Kiecolt-Glaser et al. 2003; Maggio et al. 2006; Papanicolaou et al. 1998; Yudkin et al. 2000). In depression, there is a dysregulation of the circadian rhythm of circulating IL-6 and elevated plasma IL-6 levels have been reported among individuals with sleep disorders (Alesci et al. 2005; Vgontzas and Chrousos 2002). It has been proposed that plasma IL-6 affects health through stimulating HPA axis activity thereby contributing to the development of metabolic disorders and cardiovascular disease (Yudkin et al. 2000). Despite the correlative evidence between plasma IL-6 and disease the mechanism of action for plasma IL-6 to elicit negative effects on health remains to be elucidated.

IL-6 may affect health through interactions with the HPA axis (Chesnokova and Melmed 2002; Dunn 2000; Elenkov et al. 2005; Kariagina et al. 2004; Papanicolaou et al. 1998). IL-6 has the ability to activate each level of the HPA axis (Abraham and Minton 1997; Dunn 2004; Navarra et al. 1991; Silverman et al. 2004; Spinedi et al. 1992) and a dose-dependent increase in ACTH and cortisol is seen in response to IL-6 administration in man (Tsigos et al. 1997). Studies with rat hemipituitaries provide evidence that IL-6 can increase ACTH secretion and intraventricular injection of IL-6 increases plasma ACTH within 15 min (Lyson and McCann 1991). The ability of IL-6 to activate the HPA axis occurs even in the absence of CRH (Bethin et al. 2000; Muglia et al. 2000;

Silverman et al. 2004). Although the hypothalamus expresses IL-6 (O'Connor et al. 2003) and cells of the anterior pituitary express the IL-6 receptor (Bethin et al. 2000; Chesnokova and Melmed 2002; Hanisch et al. 2000), an anatomical pathway providing access for hypothalamic-IL-6 to directly affect anterior pituitary function has not been clearly described.

In rats, IL-6 is co-expressed with vasopressin in the hypothalamic paraventricular and supraoptic nuclei, the internal zone of the SME, and the posterior pituitary (Ghorbel et al. 2003; Gonzalez-Hernandez et al. 2006). In response to dehydration IL-6 mRNA and protein content in the hypothalamus increase and IL-6 protein content in the posterior pituitary is reduced (Ghorbel et al. 2003). In this report, when we examined IL-6 expression in the pituitary of pigs we discovered IL-6 localized to the external zone of the stalk median eminence (SME).

The discovery of IL-6 in the external zone of the SME provides anatomical evidence that IL-6 could be released into the hypophyseal portal vessels, transported to the anterior pituitary, and thereby affect anterior pituitary function. This evidence, along with the evidence that plasma IL-6 increases in response to physical and psychological stressors (Zhou et al. 1993), lead us to hypothesize that IL-6 in the SME is involved in stimulating ACTH secretion in response to acute stress. If this hypothesis is correct, we may expect to find the following in response to acute stress: 1) a decrease in IL-6 content in the SME; 2) activation of the IL-6/gp130 signaling pathway at the level of the anterior pituitary; and 3) a simultaneous increase in plasma concentrations of IL-6 and ACTH. We chose to

use exhaustive exercise as a stressor of female pigs since we had previously observed robust changes in ACTH in response to this stressor in females. In addition, exercise has been reported to increase IL-6 release from the brain in humans (Nybo et al. 2002). Thus, this study tested the hypothesis that IL-6 in the SME can be directly involved in the HPA response to stress.

RESULTS

Localization of IL-6 to external zone of SME. IL-6 was found to be localized to the external zone of the SME (Fig. 1 *A* and *D-F*) demonstrating the same immunoreactive pattern as CRH (Fig. 1*B*). IL-6 was localized to the external zone in both male and female pigs and demonstrated a molecular weight of 45kDa (Fig. 1*C*). IL-6 was also found to be localized to the posterior pituitary (Fig. 1*F*).

Changes in SME IL-6 content in response to acute stress. Exhaustive exercise bouts were completed by female pigs using an incremental exercise protocol. The increasing intensity of this exercise is illustrated by the changes in blood lactate (Fig. 2*A*). We observed a significant decrease in SME content of IL-6 in response to exhaustive exercise (Fig. 2*B*) which in part supports our hypothesis that IL-6 is released from the SME in response to acute stress. In further support of our hypothesis, we observed a significant increase in the phosphorylation of STAT3 in the anterior pituitary (Fig. 2*C*), presumably due to activation of the IL-6/gp130 signaling pathway in those tissues (Auernhammer et al. 2004). Finally, plasma concentrations of IL-6 and ACTH increased simultaneously as predicted by our hypothesis (Fig. 2*D* and *E*, respectively), demonstrating a significant correlation ($r = 0.89$; $P < 0.001$) between the plasma concentrations of these two molecules. Plasma cortisol also increased in response to exercise (Fig. 1*F*).

Sex difference in SME IL-6 content and ACTH response to stress. When we evaluated the influence of gender on this relationship, we observed that female

pigs had greater IL-6 content in their SME (Fig. 3*A*) and displayed an exaggerated ACTH response to exercise (Fig. 3*B*) when compared to male pigs. We additionally note that the exercise model used in Fig. 3 involved running at sub-maximal intensities and, unlike Fig. 2, these animals were not exercised to exhaustion. Nonetheless, from pre-exercise to jogging on the treadmill, female pigs responded with a 10-fold increase in mean plasma ACTH while male pigs were unresponsive (Fig. 3*B*).

To determine whether the sex difference in ACTH response in pigs was unique to the stressor used, we subsequently incorporated two additional stressors. Similar to the sex difference found in WT mice (Bethin et al. 2000), female pigs demonstrated a greater ACTH response to restraint than male pigs (Fig. 3*C*). In contrast, we found no effect of gender on the ACTH response to surgical stress under anesthesia (Fig. 3*D*).

DISCUSSION

We discovered IL-6 localized to the external zone of the SME suggesting that IL-6 may be released into the portal vessels and directly modulate anterior pituitary function. This hypothesis gained support from two different lines of evidence: 1) IL-6 content in the SME decreases in response to acute stress along with anterior pituitary STAT3 phosphorylation and a simultaneous increase in plasma IL-6 and ACTH (Fig. 2), and 2) animals that demonstrate greater SME IL-6 content demonstrate greater ACTH response to stress (Fig. 3).

We report the molecular weight of IL-6 present in the SME was 45kDa. Depending on the level of glycosylation a monomer of IL-6 has been reported in the size range of 21-30 kDa (May et al. 1991) and is found to circulate as complexes of high molecular mass of 100-150 kDa and 400-500 kDa (Sehgal 1992). The 45 kDa dimer was also the predominant molecular weight species detected in human serum following TNF infusion or endotoxin challenge (Fong et al. 1989; Jablons et al. 1989). The 45 kDa form survived under reducing and denaturing conditions and demonstrated both hepatocyte stimulating activity and B cell differentiation activity, two cardinal features of IL-6 (Fong et al. 1989; Jablons et al. 1989). Indeed, the previous names for IL-6 (B cell differentiation factor/hepatocyte-stimulating factor) were derived from the biological activities of this cytokine.

We assert that the uniqueness of this work rests not only in the anatomical evidence for IL-6 being directly involved in stimulating HPA activity but also the

physiological evidence. The observed changes in response to acute stress (1: decrease in IL-6 content in the SME; 2: increase in anterior pituitary STAT3 phosphorylation; and 3: simultaneous increase in plasma IL-6 and ACTH) along with the observation that higher SME IL-6 content coincides with greater ACTH responsiveness; strongly supports IL-6's role in augmenting the ACTH response to stress. This hypothesis is consistent with, and strengthened by previous observations in transgenic mice where IL-6 KO animals have decreased HPA responsiveness (Bethin et al. 2000). In this latter study, investigators reported that WT female mice had a greater HPA response to restraint stress than male WT and that this sex difference was eliminated in IL-6 KO animals. In fact, female IL-6 KO mice had a 70% reduction in their corticosterone response to restraint compared to female WT, while no difference in corticosterone response was found between male WT and male IL-6 KO animals. These data provide evidence that IL-6 contributes to the greater HPA responsiveness seen in females (Bethin et al. 2000). Our data suggest that IL-6 release from the SME and activation of the anterior pituitary is the mechanism of action by which IL-6 contributes to sex differences in HPA response.

Understanding the site of production for stress-induced increases in IL-6 has clinical relevance as the magnitude of increase in plasma IL-6 in response to stress predicts future ambulatory blood pressure (Brydon and Steptoe 2005). In addition, our findings may aid in understanding the sex difference in the association between plasma IL-6 and risk factors for cardiovascular disease (Fernandez-Real et al. 2001). In women, plasma IL-6 was associated with blood

pressure, but not insulin sensitivity, whereas in men, plasma IL-6 was associated with insulin sensitivity but not blood pressure (Fernandez-Real et al. 2001). Our results describing the interaction between IL-6 and the stress axis may be of particular interest to women's health and quality of life, as Constanzo et al. (Costanzo et al. 2005), reported: "IL-6 may, therefore, be one pathway by which psychosocial factors such as depression and social support could influence disease outcomes in ovarian cancer."

Despite all associations between plasma IL-6 and disease it must be noted that chronic elevation in plasma IL-6 represents a concentration change that is a mere fraction of what plasma IL-6 levels can reach during an infection/inflammatory response. As an example, in patients with sleep apnea mean plasma concentrations of IL-6 increase to 4 pg/ml versus the 1.5 pg/ml level observed in controls (Vgontzas and Chrousos 2002). In contrast, during an inflammatory response to turpentine injection plasma concentrations of IL-6 can exceed 2,000 pg/ml (Turnbull et al. 2003). In response to injection of IL-6, which elevated plasma IL-6 for 4 h, increased ACTH was observed when IL-6 plasma concentrations were maintained at 290 and 4,050 pg/ml (Tsigos et al. 1997). However, when IL-6 injections produced plasma IL-6 concentrations of 8, 22, or 65 pg/ml there was no effect on circulating ACTH (Tsigos et al. 1997). Therefore, if peripheral production of IL-6 is the only source of circulating IL-6, the small change in circulating IL-6 that occurs as in disease, or in response to stress, would not be expected to affect pituitary function. However, if IL-6 release from the SME is partially reflected by plasma IL-6 concentrations then the impact of IL-

6 on anterior pituitary function, and thus health, may be greater than is currently appreciated. The hypothesis that IL-6 could be released from the SME and contribute to circulating concentrations gains support from the evidence that hypothalamic IL-6 content demonstrates a significant correlation with light-dark cycle changes in circulating IL-6 levels (Guan et al. 2005).

In conclusion, we have provided evidence here that IL-6 is localized to the external zone of the SME. We have also demonstrated that the content of IL-6 protein in the SME decreases in response to acute stress, accompanied by an increase in STAT3 phosporylation in the anterior pituitary, and that plasma concentrations of IL-6 and ACTH increase congruently. In addition, the content of IL-6 in the SME, as well as the ACTH response to restraint or exercise stress, is greater in female pigs compared to males. Thus, IL-6 can be involved in augmenting ACTH release in response to acute stress and this relationship may provide a mechanistic link among IL-6, stress, and disease.

MATERIALS AND METHODS

Experimental animals. Male and female adult Yucatan miniature swine were obtained from a breeder (Sinclair Research Farm, Columbia, MO) and maintained in accordance with standards set forth by the University of Missouri Institutional Animal Care and Use Committee. Pigs were sexually mature, 7-13 months of age and weighed 24-40 kg. Pigs used for blood collection during exercise had indwelling catheters placed in the jugular vein and connected to a vascular access port. Pigs were allowed to recover at least one week prior to inclusion in studies. For tissue harvest, pigs were sedated with ketamine (35 mg/kg im) and xylazine (2.25 mg/kg, im) and anesthetized with thiopental sodium (10 mg/kg im). Following euthanasia, the brain was removed and the pituitary dissected.

Immunohistochemistry. Collected tissues were immersed in 10% neutral buffered formalin, embedded in paraffin, then sectioned serially at 6 μm thickness. Sections were floated onto positively charged slides (Fisher), deparaffinized then steamed in an antigen target retrieval solution (Dako S1699). Endogenous biotin was blocked with an avidin-biotin two-step blocking solution (Vector SP-2001) and endogenous peroxidase was inhibited by placing sections in 3% hydrogen peroxide. Prior to incubation with primary antibody, a non-serum protein block (Dako X909) was applied. Sections were incubated overnight at 4 C with a monoclonal anti-porcine IL-6 antibody (25ng/ml; MAB686, R&D Systems). Additional sections were incubated overnight with anti-CRF (1:2,000; Peninsula Laboratories) plus protein A (1:200; Sigma). For negative control, sections were

incubated overnight with the antibody diluent minus primary antibody. Sections were incubated with a biotinylated link antibody (Dako LSAB+ kit) for 30 min then incubated with peroxidase-labeled streptavidin for 30 min (Dako LSAB+ kit). Diaminobenzidine (Dako) was applied for visualization of the reaction product followed by counterstaining with Mayer's hematoxylin stain, dehydration and coverslipping. Photographs were obtained with an Olympus BX40 photomicroscope and Spot Insight Color camera (Diagnostic Instruments).

Western blotting. Whole median eminence was placed in chilled homogenization buffer (50 mM Tris-HCL, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 1:200 protease inhibitor cocktail (P8340, Sigma)), homogenized (Omni GLH), and centrifuged at 100,000 × g for 45 min (Young et al. 2003). The supernatant was collected and protein concentration was determined (Coomassie Protein Assay, Pierce). A volume of supernatant containing 20 µg of protein was diluted 10:1 with trichloroacetic acid (TCA) solution, placed on ice for 10 min, then centrifuged at 16,000 × g for 10 min. The TCA was suctioned off, replaced with 500 µl acetone, and samples were centrifuged again at 16,000 × g for 10 min. The acetone was removed and the protein pellet was resuspended in 20 µl Laemmli buffer then kept at -80° C until analysis. On the day of analysis, samples were heated to 70° C for 10min, loaded (10 µg/lane) onto 4-12% NuPage Bis-Tris gel (Invitrogen), electrophoresed under reducing conditions and electrotransferred to polyvinylidene difluoride membrane (Hybond-enhanced chemiluminescence, Amersham). The membrane was blocked for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline with Tween and incubated

overnight at room temperature with primary antibody against porcine IL-6 (1 μg/ml; MAB686, R&D Systems). After another 1 h wash, the membrane was incubated with secondary antibody (1:2,500, horseradish peroxidase-conjugated anti-mouse; Sigma), washed then detected by enhanced chemiluminescence (Amersham). Exposed films were scanned and band intensity determined (NIH Image). To confirm equal loading, membranes were reprobed for α-Tubulin (1:15,000, Santa Cruz).

Plasma assays. Blood samples were collected in chilled EDTA containers, centrifuged and kept at -80° C until analysis. Lactate was measured (Sigma) spectrophotometrically. IL-6 was measured using an anti-porcine IL-6 ELISA (R&D Systems). ACTH and cortisol were measured by chemiluminescent assay (Immulite, DPC). The intra- and inter-assay coefficients of variation for cortisol were 4.5 and 5.1, and ACTH was 3.4 and 3.6, respectively.

Stress models. The exhaustive exercise bout involved incremental increases in running speed to exhaustion. Caudal electrical stimulation was used. The animals began walking on the treadmill at 3.2 km/h and then speed was increased for 5 min intervals each at 4.8, 8.0, 11.3 and 11.3 km/h with 5% incline. Between each stage the animals were allowed to walk for 5 min at 3.2 km/h at which time blood samples were collected via indwelling catheters. For gender comparisons, additional pigs were acclimated to sub-maximal treadmill jogging in the absence of electrical stimulation. Pigs walked at 3.2 km/h for 5 min then performed fast walking at 4.8 km/h for 5 min and finished with a 5 min jog at 8.0 km/h. Blood samples were drawn during the final minute of each stage via

indwelling catheters. For restraint stress conscious pigs were flipped on to their back and blood samples were obtained via jugular venipuncture. Samples for the surgical stress under anesthesia were collected approximately 30 min after the animals had been placed on anesthesia (thiopental sodium, 10 mg/kg, im) and following minor surgery including intubation.

Statistical analyses. One way ANOVA was used to compare ACTH changes during exercise. A Kruskal-Wallis one way ANOVA on ranks was used if the normality test failed. A Pearson correlation was run to compare relationship between plasma concentrations of ACTH and IL-6 during exercise. A t-test was run to compare groups and, if normality failed Mann-Whitney U-test was employed. Data are presented as mean ± SEM.

Fig. 1. Immunohistochemical detection of IL-6 in the SME. Frontal view of IL-6 (*A*) and CRH (*B*) immunoreactivity in SME showing positive staining in external zone next to hypophyseal portal vessels. Immunoblot analysis of IL-6 protein isolates from SME displaying immunoreactive bands at 45kDa (*C*). Magnified view of IL-6 in the external zone of SME (*D*) and absence of immunoreactivity in negative control (*E*). Sagittal view of pituitary showing IL-6 immunoreactivity localized to external zone of SME and posterior pituitary. 3V, third ventricle; SME, stalk median eminence; AP, anterior pituitary; PP, posterior pituitary. Bar represents 500μm (*A*, *B, F*) and 100μm (*D, E*).

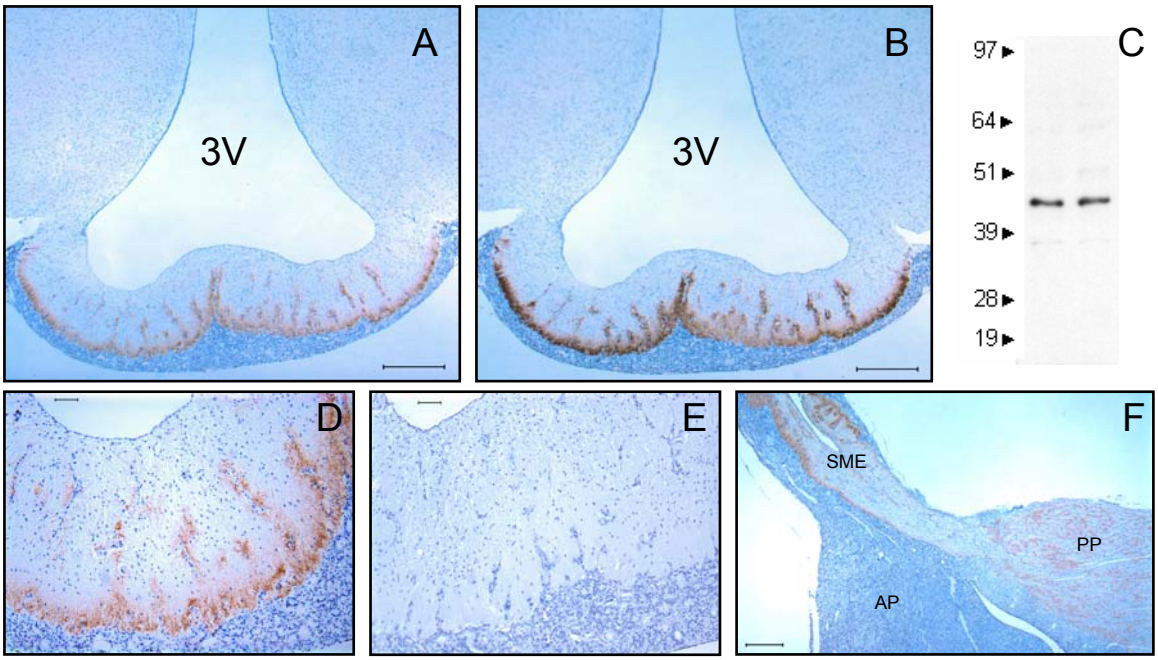

A
3V
B
3V
C
97
64
51
39
28
19
D
E
F
SME
PP
AP

Fig. 2. The effects of acute stress on IL-6 content in the SME. Increase in plasma lactate during exhaustive exercise bout demonstrates increase in intensity (*A*). (*B*) In response to exercise, IL-6 protein content in the SME decreased as revealed by Immunoblot analysis ($P = 0.008$; $n \geq 5$). (*C*) Immunoblot demonstrates increased phosphorylation of STAT3 in the anterior pituitary in response to exercise ($P = 0.018$; $n = 4$ per group). Increase in plasma IL-6 (*D*) ACTH (*E*), and cortisol (*F*) in response to exercise ($n \geq 5$). c, control; s, stress.

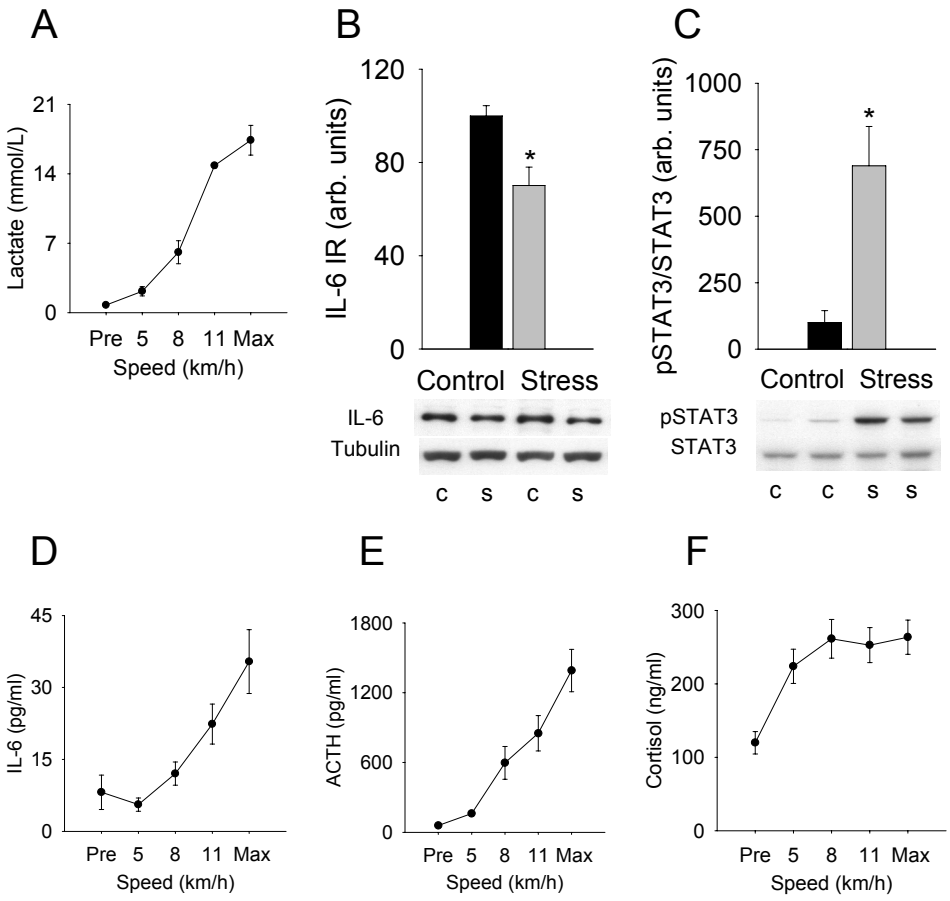
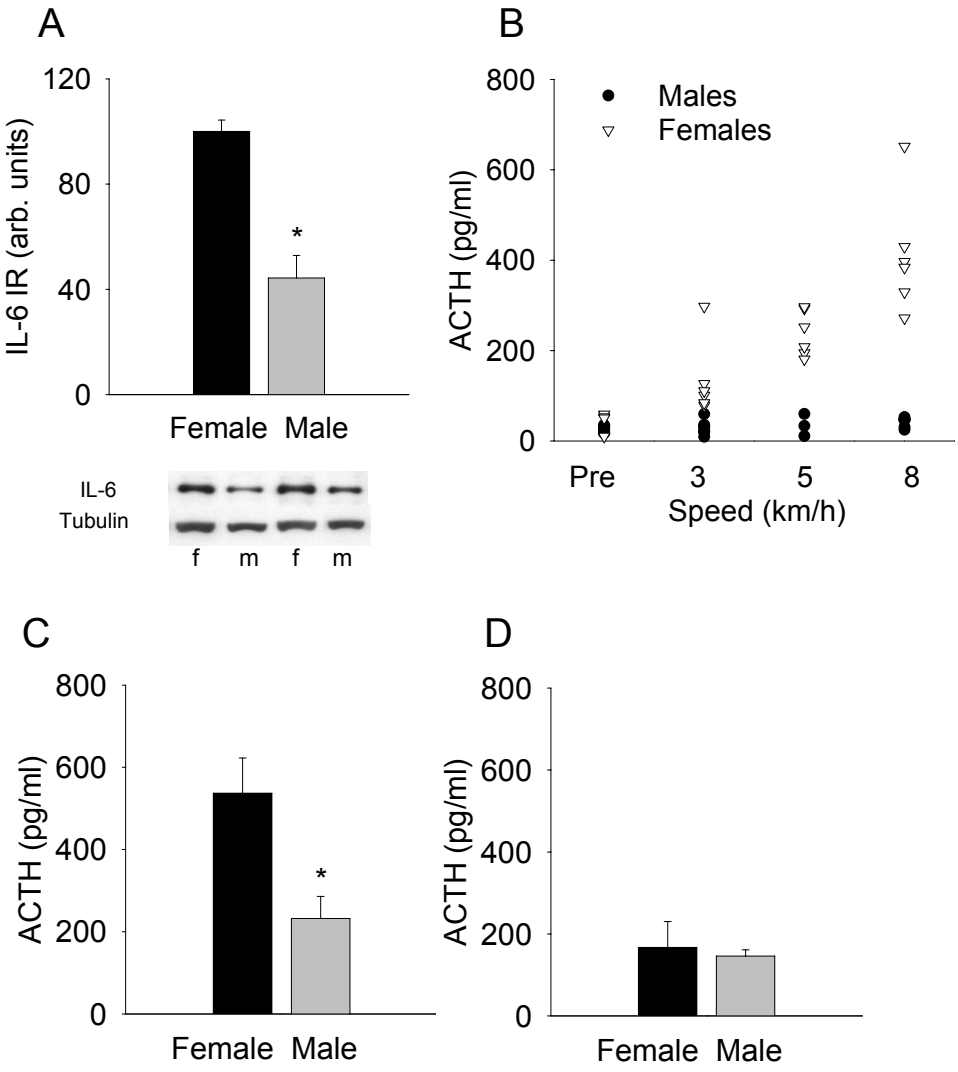

A
Lactate (mmol/L)
21
14
7
0
Pre 5 8 11 Max
Speed (km/h)
B
IL-6 IR (arb. units)
120
80
40
0
*
Control Stress
IL-6
Tubulin
c s c s
C
pSTAT3/STAT3 (arb. units)
1000
750
500
250
0
*
Control Stress
pSTAT3
STAT3
c c s s
D
IL-6 (pg/ml)
45
30
15
0
Pre 5 8 11 Max
Speed (km/h)
E
ACTH (pg/ml)
1800
1200
600
0
Pre 5 8 11 Max
Speed (km/h)
F
Cortisol (ng/ml)
300
200
100
0
Pre 5 8 11 Max
Speed (km/h)

Fig. 3. SME IL-6 protein content and ACTH response to stress in female and male pigs. (*A*) Immunoblot analysis showing lesser SME IL-6 protein content in males compared to females ($P < 0.001$; $n \geq 5$). (*B*) Females display increased ACTH at all speeds versus pre-exercise ($P < 0.05$) and compared to males ($P < 0.05$; $n \geq 6$). Results displayed as individual data points at corresponding speeds. There was no sex difference in ACTH concentration prior to initiation of exercise. There was no increase in ACTH in males in response to exercise. (*C*) Females exhibit greater ACTH response to restraint stress ($P = 0.02$; n ≥ 7). (*D*) No sex difference in ACTH is seen in response to surgical stress under anesthesia (f, $n = 3$; m, $n = 6$). Results displayed as mean ± SEM (*A, C* and *D*). f, female; m, male.


A
120
80
40
0
IL-6 IR (arb. units)
*
Female Male
IL-6
Tubulin
f m f m
B
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ACTH (pg/ml)
Males
Females
Pre 3 5 8
Speed (km/h)
C
800
600
400
200
0
ACTH (pg/ml)
*
Female Male
D
800
600
400
200
0
ACTH (pg/ml)
Female Male

CHAPTER 3

ACTH Response to Stress, Nitric Oxide and Blood Flow to the Hypothalamic-Pituitary-Adrenal Axis

ABSTRACT

Nitric oxide (NO) is involved in neural signaling and in the control of blood flow. During the stress response NO can modulate the activity of the hypothalamic-pitutiary-adrenal (HPA) axis. In this study we tested the hypothesis that nitric oxide synthase (NOS) inhibition will result in an increase in ACTH response to stress. We also tested the hypothesis that NOS inhibition will decrease blood flow to tissues of the HPA axis. Treatment with L-NAME increased mean arterial pressure and decreased vascular conductance in the hypothalamus, the anterior pituitary and the adrenal. In addition, we found that treatment of pigs with L-NAME significantly decreased the ACTH response to restraint stress but augmented the ACTH response to exercise stress. We report the circadian rhythm for ACTH and cortisol in female pigs and note how handling and treadmill walking affect the plasma concentration of these hormones. Our results suggest that NO is involved in maintaining vascular tone in tissues of the HPA axis. The divergent effects of L-NAME on the ACTH response to restraint or exercise imply that the role of nitric oxide in modulating HPA activity is stressor dependent. These findings demonstrate that NO modulates blood flow to the HPA axis and is involved in the hormonal response to stress.

INTRODUCTION

In response to stress, release of ACTH secretagogues from the hypothalamus initiates the activation of the HPA axis. The primary site of control for the HPA axis resides in a set of neurons within the hypothalamic paraventricular nucleus (PVN) (Herman et al. 2002). The neurons within the PVN produce corticotropin-releasing hormone (CRH) and vasopressin (VP) and are capable of integrating extrinsic and intrinsic information so that a glucocorticoid response appropriate for the stressor encountered is achieved (Herman et al. 2002). It has been proposed that nitric oxide can affect the activation of these PVN neurons (Riedel 2000; Rivier 2001; Stern 2004).

Brain NO may be derived from any one of three different NOS isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). Nitric oxide is involved in modulating neural activity (Stern 2004; Stern and Zhang 2005) and in the control of cerebral vascular tone (Hamel 2006; Hudetz et al. 1998; Lee 2000; Paravicini et al. 2006; Zoccoli et al. 2001). Within the hypothalamus, nitric oxide can modulate neuroendocrine function and may link neuronal-glial-vascular interactions (Stern and Zhang 2005).

Depending on the route of delivery, NOS inhibitors have been shown to increase or decrease ACTH release in response to stress. The i.p. delivery of L-NAME, a NOS inhibitor, augments the ACTH response to nicotine while i.p. delivery of L-arginine, a NOS substrate, has the opposite effect (Gadek-Michalska and Bugajski 2004). The i.v. administration of L-NAME also augments

the ACTH response to IL-1β, VP and oxytocin (Rivier and Shen 1994). In contrast to the effects of systemic L-NAME, i.c.v. L-NAME decreases the ACTH response to electroshock along with a concurrent decrease in hypothalamic NOS activity (Kim and Rivier 2000). Studies using NO donors have shown that both i.c.v. and i.v. injection of a NO donor increases ACTH while only the i.c.v. injection results in the induction of immediate early gene expression in the PVN (Seo et al. 2002). Further support for the role of nitric oxide in modulating the stress response comes from evidence showing that microinfusion of NO into the PVN results in release of ACTH (Seo and Rivier 2001).

In addition to neuronal signaling, brain nitric oxide is involved in the control of cerebral blood flow. Nitric oxide is the major contributor to cerebral blood flow differences in the sleep-wake states (Zoccoli et al. 2001). Nitric oxide has been suggested as a coupling link between neural activity and cerebral blood flow (Hamel 2006; Iadecola 1993). Thus, if NO from activated neurons diffuses to surrounding vascular tissue this may lead to vasodilation and a concurrent increase in tissue blood flow. The purpose of this study was to test the hypothesis that chronic L-NAME treatment will augment the ACTH response to stress and decrease vascular conductance to tissues of the HPA axis. If L-NAME has a central effect, the decrease in hypothalamic NOS activity may be reflected by a decrease in ACTH response to stress along with a decrease in vascular conductance.

MATERIALS AND METHODS

Animals and Surgery. Female Yucatan miniature swine (Sinclair Research Farm, Columbia, MO) were used for this study. All animals were sexually mature, weighed between 24-40 kg and were between 7-13 months of age. Animals involved in blood collection and blood flow determination studies underwent surgery to have catheters implanted. Pigs used for blood collection experiments had a catheter placed in the jugular vein and connected to a vascular access port positioned between the scapulae. Pigs that were used for blood flow determination studies had two catheters surgically placed, one in the left atrium and one in the aorta. Pigs were allowed to recover from surgery for at least five days before being included in any experiments.

In the treatment group, L-NAME was added to the drinking water (.5 g per 5 L) for 30 days prior to inclusion in studies. On date of tissue collection, animals were sedated with ketamine (35 mg/kg) and xylazine (2.25 mg/kg) and anesthetized with thiopental sodium (10 mg/kg). Tissues were dissected following euthanasia. Pigs were housed in a 12:12 hr light/dark cycle and maintained in accordance with standards set forth by the University of Missouri Institutional Animal Care and Use Committee.

Blood Collection. To determine the circadian rhythm of ACTH and cortisol in female pigs, blood samples were collected with automated blood sampling equipment (Accusampler, DiLab). Pigs were individually placed in the sampling pen (1.2 x 1.8 m) for at least 4 days prior to initiation of blood collection. Pigs

were connected and sampling commenced 2 hrs post handling. Connection of pigs to sampling equipment did not restrict the pig's movement within the pen. Collected samples were placed in tubes with EDTA and kept at 4° C during the 24 hr collection.

To determine the hormone response of female pigs to handling, blood samples were manually collected while the animal was in pen and after moving the animal out of the pen. Pigs were placed on a scale that prevented their movement for blood collection. Pigs were also placed on the treadmill and blood samples were collected prior to the start of the treadmill belt and after 10 min of treadmill walking. During treadmill walking no electrical stimulation was used.

Stressors. For restraint stress, pigs were removed from their pen, picked up by their hind limbs and placed flat on their back. The pigs were restrained by several investigators while blood samples were obtained via jugular venipuncture. Blood samples were collected in chilled EDTA vacutainers within 5 min of placing the animal on its back.

The exercise stress involved incremental exercise to exhaustion. Every 5 min throughout the protocol the treadmill speed was changed. Animals began walking at 3.2 km/hr and this walking stage was repeated between 5 min stages of 4.8, 8.0, 11.3 km/hr. If the pig was able to complete the 11.3 km/hr stage a 5% treadmill grade was then added to the speed of 11.3 km/hr following the walking period. An agricultural prodder was used on the rear to encourage running for the

maxmimal exercise test. Between running stages blood samples for hormone measurements were collected in chilled EDTA vacutainers.

Blood flow determination. The determination of blood flow at rest and during exercise was accomplished with the use of radiolabeled microspheres of 15 μm diameter (Perkin Elmer) (Armstrong et al. 1987; Delp et al. 2001). Isotopes used included ^{103}Ru, ^{46}Sc and ^{57}Co. Microspheres were suspended in saline with 0.5% Tween 80 and sonicated then vortexed prior to infusion. The infusion of microspheres into the left atria was followed with a 37° C saline flush and accompanied by the withdrawal of a reference blood sample from the aorta at a rate of 4.12 ml/min. The collection of the reference sample started prior to infusion of micropheres and continued 2 min after the saline flush. To ensure that a large enough volume of microspheres were infused to obtain accurate blood flow measurements only experimental animals where at least 400 microspheres were collected in the reference blood sample were used for analysis (Buckberg et al. 1971). Between infusions the externalized aortic catheter was connected to a blood pressure analyzer (MicroMed) to obtain mean arterial pressure. After completion of the exercise protocol, animals were sedated and placed on anesthesia. Following euthanasia, tissues were dissected and weighed and radioactivity was determined with a gamma counter (LKB Wallac 1282). Blood flow was normalized to tissue mass and vascular conductance was calculated by dividing blood flow by mean arterial pressure.

Hormone Assays. All blood samples were collected in chilled EDTA containers, centrifuged and plasma was stored at -80° C until analysis. Both

ACTH and cortisol were measured by chemiluminescent assays (Immulite, DPC). The intraassay and interassay coefficients of variation were 3.4 and 3.6 for ACTH, respectively. For cortisol, the intrassay and interassay coefficients of variation were 4.5 and 5.1, respectively.

Statistical Analyses. Data are presented as mean ± SE. One-way repeated measures ANOVA was used to determine the effect of time on plasma ACTH and cortisol. A 2-way repeated measures ANOVA and Student-Newman-Keuls pairwise multiple comparison method was used to compare the effects of treadmill speed and L-NAME on the ACTH and cortisol response to exercise. A *t*-test was run for other group comparisons.

RESULTS

Circadian Rhythm. The circadian rhythm for ACTH and cortisol in female pigs is presented in Fig. 1. During this 24 hr period blood sample collection was automated and no animal handling performed. There was a significant effect of time on ACTH and cortisol. Animals were fed around 10:00 AM. The increase in morning ACTH and cortisol began prior to the start of the light phase.

Effects of animal handling. The effect of manual blood collection and animal handling on hormone response is presented in Fig. 2. Compared to automated sampling, manual sampling in pen significantly increased ACTH but did not affect cortisol (comparison not shown). Compared to blood collection in pen, removal of the animal from its pen significantly increased ACTH and cortisol regardless of whether the animal was placed on the treadmill or on a scale. No difference in ACTH or cortisol was seen between standing on the scale or standing on the treadmill. Treadmill walking significantly increased ACTH and cortisol compared to just standing on the treadmill prior to exercise.

L-NAME

Restraint. Animals that had been on chronic L-NAME treatment had a significant reduction in their ACTH response to restraint (P = 0.003, Fig. 3). There was no effect of L-NAME on the cortisol reponse to restraint.

Exercise. Treadmill running significantly increased ACTH and cortisol (Fig. 4). There was a significant interaction between L-NAME and treadmill speed on

the ACTH response to exercise (P = 0.016). During exercise, L-NAME significantly increased the ACTH response at maximal exercise (*P = 0.007). L-NAME treatment had no effect on the cortisol response to exercise.

Blood flow and vascular conductance. Table 1 presents the effects of L-NAME on mean arterial pressure and tissue blood flow. L-NAME significantly increased mean blood pressure. L-NAME decreased blood flow to frontal cortex but did not significantly decrease blood flow to other tissues examined. The effect of L-NAME on vascular conductance is presented in Fig. 5. In response to L-NAME treatment, there was a significant decrease in vascular conductance in the frontal cortex, the hypothalamus, the anterior and posterior pituitary and the adrenal gland.

DISCUSSION

Our findings indicate that nitric oxide is involved in modulating the HPA response to stress and in the control of vascular conductance in tissues of the HPA axis. Of particular interest was the finding that the role of nitric oxide in modulating the ACTH response is stressor dependent. In addition, we report the effects of handling and treadmill walking on ACTH and cortisol concentrations in female pigs.

Female pigs demonstrated a circadian rhythm for ACTH and cortisol (Fig. 1). The increase in ACTH and cortisol began prior to the start of the light cycle, was highest throughout the early morning and steadily decreased from noon until lights out. There also appeared to be an increase in ACTH and cortisol at 10:00 AM that coincided with the daily meal. The lowest circulating levels of ACTH and cortisol were seen between 6:00 PM and 2:00 AM, coinciding with the start of the dark period.

If the pig is cooperative you can enter the pen and collect blood samples with no change in cortisol and an increase in ACTH. However, frequent handling would not be ideal for blood sample collection over a 24 hr period. Moving the animal out of the pen induces a stress response and being placed on the treadmill induced no greater hormonal change than being placed on the scale (Fig. 2). This increase in hormones in response to exercise was still apparent even after a few weeks of acclimation to being on the treadmill (data not shown).

The addition of L-NAME to the drinking water of pigs resulted in a significant increase in blood pressure (Table 1). This was likely the result of a decrease in tissue vascular conductance (Musch et al. 2001). Changes in blood pressure affect baroreceptor activity and it is possible that this could influence our results (Purinton and Wood 2002). However, given that the L-NAME treatment lasted several weeks there was enough time for resetting of the baroreceptors to occur (Krieger 1988).

Consistent with previous reports suggesting NO modulates cerebral blood flow (Hudetz et al. 1998; Lee 2000; Paravicini et al. 2006; Zoccoli et al. 2001), we found that NO blockade decreased vascular conductance of the frontal cortex and hypothalamus. This suggests that NO production helps maintain cerebral blood flow at rest. Interestingly, the pituitary stalk median eminence was the only tissue examined in this study that did not display a significant decrease in vascular conductance in response to L-NAME, suggesting that NO plays a lesser role in maintaining blood flow to the median eminence in comparison to other tissues.

The divergent effects of L-NAME on the ACTH response to stress were not predicted by our hypothesis a priori. If L-NAME had only affected peripheral tissues (including median eminence and pituitary) we would have expected to see an increase in the ACTH response to restraint stress (Rivier 2001). Since the ACTH response during L-NAME was decreased it is likely that the most prominent site of influence for L-NAME on the HPA response to stress was central. This would fit with previous reports that i.c.v. L-NAME decreases the

ACTH response to electroshock while microinfusion of NO to the PVN results in ACTH release (Kim and Rivier 2000; Seo and Rivier 2001). The decrease in ACTH response to stress suggests that L-NAME decreased hypothalamic NOS activity, as has been previously shown (Kim and Rivier 2000).

In further support of a decrease in hypothalamic NOS activity in response to L-NAME was the decrease in resting vascular conductance in the hypothalamus (Fig. 5). The decrease in vascular conductance in the hypothalamus, as well as the decrease in the response to restraint stress, implies that L-NAME was acting centrally. Our data are consistent with the notion that tonic NOS activity is involved in maintaining hypothalamic vascular conductance and in the activation of the PVN response to stress. The production of NO in the hypothalamus can be derived from several different cell types and different NOS isoforms (Stern and Zhang 2005). We can't determine from our data which cell type or NOS isoform the L-NAME treatment affected. We also do not know if the effects of L-NAME on vascular conductance and ACTH response are the result of decreased NO production from the same or unique sources.

In contrast to restraint stress, L-NAME augmented the ACTH response to stress during exercise. Although not significant, at pre-exercise and during treadmill walking at 3.2 km/hr the L-NAME group tended to show lower ACTH concentrations. However, once animals were jogging at 8.0 km/hr the trend was for ACTH concentrations to be higher in the L-NAME animals and this difference became significant at maximal exercise (Fig. 4). In addition to being different stressors, the time difference in stressor duration between restraint (<5 Min) and

exercise (45 min) may partly explain the differential effects of L-NAME on ACTH response (Rivier 2001). It has been shown that in response to s.c. injection of L-NAME the ACTH response to shocks in rats was initially decreased during the first 5-20 min but was increased after 30-60 min compared to control (Rivier 2001). In addition to this data, it has been proposed that NO in the hypothalamus plays an inhibitory role during states of increased neuronal activity (Stern and Zhang 2005). Thus, the role for NO in PVN activation may change during continued exposure to stress.

In summary, we have shown that NO affects the ACTH response to stress. We report here that NOS inhibition decreases the ACTH response to restraint but augments that ACTH response to maximal exercise. In addition, we report that NOS generated NO is involved in maintenance of basal blood flow to tissues of the HPA axis.

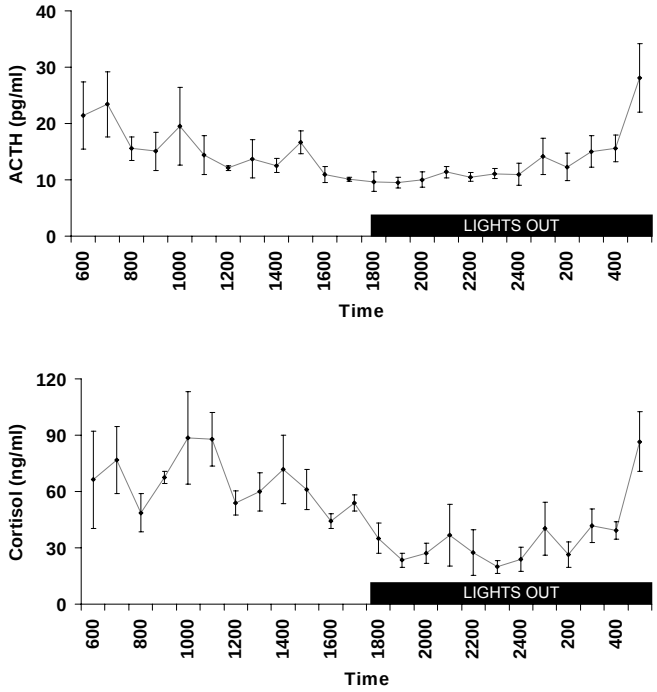
Table 1. Effects of L-NAME on mean arterial pressure and blood flow.

	MAP	Frontal Cortex	Hypothalamus	Pituitary Stalk	Anterior Pituitary	Posterior Pituitary	Adrenal
	mmHg	ml/min/100g	ml/min/100g	ml/min/100g	ml/min/100g	ml/min/100g	ml/min/100g
Control	81 ± 5	126 ± 17	73 ± 13	334 ± 66	31 ± 3	452 ± 52	194 ± 26
L-NAME	119 ± 8*	83 ± 9*	49 ± 8	351 ± 64	19 ± 3	350 ± 46	149 ± 10

Values presented as mean ± SE. *P < 0.05 vs. control.

Figure 1. Circadian rhythm for ACTH (*top*) and cortisol (*bottom*) in female pigs (*n* = 4), There was a significant effect of time on ACTH and cortisol. During this 24 hr period animals were not handled and blood samples were collected by automated sampling equipment. Animals received their daily meal around 10:00 AM.

Figure 1


ACTH (pg/ml)
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30
20
10
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600
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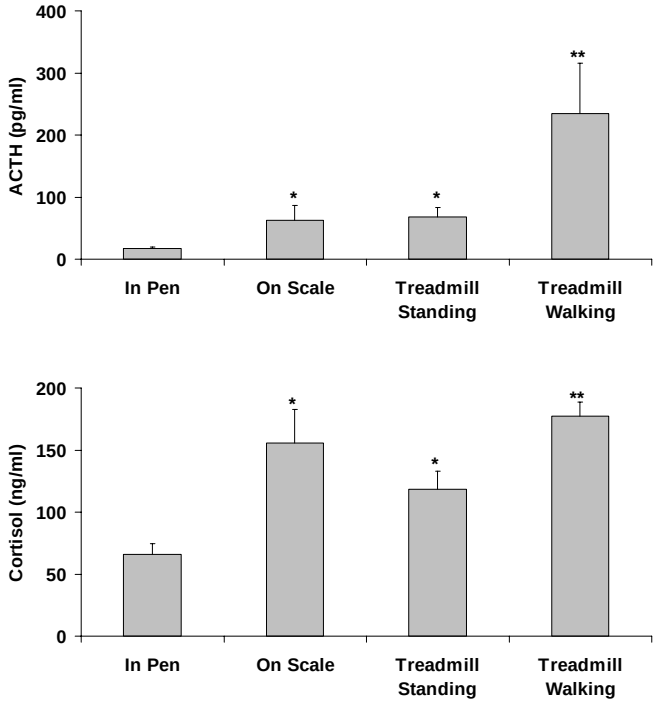
1000
1200
1400
1600
1800
2000
2200
2400
200
400
Time
Cortisol (ng/ml)
120
90
60
30
0
LIGHTS OUT
600
800
1000
1200
1400
1600
1800
2000
2200
2400
200
400
Time

Figure 2. Plasma ACTH (*top*) and cortisol (*bottom*) response to manual blood collection. Pen refers to samples collected while animals remained in their pen. Female pigs were removed from pen and placed on scale or treadmill. Treadmill standing is pre-exercise while treadmill walking is after the completion of 10 min of treadmill walking. All samples were collected via vascular access ports. *P < 0.05 vs. pen; **P < 0.05 vs. treadmill standing; n = 10, pen; n = 4, scale; n = 12, treadmill standing and treadmill walking.

Figure 2


ACTH (pg/ml)
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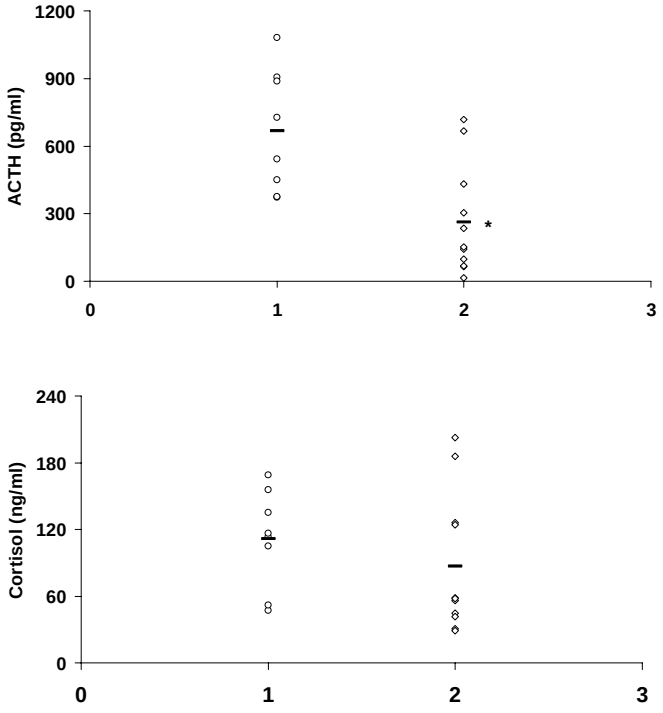
In Pen
On Scale
Treadmill Standing
Treadmill Walking
Cortisol (ng/ml)
200
150
100
50
0
*
*
**
In Pen
On Scale
Treadmill Standing
Treadmill Walking

Figure 3. The effect of L-NAME on the plasma ACTH (*top*) and cortisol (*bottom*) response to restraint stress. Data are displayed as individual data points with a bar representing the mean. *$P < 0.01$ for L-NAME ($n = 11$) vs. control ($n = 8$).

Figure 3


ACTH (pg/ml)
1200
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300
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3
*
Cortisol (ng/ml)
240
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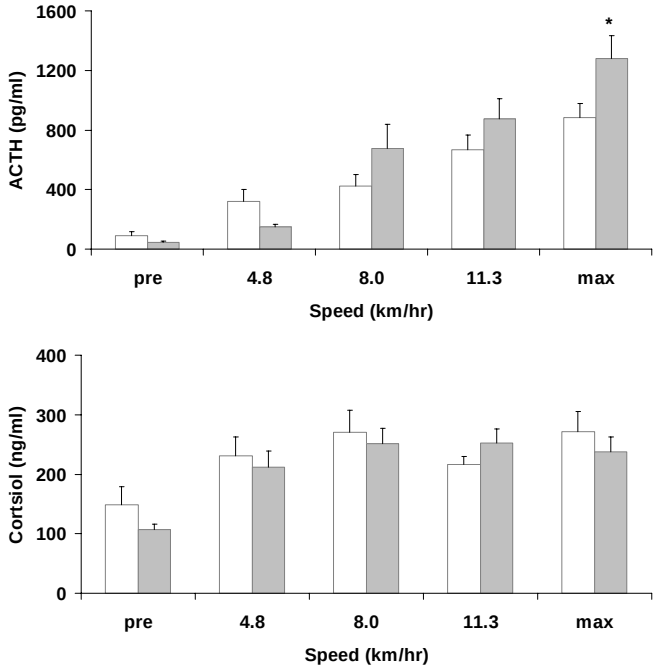
0
1
2
3

Figure 4. The effect of L-NAME on the plasma ACTH (*top*) and cortisol (*bottom*) response to treadmill exercise (n = 7 per group). Open bars represent control data and filled bars represent L-NAME data. Blood samples were collected prior to initiation of exercise and immediately following displayed treadmill speeds. Exercise significantly increased ACTH and cortisol and there was a significant interaction between L-NAME and treadmill speed for ACTH ($P < 0.05$). *$P < 0.05$ vs. control.

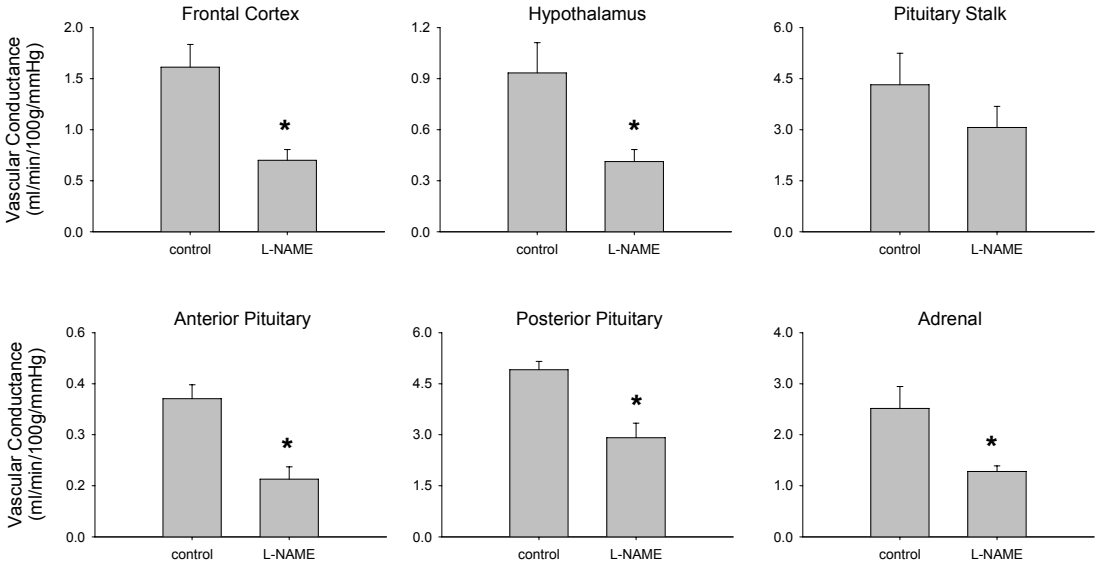
Figure 4


1600
1200
800
400
0
ACTH (pg/ml)
*
pre
4.8
8.0
11.3
max
Speed (km/hr)

400
300
200
100
0
Cortsiol (ng/ml)
pre
4.8
8.0
11.3
max
Speed (km/hr)

Figure 5. The effect of L-NAME on tissue vascular conductance. At rest, L-NAME treatment significantly reduced vascular conductance in the frontal cortex, the hypothalamus, the anterior and posterior pituitary and the adrenal. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control; $n \geq 4$ per bar.

Figure 5


Frontal Cortex
Hypothalamus
Pituitary Stalk
Anterior Pituitary
Posterior Pituitary
Adrenal
Vascular Conductance (ml/min/100g/mmHg)
control
L-NAME
*

CHAPTER 4

Exercise training reverses the effects of high fat diet on free fatty acids and ACTH response to stress.

ABSTRACT

Modifiable behaviors such as physical activity and diet can impact the activity of the hypothalamic-pituitary-adrenal (HPA) axis. Exercise training can affect HPA function so that the HPA response to non-exercise stressors is modified. A high fat diet has been shown to enhance the stress response in rodents while lipid infusion in humans decreases HPA activity. The purpose of this study was to test the hypothesis that changes in serum free fatty acids (FFA) due to diet and exercise are accompanied by alterations in the ACTH response to stress. To test this hypothesis we used a porcine model of cardiovascular disease. We found that a high fat diet significantly increased serum free fatty acids (FFA) and decreased the ACTH response to the stress associated with anesthesia and surgery. We found that exercise training was able to reduce the increase in serum FFA in response to high fat feeding and increased the ACTH response to stress. We found that the high fat diet increased serum glucose, insulin and IGF-1, but none of these factors were affected by exercise training. In addition, the clinical chemistry profiles of our groups of animals revealed that the high fat diet had affected liver function, kidney function and serum albumin and phosphate. In animals on the normal diet, exercise training did not alter the HPA response to stress or serum insulin or IGF-1. Thus, a high fat diet increases FFA and decreases the ACTH response to stress and both of these perturbations are reversed with exercise training.

INTRODUCTION

Disruptors of homeostasis, stressors, result in the activation of the HPA axis and an increase in circulating glucocorticoids (Miller and O'Callaghan 2002). The activity of the HPA axis is under the control of neurons in the paraventricular nucleus that produce CRH in response to stress (Miller and O'Callaghan 2002). Activation of the HPA axis occurs in response to both real and perceived threats and neurons within the PVN must integrate information from various sources when determining the magnitude of response (Herman et al. 2002).

Acute exercise is a stressor that can increase the activity of the HPA axis in an intensity-dependent manner (Davies and Few 1973; Luger et al. 1987). Acute exercise does not always induce an HPA response and a decrease in cortisol can be seen during low intensity (<50% VO2max) exercise (Davies and Few 1973). In response to exercise training, the HPA response to the same absolute workload is decreased (Buono et al. 1987). Interestingly, exercise training can also alter the HPA response to non-exercise stressors in rodents (Droste et al. 2003; Fediuc et al. 2006).

Food intake is also a stressor that can increase the activity of the HPA axis (Vicennati et al. 2002). The HPA response to food intake is dependent on macronutrient composition (Vicennati et al. 2002). A diet of high-fat meals results in changes in both basal and stress-induced activity of the HPA axis (Legendre and Harris 2006; Tannenbaum et al. 1997). Although a high-fat diet may induce multiple effects, one mechanism by which high-fat feeding may affect HPA

activity is through circulating free fatty acids (FFA) (Lanfranco et al. 2004). It has been shown that infusion of a lipid-heparin emulsion increases FFA and inhibits ACTH and cortisol secretion in humans (Lanfranco et al. 2004).

The purpose of this study was to determine the effects of exercise training on diet-induced changes in HPA function. We initially discovered differences in ACTH concentrations in our swine model of early stage cardiovascular disease (Thompson et al. 2004; Turk et al. 2003) in blood samples collected prior to euthanasia. In this model, pigs placed on a high fat diet develop atherosclerotic plaques and vascular lesions (Turk and Laughlin 2004). We hypothesized that the differences seen in ACTH between dietary groups were the result of differences in circulating FFA. In addition, we also examined the effects of this atherogenic diet, as well as the influence of exercise, on other metabolic and hormonal indices.

MATERIALS AND METHODS

Animals. Male Yucatan miniature swine (Sinclair Research Farm, Columbia, MO) were used for this study. Pigs were around 6 mo of age upon arrival to our animal facility and between 11-13 mo of age at time of blood and tissue collection. Pigs were placed in one of 4 different groups (normal diet, sedentary; normal diet, exercise training; high fat diet, sedentary; high fat diet, exercise training). Animals were placed on their respective dietary and activity treatments for 16-20 weeks. Prior to initiation of treatments animals completed a maximal exercise test to determine endurance capacity. This maximal exercise test was also completed at the end of the treatment. Body weights, blood samples and heart weights were obtained post treatment.

Diets. The pigs on the normal diet received standard mini-pig chow (Laboratory Mini-Pig Breeder diet, Constant Nutrition) with macronutrient composition as follows: carbohydrate (69%), protein (23%) and fat (8%). In addition to this standard mini-pig chow animals on the high fat diet had coconut oil (17%), corn oil (2%), cholesterol (2%) and sodium cholate (0.7%) added with their mini-pig chow resulting in the following macronutrient composition: carbohydrate (41%), protein (13%) and fat (46%). Animals were fed once daily.

Exercise. Pigs undergoing exercise training completed treadmill running 5 days per week for about 1 hr throughout the exercise training period. Duration and treadmill speed were increased throughout the training period and by the

end of the study animals were running around 8 km per day. Exercise was completed the day before the collection of blood and tissues.

Blood sample collection. Following an overnight fast, animals were sedated with ketamine (35 mg/kg, im) and xylazine (2.25 mg/kg, im) and anesthetized with thiopental (35 mg/kg). After surgical intubation blood samples were collected. Animals had been placed on anesthesia approximately 30 min before blood samples were collected. Vacutainers were used for the collection of plasma (EDTA) and serum. Following centrifugation, samples were aliquoted and placed at -80° C until analysis. Automated sample collection (Accusampler, DiLab) in another group of Yucatan miniature swine with vascular access ports was used to collect undisturbed blood samples to determine basal ACTH and cortisol in our pigs.

Blood assays. Blood glucose (Infinity) and non-esterified fatty acids (NEFA; Wako) were measured by absorbance assays. ACTH and cortisol were measured by chemiluminescent assays (Immulite, DPC). Serum was delivered to University of Missouri Veterinary Medical Diagnostic Laboratory (University of Missouri) for clinical chemistry profiles. Insulin and IGF-1 were measured by radioimmunoassay at Diagnostic Center for Population and Animal Health (Michigan State University).

Statistical Analyses. One-way and two-way ANOVAs with Holm-Sidak pairwise multiple comparison method were used to determine effects of diet and activity. Data presented as mean ± SE, $P < 0.05$.

RESULTS

Body weight and exercise capacity. The body weight in the normal diet sedentary group was not different from any other group (Table 1). The normal diet exercise group had lower body weight than both sedentary and exercise animals on the high fat diet. The high fat diet exercise group had increased heart weights. Both exercise groups had greater heart weight/body weight ratios than the sedentary groups. Compared to the sedentary animals, exercise trained animals had an increase in their treadmill time to exhaustion.

High fat diet induces hypercholesterolemia. The high fat diet increased serum cholesterol in both sedentary and exercise trained animals (Fig. 1). Exercise training had no effect on serum cholesterol. The high fat diet increased FFA and exercise training decreased FFA in both the normal diet and high fat diet animals (Fig. 1).

Clinical chemistry profiles. Table 2 presents the clinical chemistry profiles on the four different study groups. The high fat diet significantly affected serum albumin, BUN, alanine transaminase, alkaline phosphatase and phosphate. Exercise training had significant effects on alanine transaminase, alkaline phosphatase and phosphate. There were no significant interactions between activity and diet.

Hypothalamic-pituitary-adrenal response to stress. The ACTH and cortisol response to the stress of anesthesia and surgery are presented in Figure 2. Compared to mean basal daytime concentrations of ACTH (16.9 ± 0.9) and

cortisol (77.3 ± 5.6) the stress of anesthesia and surgery increased ACTH and cortisol by 9- and 1.5-fold, respectively. Exercise training did not affect the ACTH response to stress in animals on the normal diet. In the sedentary animals the ACTH response to this stress was decreased by the high fat diet. The decrease in ACTH response to stress in the high fat diet sedentary animals was reversed by exercise training.

Glucose regulation. The high fat diet significantly increased serum glucose, insulin and IGF-1 (Fig.3). Exercise training did not affect serum glucose, insulin or IGF-1 in animals on the normal diet or in animals on the high fat diet.

DISCUSSION

In this study we have shown some of the metabolic and hormonal effects of our high fat diet which induces an early stage of cardiovascular disease (Thompson et al. 2004). We show that the ACTH response to the stress of anesthesia and surgery is decreased with a high fat diet and that exercise training reverses this effect. We also show that the high fat diet increases serum glucose, insulin and IGF-1 and that these factors were unaffected by exercise training.

The high fat diet had significant effects on some of the clinical chemistry indices and most of the metabolic and hormonal indices assessed here. As expected, there was a large increase in serum total cholesterol. Previously, our group had shown that this high fat diet significantly increases blood triglyceride, HDL and LDL cholesterol (Thomas et al. 2002). In this previous study no effect of exercise or exercise/diet interactions were seen on blood triglyceride or lipoproteins. In our study, we found no effect of exercise on total cholesterol but we did find a significant effect of exercise on FFA.

The increases in glucose and FFA show that the metabolism of animals placed on the high fat diet had been perturbed. In further support of this we see that there is a significant increase in insulin and IGF-1. The clinical chemistry profile provides evidence that this high fat diet affected both kidney and liver function and we see a significant effect of the high fat diet on serum albumin and phosphate. Interestingly, low serum albumin has been proposed as an indicator

of cardiovascular disease risk (Schalk et al. 2006) and our high fat diet significantly decreased serum albumin levels.

In pigs on the normal diet exercise training increased the heart weight/body weight ratio and exercise capacity and decreased FFA. These effects of exercise training were also seen in animals on the high fat diet. Exercise training in animals on the normal diet did not alter body weight, cholesterol, ACTH or cortisol response to stress, glucose, insulin or IGF-1. The effect of exercise training on ACTH response to stress was only seen in animals on the high fat diet, where diet had caused a significant reduction in the ACTH response.

We began this study to test the hypothesis that the differences we observed in ACTH response to stress (Fig. 2) were the result of differences in blood FFA concentrations. Consistent with our hypothesis we found that animals on the high fat diet had increased FFA and a decreased ACTH response to stress and that exercise training decreased FFA and increased the ACTH response to stress. In further support of our hypothesis, other factors by which exercise could have affected the ACTH response, such as serum glucose, insulin or IGF-1, were unaffected by exercise training. Thus, it appears that the effect of exercise on HPA activity in animals on a high fat diet may be partially mediated by the effects of exercise on serum FFA levels.

Our study is in agreement with previous evidence in humans demonstrating that increasing blood FFA decreases HPA activity (Lanfranco et

al. 2004). In rats, many reports have shown an enhanced stress response with high fat feeding, although that is not always the case (la Fleur et al. 2005; Legendre and Harris 2006; Pascoe et al. 1991; Pecoraro et al. 2006; Tannenbaum et al. 1997). One possible reason for the difference between our study that demonstrated a decreased HPA response and rodent studies that demonstrated an enhanced response is the fact that our pigs were under anesthesia during stressor exposure. Thus, the stress associated with anesthesia and surgery represents a physical stressor whereas restraint, the primary stressor utilized in the rodent studies, has a large psychological component. The fact that humans also demonstrate a decrease in HPA activity in response to circulating FFA may in part be due to the fact that humans voluntarily choose to participate while animal procedures require forced participation. Therefore, the effect of high fat feeding on HPA responsiveness is likely stressor dependent.

In conclusion, we have shown that exercise and diet can modulate the HPA response to stress and this modulation appears, at least in part, to be due to effects on circulating FFA levels. Exercise training in pigs on a normal diet did not alter the HPA response to stress. Feeding pigs a high fat diet increased serum FFA and decreased the ACTH response to stress while exercise training reduces the increase in circulating FFA and enhances the ACTH response to stress. Thus, the increased FFA in animals on the high fat diet could be the factor inhibiting the ACTH response to stress and exercise training, through lowering blood FFA, is able to reverse this.

Table 1. Effects of diet and exercise on body weight and exercise capacity.

	Normal Diet				High Fat Diet			
	Sedentary		Exercise		Sedentary		Exercise	
Body Weight (kg)	45.8 ± 2.3	a,b	39.7 ± 1.0	b	49.6 ± 2.2	a	47.7 ± 2.0	a
Heart Weight (g)	191.7 ± 6.4		206.0 ± 5.4		195.4 ± 6.1		234.6 ± 8.7	a
Heart/Body Weight Ratio (g/kg)	4.2 ± 0.1		5.2 ± 0.2	a	4.0 ± 0.1		4.9 ± 0.1	a
Exercise Capacity								
Pre (min)	26.2 ± 1.0		28.4 ± 0.9		25.9 ± 1.1		25.3 ± 1.5	
Post (min)	26.6 ± 0.8	a	39.7 ± 1.4	b	22.4 ± 1.5	c	33.3 ± 0.7	d
Change (min)	0.4 ± 0.8		11.3 ± 1.7	a	-3.6 ± 1.3		8.1 ± 1.5	a

Values are presented as mean ± SE, n = 8 per group. Pre, Treadmill exercise test to exhaustion completed prior to initiation of exercise training. Post, Treadmill exercise test time to exhaustion completed after 20 weeks of diet and exercise treatments. Change, difference in time to exhaustion from pre to post. Weights were collected after 20 weeks of diet and exercise treatments. Different letters denotes significant difference between groups, one-way ANOVA, $P < 0.05$.

Table 2. Effects of diet and exercise on clinical chemistry profiles.

	Normal Diet		High Fat Diet		Significant Effects		
	Sedentary	Exercise	Sedentary	Exercise	Exercise	Diet	Interaction
Albumin (g/dL)	3.7 ± 0.1	3.7 ± 0.2	3.4 ± 0.1	3.3 ± 0.1	0.78	0.012*	0.78
Anion GAP	11.0 ± 1.2	14.0 ± 1.7	12.6 ± 1.1	13.4 ± 1.0	0.16	0.70	0.41
CO2 (mEq/L)	30.8 ± 1.0	29.6 ± 0.4	29.0 ± 0.8	29.6 ± 0.2	0.67	0.22	0.22
Globulin	2.8 ± 0.3	3.2 ± 0.2	3.4 ± 0.3	3.4 ± 0.1	0.30	0.09	0.30
Total Protein (g/dL)	6.5 ± 0.2	6.9 ± 0.2	6.7 ± 0.3	6.7 ± 0.2	0.39	0.96	0.23
Kidney function							
BUN (mg/dL)	21.2 ± 3.6	23.6 ± 2.7	12.2 ± 2.1	11.6 ± 1.4	0.73	<0.001*	0.57
Creatinine (mg/dL)	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.0	1.1 ± 0.1	0.09	0.89	0.23
Liver function							
Alanine Transaminase (U/L)	39.2 ± 1.7	49.8 ± 2.9	32.8 ± 2.3	34.0 ± 2.7	0.028*	<0.001*	0.07
Alkaline Phosphatase (U/L)	39.4 ± 1.3	61.4 ± 9.7	86.8 ± 9.9	98.8 ± 7.6	0.048*	<0.001*	0.54
Pancreatic Function							
Amylase (U/L)	944 ± 110	1043 ± 41	930 ± 190	1118 ± 129	0.28	0.82	0.74
Electrolytes							
Na (mEq/L)	132.8 ± 3.0	137.6 ± 2.9	133.0 ± 3.4	135.6 ± 2.1	0.22	0.76	0.71
K (mEq/L)	3.6 ± 0.2	3.9 ± 0.2	3.9 ± 0.2	3.9 ± 0.1	0.39	0.58	0.51
Cl (mEq/L)	94.8 ± 2.5	98.0 ± 1.9	95.2 ± 2.0	96.6 ± 1.3	0.26	0.80	0.66
Phosphate (mg/dL)	5.4 ± 0.2	5.9 ± 0.2	5.9 ± 0.2	6.4 ± 0.3	0.047*	0.047*	0.86
Ca (mg/dL)	10.1 ± 0.3	10.2 ± 0.3	9.9 ± 0.2	9.8 ± 0.2	0.94	0.27	0.71

Values are presented as mean ± SE under normal diet and high fat diet categories. Under significant effects heading numbers represent P value from two-way ANOVA. *P < 0.05, n = 5 per group.

Figure 1

The high fat diet significantly increased serum cholesterol (top, n = 5) and FFA (bottom, n = 8). Exercise training lowered FFA in both dietary groups but did not affect cholesterol. $^{\S}P < 0.05$ demonstrating main effect of high fat diet. *$P < 0.05$ demonstrating effect of exercise within dietary group.

FIGURE 1

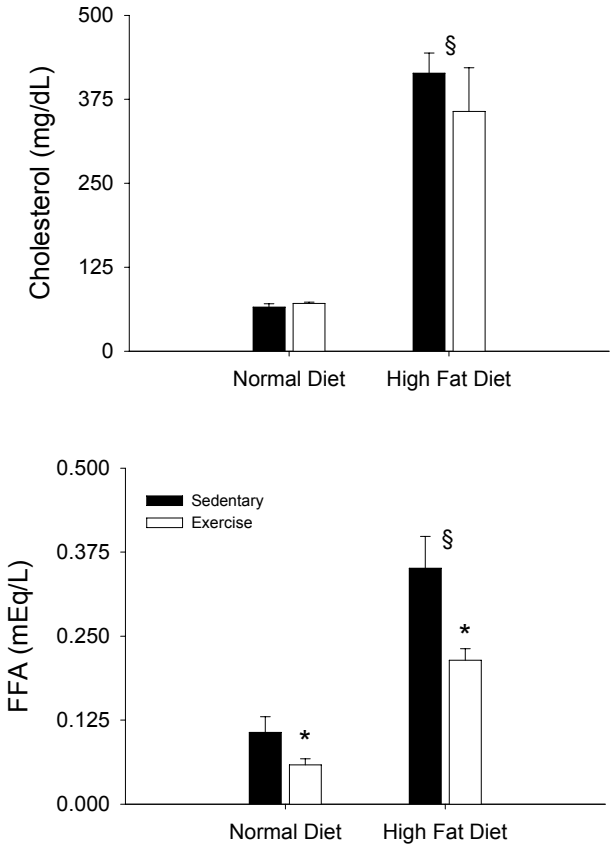

Cholesterol (mg/dL)
500
375
250
125
0
§
Normal Diet
High Fat Diet
FFA (mEq/L)
0.500
0.375
0.250
0.125
0.000
Sedentary
Exercise
§
*
*
Normal Diet
High Fat Diet

Figure 2

Effect of high fat diet on the hypothalamic-pituitary-adrenal response to the stress associated with anesthesia and surgery. The ACTH response (top) to stress was decreased by the high fat diet within the sedentary group. Exercise training significantly increased the ACTH response to stress within the high fat diet group. The cortisol response (bottom) to stress was unaffected by diet or exercise. $^{\dagger}P < 0.05$ demonstrating effect of diet within activity group. $^{*}P < 0.05$ demonstrating effect of exercise within dietary group, n = 8.

FIGURE 2

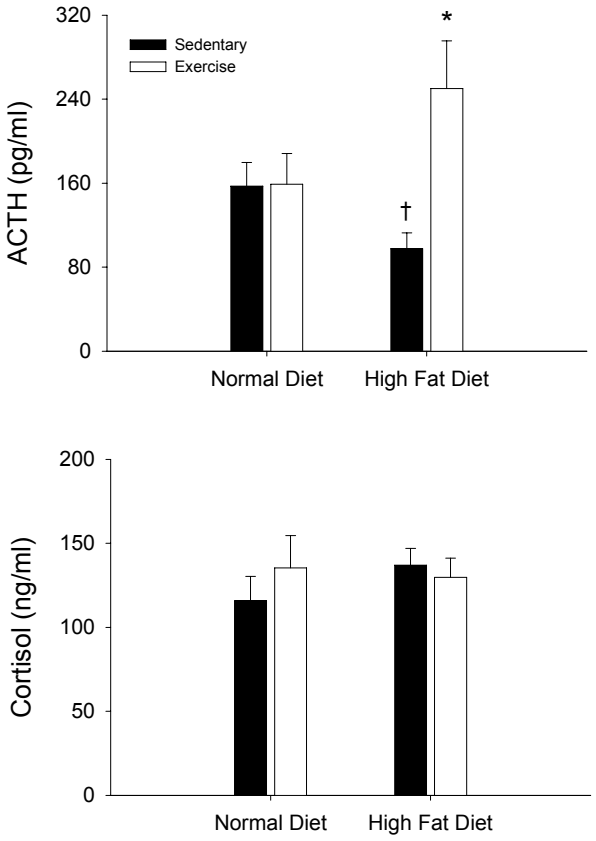
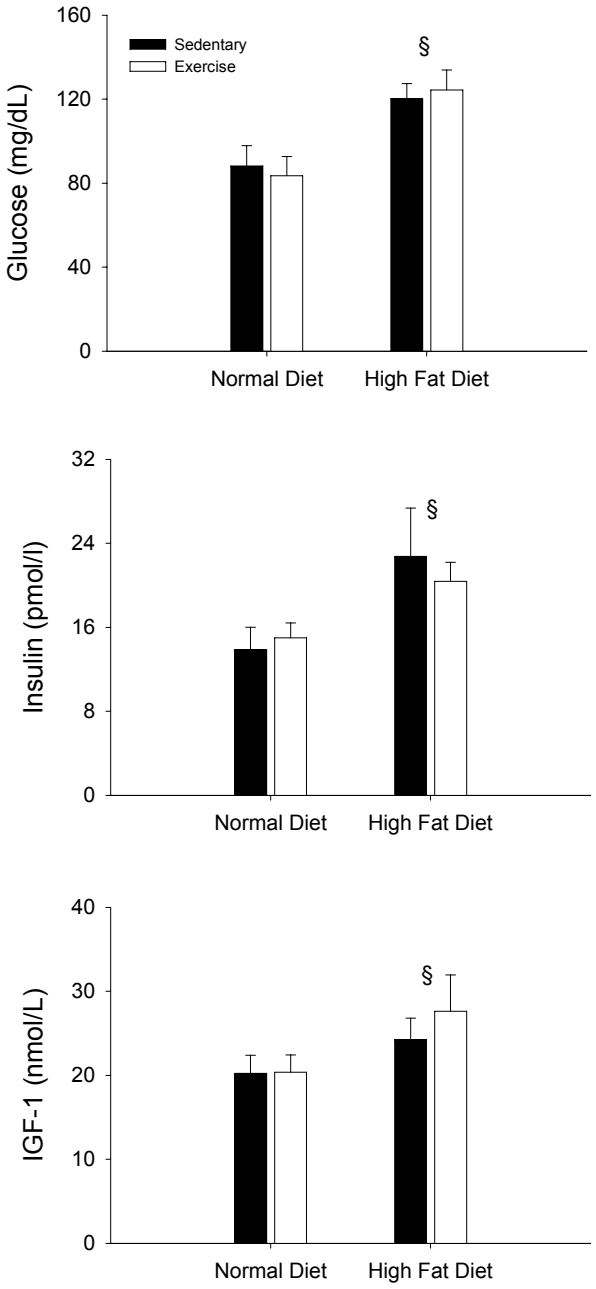

320
240
160
80
0
ACTH (pg/ml)
Sedentary
Exercise
*
†
Normal Diet
High Fat Diet
200
150
100
50
0
Cortisol (ng/ml)
Normal Diet
High Fat Diet

Figure 3

Effect of high fat diet on glucose homeostasis. The high fat diet resulted in significant increases in serum glucose (top), insulin (middle) and IGF-1 (bottom). Exercise did not affect serum glucose, insulin or IGF-1. $^{\S}P < 0.05$ demonstrating main effect of high fat diet, n = 8.

FIGURE 3


Glucose (mg/dL)
160
120
80
40
0
Sedentary
Exercise
§
Normal Diet
High Fat Diet
Insulin (pmol/l)
32
24
16
8
0
§
Normal Diet
High Fat Diet
IGF-1 (nmol/L)
40
30
20
10
0
§
Normal Diet
High Fat Diet

CHAPTER 5

DISCUSSION

INTRODUCTION

Important decisions should be weighed. Such is the case in life. Such is the case for maintaining life. One of the cardinal features of maintaining life is the ability to adapt to the environment, a process that involves glucocorticoid production from the HPA axis. Thus, it should be of no surprise that the system required for biological adaptation to the environment weighs various inputs of information when determining its output. This thesis, through the experimental studies on HPA function, examined the biological activity of this adaptation system that the body uses for maintaining life. The meaning of the findings from chapters 2-4 along with their possible implications for health will be expanded upon in this discussion.

This thesis focused on the release of ACTH. The discovery of IL-6 in the external zone of the median eminence in chapter 2 provided a novel hypothalamic releasing factor that could be secreted into the hypophyseal portal vessels to affect ACTH release. In chapter 3, evidence was provided that nitric oxide is involved in affecting the ACTH response to stress. Finally, in chapter 4, evidence was provided showing the effects of modifiable lifestyle behaviors on the ACTH response to stress. Thus, the results provided in this thesis show that IL-6, nitric oxide, diet and exercise can all influence the activity of the HPA axis.

IL-6 and Stress

The most important finding in this thesis was the discovery of IL-6 in the external zone of the median eminence. This discovery provided evidence that IL-6 could be directly involved in modulating HPA activity. Thus, instead of IL-6 being produced by other tissues throughout the body, traveling through the circulation and then affecting HPA function IL-6 could be produced by the hypothalamus to be secreted into the hypophyseal portal vessels to affect ACTH release from the anterior pituitary. Thus, IL-6 could affect HPA activity without large increases in circulating IL-6 concentrations.

Although novel, the description of a mechanistic role for IL-6 in HPA activity is not surprising. The mRNA and protein for IL-6 were already found expressed in the hypothalamus (Ghorbel et al. 2003; Shizuya et al. 1998). The ability of IL-6 to stimulate ACTH release from the anterior pituitary had been shown as well as the presence of IL-6 receptors within the anterior pituitary

(Ghorbel et al. 2003; Hanisch et al. 2000; Lyson and McCann 1991). In addition, physical and emotional stressors increase HPA activity and IL-6 (Zhou et al. 1993). Despite this evidence, elucidating the relationship between IL-6 and HPA activity has remained evasive. Chapter 2 provided evidence that IL-6 can be directly involved in modulating HPA activity through its release into the hypophyseal portal vessels.

The insight into the relationship between IL-6 and HPA activity provided by the findings in chapter 2 is of value for human health as many diseases, such as obesity, insulin resistance and high blood pressure, are associated with increased plasma IL-6 and increased activity of the HPA axis (Bastard et al. 2002; Bjorntorp and Rosmond 2000; Chan et al. 2002; Fernandez-Real et al. 2001; Pickup et al. 1997; Wernstedt et al. 2004). Additionally, hypothalamic dysfunction and dysregulation of IL-6 are also present in disorders of the central nervous system such as depression and sleep disorders (Alesci et al. 2005; O'Brien et al. 2004; Spath-Schwalbe et al. 1998; Vgontzas and Chrousos 2002; Vgontzas et al. 2003).

Cause or Effect?

IL-6 release into the hypophyseal portal vessels could directly impact anterior pituitary function thereby modulating the hormonal milieu to which the body is exposed. IL-6 can affect ACTH release (chapter 2) and through this manner IL-6 could result in an increase in circulating glucocorticoids. Elevated glucocorticoids contribute to the development of obesity, insulin resistance and

hypertension (Bjorntorp and Rosmond 2000). Thus, increased IL-6, in the presence of CRH and/or vasopressin, could be the cause of increased activity of the corticotrophs (ACTH producing cells in anterior pituitary) leading to elevated glucocorticoids and disease development.

Increased activity of the HPA axis may result in increased release of IL-6 from the hypothalamus which is reflected by increased plasma IL-6. Although IL-6 could be released from the hypothalamus and this release reflected by increased plasma IL-6 it could be that CRH or vasopressin are the culprits for causing overproduction of ACTH release, leading to increased glucocorticoids and the following disease developments. In this scenario, IL-6 is an effect, a by-product of hypothalamic dysregulation that is not the cause of increased ACTH release but merely a reflection of altered hypothalamic activity.

The above scenarios both describe situations which would result in increased HPA activity and increased plasma IL-6. The common pathophysiological pathway in both situations is a dysregulation of hypothalamic output. Therefore, excluding individuals with genetic disorders that result in hypothalamic dysfunction, the incoming information received by the hypothalamus is ultimately the cause of altered HPA activity. In addition, continuous input exposure, or lack thereof, will affect how hypothalamic neurons respond to incoming information. One way by which this can occur is through changes in the expression of various receptors leading to an altered sensitivity to specific inputs (Porcher et al. 2004). An example of input modulation of HPA

activity is provided in chapter 4 where exposure to diet or exercise altered the HPA response to stress.

Hypertension

The hypertensive response to acute stress is attenuated in IL-6 KO animals (Lee et al. 2004; Lee et al. 2006). Evidence demonstrated that the hypertensive response to an acute stressor is decreased in IL-6 KO mice (Lee et al. 2004) and Angiotensin II induced hypertension is also reduced in IL-6 KO animals (Lee et al. 2006). Thus, the blood pressure response to acute stress is modulated by IL-6.

The magnitude of IL-6 response to an acute stressor predicted ambulatory blood pressure at a 3 yr follow-up (Brydon and Steptoe 2005). Chronic elevations in IL-6 are also associated with hypertension (Fernandez-Real et al. 2001). These data suggest that understanding the source of IL-6 during an acute stress response has the potential to provide insight into the association between chronic IL-6 elevations and hypertension.

Of significant interest is the evidence that prenatal exposure to IL-6 leads to hypertension in adulthood (Samuelsson et al. 2004). In addition, prenatal exposure to IL-6 altered the activity of the HPA axis in adulthood. Thus, rats *in utero* that were exposed to elevated IL-6 developed hypertension in adulthood, had altered stress responsive HPA activity and an altered circadian rhythm in ACTH and corticosterone (Samuelsson et al. 2004). The authors suggested that

IL-6 induced these effects by affecting the developing brain. The implications of this will be discussed further in the next section on IL-6 and sex.

Sex

In chapter 2, evidence was provided that IL-6 expression in the median eminence is greater in females compared to males. In healthy humans blood pressure and plasma IL-6 are correlated in females, but not males (Fernandez-Real et al. 2001). Thus, the findings in chapter 2 may have clinical relevance for understanding the development of hypertension in females.

As brought up in the previous section prenatal exposure to IL-6 leads to hypertension and altered HPA activity in adulthood (Samuelsson et al. 2004). Since stress increases circulating IL-6, pregnant women under high stress may predispose their children to disease development by increasing the fetus' exposure to IL-6. If this is true, the possibility exists that the increasing prevalence of metabolic diseases in children today could be directly related to the stress load their mothers carried while the child was *in utero*. Thus, *in utero* is the first time period, and may be one of the most important time periods, for preventative medicine treatment of some conditions such as metabolic syndrome and hypertension.

The greater expression of IL-6 in the median eminence of female pigs suggests that IL-6 plays a greater role in modulating ACTH in females. Our data are consistent with evidence in humans that males had a greater ACTH response to intravenous infusion of IL-6 compared to females (Silva et al. 2002). Although

it may not be initially intuitive the greater ACTH response to IL-6 in males makes sense. Our data suggest that IL-6 is more involved in ACTH release in females and thus the exposure of the corticotrophs to IL-6 in females would be greater. The greater exposure to IL-6 by the corticotrophs would result in a decrease in receptors for IL-6 leading to a decrease in the sensitivity of the anterior pituitary to IL-6. Thus, the ACTH response to a given concentration of IL-6 would be less in animals that have higher IL-6 exposure over the long term. Therefore, IL-6 infusion should induce a greater ACTH response in males, as was shown (Silva et al. 2002).

Sex steroids also can modulate the cytokine response and HPA activity. Estrogen significantly reduced the IL-6 and ACTH response to endotoxin, which the authors suggested was by the action of estrogen in restraining the cytokine response thereby limiting the HPA response (Puder et al. 2001). Testosterone also limits the ACTH response to IL-6 administration and decreases the HPA response to endotoxin (Papadopoulos and Wardlaw 2000). The role for IL-6 in the HPA response to an inflammatory stressor is further seen in evidence demonstrating that IL-6 KO animals, or administration of IL-6 antiserum, results in an attenuation of the HPA response (Turnbull et al. 2003).

Gonadectomy alters the ACTH response to LPS and replacement of testosterone or estrogen restores the alteration in male and female rats, respectively (Watanobe and Yoneda 2003). The gender difference in ACTH response to LPS was removed when animals were gonadectomized. Interestingly, gonadectomy increased the ACTH response to stress in males

while gonadectomy in females resulted in a decreased ACTH response to stress. In this study no difference was seen in plasma IL-6 concentrations between any of the groups (Watanobe and Yoneda 2003).

Our results provide a mechanism to explain the role of IL-6 in contributing to the sex difference in stress responsiveness (Bethin et al. 2000). Bethin et al. 2000, provided insight, suggesting an important role, for the involvement of IL-6 in the female stress response. They demonstrated that female IL-6 KO mice demonstrated a 70% reduction in the HPA response to restraint stress compared to wild type animals. This effect was not seen in males and, importantly, the gender difference in HPA response in the wild type animals was removed in the IL-6 KO animals. This was the first data to suggest that IL-6 plays a greater role in females with regards to HPA responsitivity. Our data further our knowledge by providing evidence that the differential expression of IL-6 in the median eminence is the site of the gender difference in IL-6 that contributes to this gender difference in HPA response to stress.

Summary

Increased activity of the HPA axis and increased circulating plasma IL-6 are common co-occurrences in diseases such as hypertension, obesity and insulin resistance. The evidence in chapter 2 provides a novel mechanism for the relationship between IL-6 and HPA activity by demonstrating that IL-6 is expressed in the median eminence and is released in response to acute stress. This provides a direct link between IL-6 and HPA activity that doesn't require

increases in plasma IL-6. This finding has clinical significance as IL-6 is involved in the acute hypertensive response to stress and is associated with the development of hypertension. It was also discovered that females demonstrate a greater IL-6 expression in their median eminence and demonstrate a greater response to stress. This finding provides further insight into the work by Bethin et al., 2000 where it was demonstrated that removal of IL-6 in KO mice eliminated the gender difference in stress response to restraint. Thus, the evidence in chapter 2 provides a mechanistic answer to the question posed by Yudkin et al., 2000, “Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link?”

Nitric Oxide and Stress

Nitric oxide (NO) is a biological signaling molecule serving to communicate information within and between cells. Although more commonly thought about in its role in control of vasomotor tone it is also involved in neural signaling. In the brain, NO is involved in control of cerebral blood flow and in modulating neural activity (Hamel 2006; Hudetz et al. 1998; Lee 2000; Paravicini et al. 2006; Stern 2004; Stern and Zhang 2005; Zoccoli et al. 2001). It has also been suggested that NO may serve as a coupling link between neural activity and cerebral blood flow (Hamel 2006; Iadecola 1993). In this manner, increased neural activity increases NO production which diffuses to surrounding areas to induce vasodilation thereby increasing substrate delivery.

The experiments in chapter 3 provided evidence of a role for NO in modulating blood flow to the HPA axis and in modulating the hormonal activity of the HPA axis. It was demonstrated that blockade of NO with chronic L-NAME treatment resulted in a significant decrease in tissue vascular conductance at rest. In addition, L-NAME treatment decreased the ACTH response to a short stressor exposure (< 5 min) but augmented the ACTH response to maximal exercise, a stressor lasting ~45 min. Thus, the role of NO in hormonal activity of the HPA axis changes during exercise.

Blood Flow

Inhibition of nitric oxide synthase (NOS) with L-NAME decreased tissue vascular conductance in the frontal cortex, the hypothalamus, the anterior and

posterior pituitary and the adrenal gland (chapter 3). This decrease in vascular conductance resulted in a significant decrease in blood flow to the frontal cortex and anterior pituitary at rest. Treatment with L-NAME did not alter vascular conductance or blood flow to the median eminence. Thus, NO contributes to maintaining blood flow by increasing vascular conductance in most, but not all tissues.

The involvement of NO in maintaining cerebral blood flow at rest is consistent with previous reports (Hudetz et al. 1998; Lee 2000; Paravicini et al. 2006; Zoccoli et al. 2001). Changes in cerebral blood flow during exercise can include a redistribution of regional blood flow which has been shown in ponies (Sikkes et al. 1992), horses (Manohar and Goetz 1998) and pigs (Delp et al. 2001; Foreman et al. 1976).The changes in cerebral regional blood flow include increased blood flow to brain regions activated during exercise and while other brain regions that would not be expected to have increased activation receive the same amount of blood flow as they do at rest (Delp et al. 2001).

HPA Activity

Nitric oxide modulates neuroendocrine function (Stern and Zhang 2005). Microinfusion of NO into the PVN induces ACTH release suggesting that NO plays a stimulatory role for the release of corticotropin releasing factors into the hypophyseal portal vessels (Seo and Rivier 2001). This increase in ACTH release is also seen when an NO donor is administered icv and is coupled with immediate early gene expression in the PVN neurons (Seo et al. 2002).

Consistent with this data is our finding in chapter 3 that L-NAME treatment reduced the ACTH response to restraint stress. These data suggest that during resting conditions NO plays a stimulatory role in the hypothalamus in the activation of the HPA axis.

Interestingly, the effect of L-NAME on the ACTH response to stress changed during exercise. Instead of L-NAME reducing the ACTH response, which we would have predicted based upon the stimulatory role for NO in the hypothalamus, NOS inhibition resulted in a greater ACTH response to stress. One important difference between these two stressors was the duration of the stressor. The restraint stress lasted only 5 min while the maximal exercise stress lasted approximately 45 min. This was not the first report of a time difference in the effect of NOS blockade on the stress response. Consistent with our study, sc injection of L-NAME decreased the ACTH response to shocks during the first 5-20 min of stressor exposure but increased the ACTH response when the stressor lasted 30-60 min (Rivier 2001). These data suggest that duration of stressor exposure determines the role of NO in modulating the HPA response to stress. This proposal gains support as it has been suggested that in the hypothalamus NO plays an inhibitory role during states of increased neuronal activity (Stern and Zhang 2005). Thus, during continued stressor exposure with continued neural activity the role of NO in the hypothalamus switches to inhibit neural activity to thereby limit the ACTH response to stress. Our data, and the report by Rivier, 2001 are consistent with this proposal.

Summary

Chapter 3 provides evidence that NO is involved in modulating vascular conductance and HPA activity. Inhibition of NOS resulted in a decrease in resting vascular conductance and a decrease in the ACTH response to a short duration stressor. During exercise, NOS inhibition augmented the ACTH response to stress. The simultaneous measurements of both blood flow and hormonal response strengthen the interpretation of the other. For example, at rest, NOS inhibition decreased hypothalamic blood flow and decreased the ACTH response to stress providing two lines of evidence that the biological availability of NO in the hypothalamus was decreased in response to L-NAME. Thus, at rest or during exposure to short duration stressors NO maintains hypothalamic blood flow and is involved in the hypothalamic activation of the HPA axis. On the other hand, during stressor exposure over a longer duration NOS inhibition did not affect hypothalamic blood flow and resulted in an augmentation of the ACTH response to stress. Thus, NO is involved in modulating hypothalamic blood flow at rest and the modulation of the hypothalamus by NO during HPA activation is dependent upon stressor duration.

Lifestyle Behaviors and Stress

Exercise training or diet can alter the basal and stress-induced activity of the HPA axis (Droste et al. 2003; Fediuc et al. 2006; Legendre and Harris 2006; Tannenbaum et al. 1997). The mechanism by which this occurs is unclear but likely involves the adaptation to the acute stressor exposures of food intake and exercise bouts. The experiments in Chapter 4 examined the chronic effects of these repeated acute exposures on the response of the HPA axis to stress. Evidence in chapter 4 demonstrated that a high fat diet decreased the ACTH response to stress and this was reversed by exercise training.

High Fat Diet

In chapter 4 we provided evidence that the high fat diet reduced the ACTH response to surgical stress in pigs. One of the fortunate outcomes of using surgical stress was that our animals were under anesthesia so that the stressor was primarily physical in nature. After the observation of the diet effect was made we hypothesized that the reason for the decrease was the result of an inhibitory effect of free fatty acids (FFA) on HPA activity (Lanfranco et al. 2004). In follow-up analyses it was determined that pigs on the high fat diet had significantly increased FFA even after an overnight fast.

There is not a consistent effect of high fat diet on the HPA response to stress in rodents. Studies have shown that a high fat diet can augment (Pascoe et al. 1991; Tannenbaum et al. 1997) or decrease (Pecoraro et al. 2006) the HPA response to stress. Adding to the confusion is evidence that different strains of

rats respond differently and the same strain of rat from different vendors also respond differently (Pecoraro et al. 2006). To make this area even more complex is the evidence that choice of lard, not total lard calories, was the factor contributing to the decreased ACTH response (la Fleur et al. 2005).

The modulation of HPA activity by high fat diet can be through changes in circulating metabolic substrates (Lanfranco et al. 2004) or as the result of changes in body composition (Bjorntorp and Rosmond 2000; Duclos et al. 2001; Katz et al. 2000). In response to physical stress the body's metabolic needs increase and glucocorticoid production is one way in which the body attempts to meet the increased energy demand. Therefore, if the system responsible for glucocorticoid production detects high availability of circulating energy supplies, for example FFA and glucose, than the need to increase glucocorticoids is lessened. Thus, in the presence of elevated FFA there should be a decrease in the ACTH response to stress, as was shown in chapter 4 and previously demonstrated in humans (Lanfranco et al. 2004).

Exercise

Acute exercise results in activation of the HPA axis in an intensity-dependent manner although during low intensity exercise a decrease in cortisol can be seen (Davies and Few 1973; Luger et al. 1987). In support of modulation of HPA activity by acute exercise bouts is the evidence that shows that exercise training lowers the HPA response to the same absolute workload and can alter

the HPA response to different stressors (Buono et al. 1987; Droste et al. 2003; Fediuc et al. 2006).

In chapter 4 we found that in animals on the normal diet exercise training did not alter the HPA response to surgical stress. Exercise training did, however, significantly reduce FFA in comparison to the sedentary group. In contrast, exercise training in animals placed on the high fat diet group resulted in a significant increase in the ACTH response to stress. Thus, the effects of exercise training on HPA activity may only be apparent when there is a dysregulation in HPA function as occurs during high fat feeding.

After observing the effect of exercise training on the ACTH response to stress we hypothesized that this could be due to exercise affecting circulating FFA. As predicted by our hypothesis, exercise training in the animals on the high fat diet did result in a significant reduction in circulating FFA. Thus, it appears that the metabolic effects of exercise (Jensen 2003; Jeukendrup 2002) may be one mechanism by which exercise can affect neuroendocrine activity.

Besides metabolic effects evidence also shows that exercise results in changes in gene expression in brain regions involved in the HPA response to stress (Droste et al. 2003; Park et al. 2005). Evidence has shown that exercise training decreased expression of the mineralocorticoid receptor in the hippocampus and decreased CRH mRNA in the PVN (Droste et al. 2003). These changes were accompanied by a decreased response to novel environment and an increased response to forced swimming in the exercise trained animals

(Droste et al. 2003). In humans, it has been shown that basal ACTH levels are increased in highly trained, but not moderately trained, men (Luger et al. 1987). Evidence in humans also shows that in endurance trained athletes the corticotrophs become less sensitive to feedback inhibition of cortisol (Duclos et al. 1998). The decrease in sensitivity to feedback inhibition is consistent with the high basal ACTH in trained men (Luger et al. 1987) and provides another possible explanation for the increased ACTH seen in exercise trained animals placed on the high fat diet.

Summary

Lifestyle behaviors play an important role in modulating the activity of the HPA axis. The commonly spoken advice, “Eat well and exercise” does have important ramifications for neuroendocrine function as both of these factors, diet and activity, acutely and chronically impact the biology of the HPA axis. A high fat diet increased FFA and resulted in a decreased ACTH response to surgical stress. Both of these changes were reversed by exercise training. The reversal of the effects of the high fat diet on HPA response may be through the effect of exercise on metabolism or through changes in gene expression in the HPA axis. Most likely, it is a combination of these effects that results in the final biological state of HPA function.

CONCLUSIONS

The hypothalamic-pituitary-adrenal (HPA) axis is a dynamic system whose control is influenced by the information it receives. The studies on IL-6 provide a novel factor that can be produced by the hypothalamus to directly augment the ACTH response to stress. The studies on nitric oxide (NO) further expanded our understanding of the role NO plays in the hypothalamus suggesting that at rest NO maintains cerebral blood flow and is involved in PVN activation. In addition, we found that the role of NO in the hypothalamus changes during exercise in that it is not required for maintaining cerebral blood flow and it begins to inhibit PVN activation as a result of continued neural activity in response to continued stressor exposure. Finally, we found that the ability of the HPA axis to respond to a stressor can be altered by placing animals on a high fat diet or by having animals undergo exercise training.

Future Questions to be Addressed

1. Does exercise exert its effects on HPA activity by altering the role of IL-6 or NO in the HPA response to stress?

2. Does a high fat diet exert its effects on HPA activity by altering the role of IL-6 or NO in the HPA response to stress?

3. Will selective KO of hypothalamic IL-6 result in a change in plasma IL-6 levels and a reduction in the IL-6 response to physical and/or emotional stressors?

4. Both exercise trained and sedentary animals showed elevated glucose concentrations. Is there a difference in insulin levels between these groups?

5. Is the circadian rhythm of ACTH and cortisol altered with a high fat diet? Would changes be reflected in circulating glucose and/or FFA?

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VITA

Ryan Jankord was born in Sioux City, Iowa in 1977. He graduated from Lennox High School, Lennox, South Dakota, in 1996. He studied exercise physiology at the University of Nebraska-Lincoln, graduating in 2000. He began his graduate studies at the Human Performance Laboratory at Ball State University completing his master's degree in exercise science in the year 2002. Ryan spent another year at Ball State teaching undergraduate physiology labs and completing coursework in the basic sciences while applying for PhD programs. Ryan joined Dr. Laughlin's lab in May of 2003 and expects to graduate with his PhD in Biomedical Sciences from the University of Missouri in December, 2006. Ryan has been married to Rekha for six years and they have a 1 ½ year-old daughter named Hailey. Ryan plans to continue his research training by joining Dr. Jim Herman's lab at the University of Cincinnati.

APPENDIX

Validation of Immulite Assay

	Cortisol			ACTH				
	low	mid	high	low	mid	high		
Intraassay CV	4.3	5.2	4.1	5.1	2.0	3.0	4.5	3.4
Interassay CV	11.5	2.1	1.8	3.7	5.1	2.0	5.1	3.6

DAY		0	2	4	7	9			Mean	SD	CV
Cortisol	low	6.9	7.1	8.6	6.7				7.3	0.8	11.5
Cortisol	mid	18.6	18.6	19.1	18.2				18.6	0.4	2.1
Cortisol	high	27.6		28.6	28.0				28.0	0.5	1.8
ACTH	low	12.1	11.7	11.3	12.3				11.8	0.4	3.7
ACTH	mid	312.6	286.5	298.3	321.5				304.7	15.5	5.1
ACTH	high	1045.1	1018.0	999.3	1035.0				1024.3	20.1	2.0

Day 0	15-Aug	1	2	3	4	5	6	7	8	Mean	SD	CV
Cortisol	low	6.8	7.2	6.3	7.4					6.9	0.5	7.0
Cortisol	mid	18.3	20.5	18.8	18.3	19.1	17.1	18.8	18.2	18.6	1.0	5.2
Cortisol	high	28.8	29.1	26.5	28.6	27.3	27.5	26.4	26.4	27.6	1.1	4.1
ACTH	low	11.9	10.9	14.8	10.6					12.1	1.9	15.9
ACTH	mid	317	320	309	300	309	317	314	315	312.6	6.4	2.0
ACTH	high	1018	1024	997	1035	1072	1067	1064	1084	1045.1	30.9	3.0

Day 2	17-Aug	1	2	3	4	5	6	7	8	Mean	SD	CV
Cortisol	low	7.4	6.7	7.5	6.8					7.1	0.4	5.7
Cortisol	mid	21.1	18.7	17.1	17.6					18.6	1.8	9.6
Cortisol	high									#DIV/0!	#DIV/0!	#DIV/0!
Cortisol	lowCV	7.1	7.2	6.7	6.5	6.6	7	6.4	6.7	6.8	0.3	4.3

ACTH	low	12.1	11	11.6	12.2					11.7	0.5	4.7
ACTH	mid	280	283	299	284					286.5	8.5	3.0
ACTH	high	1014	1036	1015	1007					1018.0	12.5	1.2
ACTH	lowCV	17.7	16.9	18.3	16.8	16.6	17.5	16.9	15.4	17.0	0.9	5.1
Day 4	19-Aug	1	2	3	4	5	6	7	8	Mean	SD	CV
Cortisol	low	9.4	7.9	8.2	8.8					8.6	0.7	7.8
Cortisol	mid	18.2	20.2	19.5	18.6					19.1	0.9	4.7
Cortisol	high	27.2	29.9	27.1	30.2					28.6	1.7	5.9
ACTH	low	11.4	12.6	10.2	10.8					11.3	1.0	9.1
ACTH	mid	307	281	301	304					298.3	11.8	3.9
ACTH	high	1010	1001	1000	986					999.3	9.9	1.0
Day 7	22-Aug	1	2	3	4	5	6	7	8	Mean	SD	CV
Cortisol	low	6.8	6.5	6.9	6.7					6.7	0.2	2.5
Cortisol	mid	17.8	17.9	17.7	19.3					18.2	0.8	4.2
Cortisol	high	25.5	28.2	28.3	29.8					28.0	1.8	6.4
ACTH	low	12.2	11.1	13.8	11.9					12.3	1.1	9.2
ACTH	mid	335	319	317	315					321.5	9.1	2.8
ACTH	high	1012	1039	1014	1075					1035.0	29.4	2.8

	ACTH H	ACTH H	ACTH H	Pig	Pig	ACTH H	ACTH H	Pig
	Expected	Diluent	Serum	actual	expected	diluent	Serum	diluent
100%	1030.5	1030.5	1030.5	759.5	759.5	**100**	**100**	**100**
75%	772.875	809	866.75	579.5	569.625	**105**	**112**	**102**
50%	515.25	527.5	575.5	421.5	379.75	**102**	**112**	**111**
25%	257.625	260.5	276.75	199	189.875	**101**	**107**	**105**

	Cort H	Cort H	Cort H	Pig	Pig	Cort H	Cort H	Pig
	Expected	Diluent	Serum	Actual	Expected	Diluent	Serum	Diluent
100%	56	56	56	35.1	35.1	**100**	**100**	**100**
75%	42	48	46.675	21.95	26.325	**114**	**111**	**83**
50%	28	32	33.4	12.35	17.55	**114**	**119**	**70**
25%	14	14.5	14.625	5.85	8.775	**104**	**104**	**67**

IL-10

INTRODUCTION

Interleukin-10 has been suggested as a mediator of the HPA axis (Smith et al., 1999). Previous studies have shown that IL-10 has the ability to stimulate ACTH release from the pituitary gland and to stimulate CRH release from the median eminence (Stefano et. al., 1998). Although IL-10 has been shown to be expressed in the hypothalamus the localization of IL-10 within the hypothalamus has not been demonstrated.

HYPOTHESIS

Based on the potential role of IL-10 as an ACTH-releasing factor we hypothesized that IL-10 protein expression in the hypothalamus would be localized to the paraventricular (PVN) and supraoptic (SON) nuclei.

METHODS

Five male post-pubertal Yucatan miniature swine were used for this study. Animals were euthanized and the brain was removed and placed in formalin for one week. After one week the hypothalamus was dissected and placed in fresh formalin for another week. Following fixation and processing of the tissue, slides were made of the PVN, the SON and the median eminence. Vasopressin (VP) and corticotropin releasing hormone (CRH) antibodies were used to identify magnocellular and parvocellular neurons within the PVN and SON. A porcine-specific IL-10 antibody was used to examine IL-10 protein expression.

RESULTS

Supraoptic Nucleus: The magnocellular neurons within the SON stained positive for VP and IL-10. (Figure 1)

Paraventricular Nucleus: The parvocellular neurons stained positive for CRH while the magnocellular neurons stained positive for VP. Neither the parvocellular or magnocellular neurons within the PVN stained positive for IL-10. (Figure 2)

Median Eminence: CRH expression was localized to the external zone of the median eminence. VP expression was seen in both the internal and external zones of the median eminence. IL-10 expression occurred in the ependymal cells and was seen in both internal and external zones of the median eminence (Figure 3).

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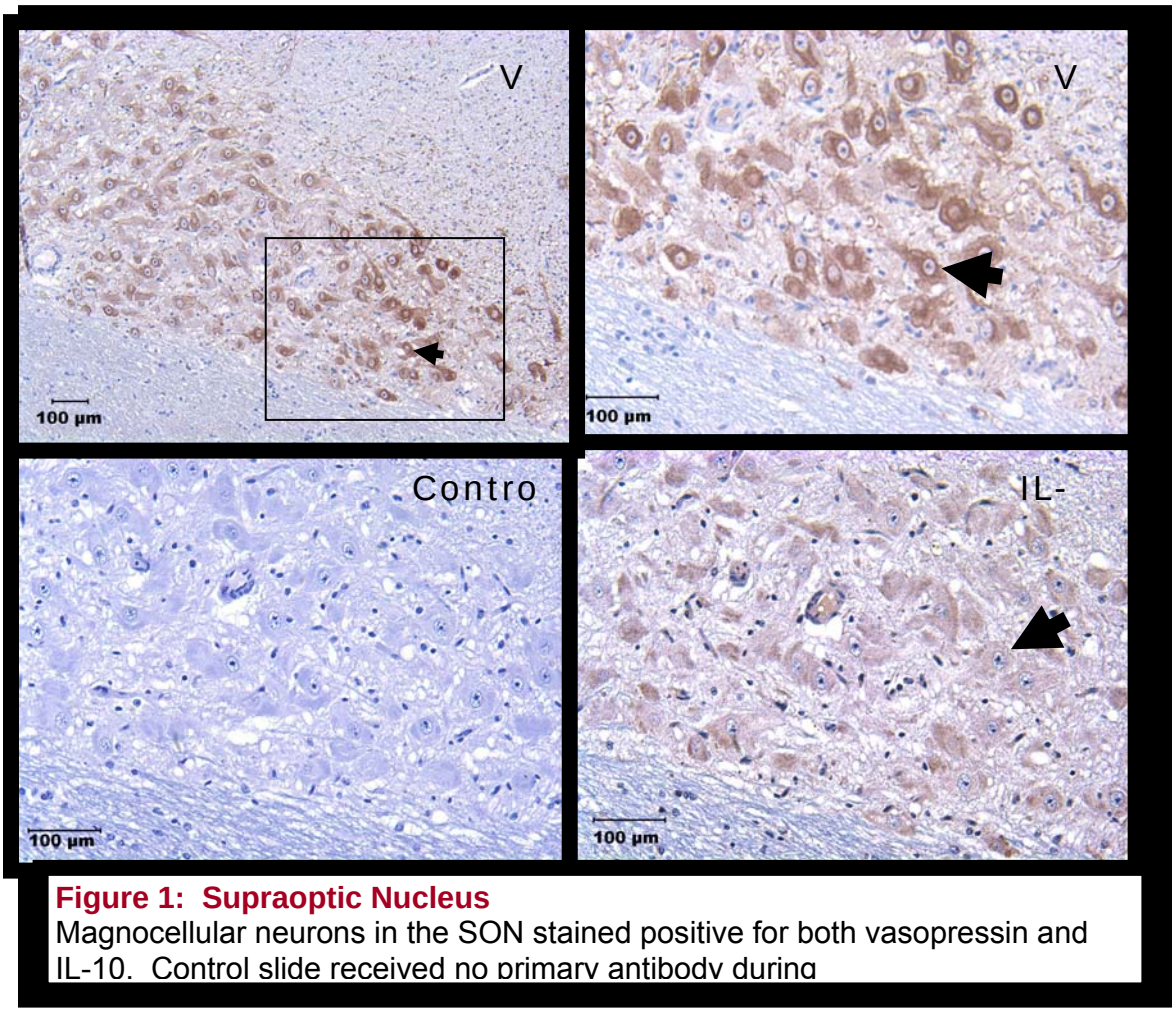


Figure 1: Supraoptic Nucleus
Magnocellular neurons in the SON stained positive for both vasopressin and IL-10. Control slide received no primary antibody during

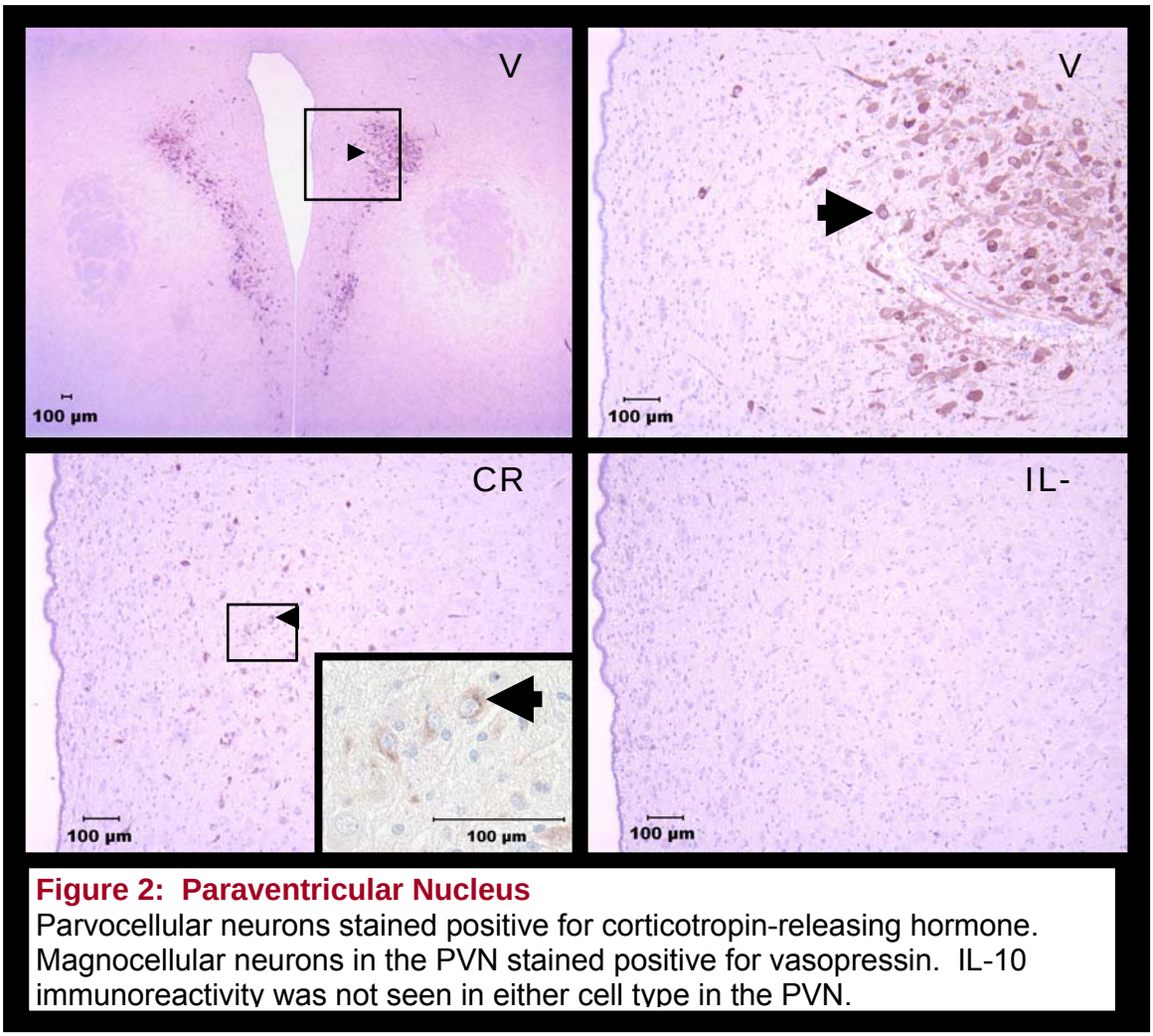


Figure 2: Paraventricular Nucleus
Parvocellular neurons stained positive for corticotropin-releasing hormone. Magnocellular neurons in the PVN stained positive for vasopressin. IL-10 immunoreactivity was not seen in either cell type in the PVN.

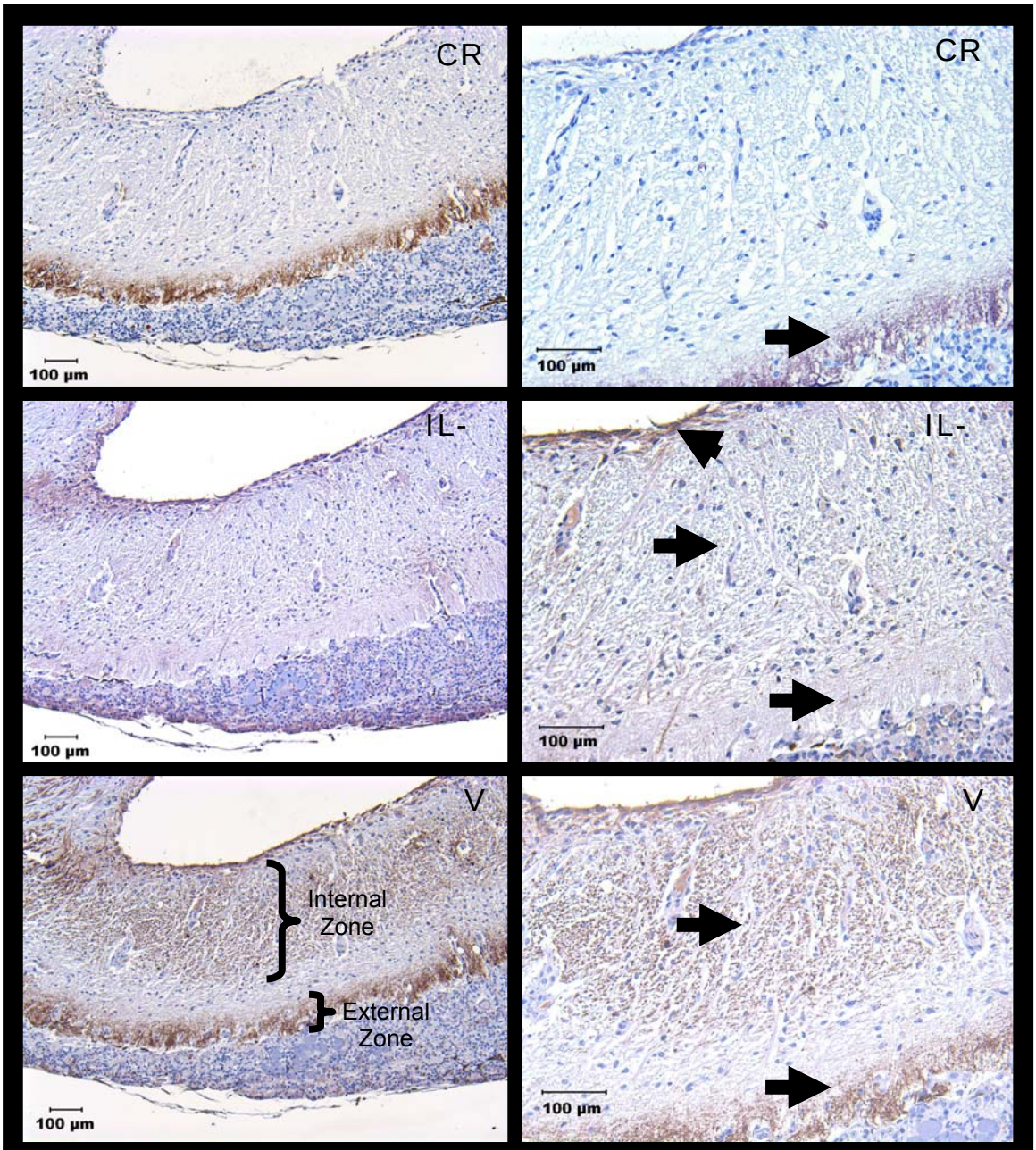
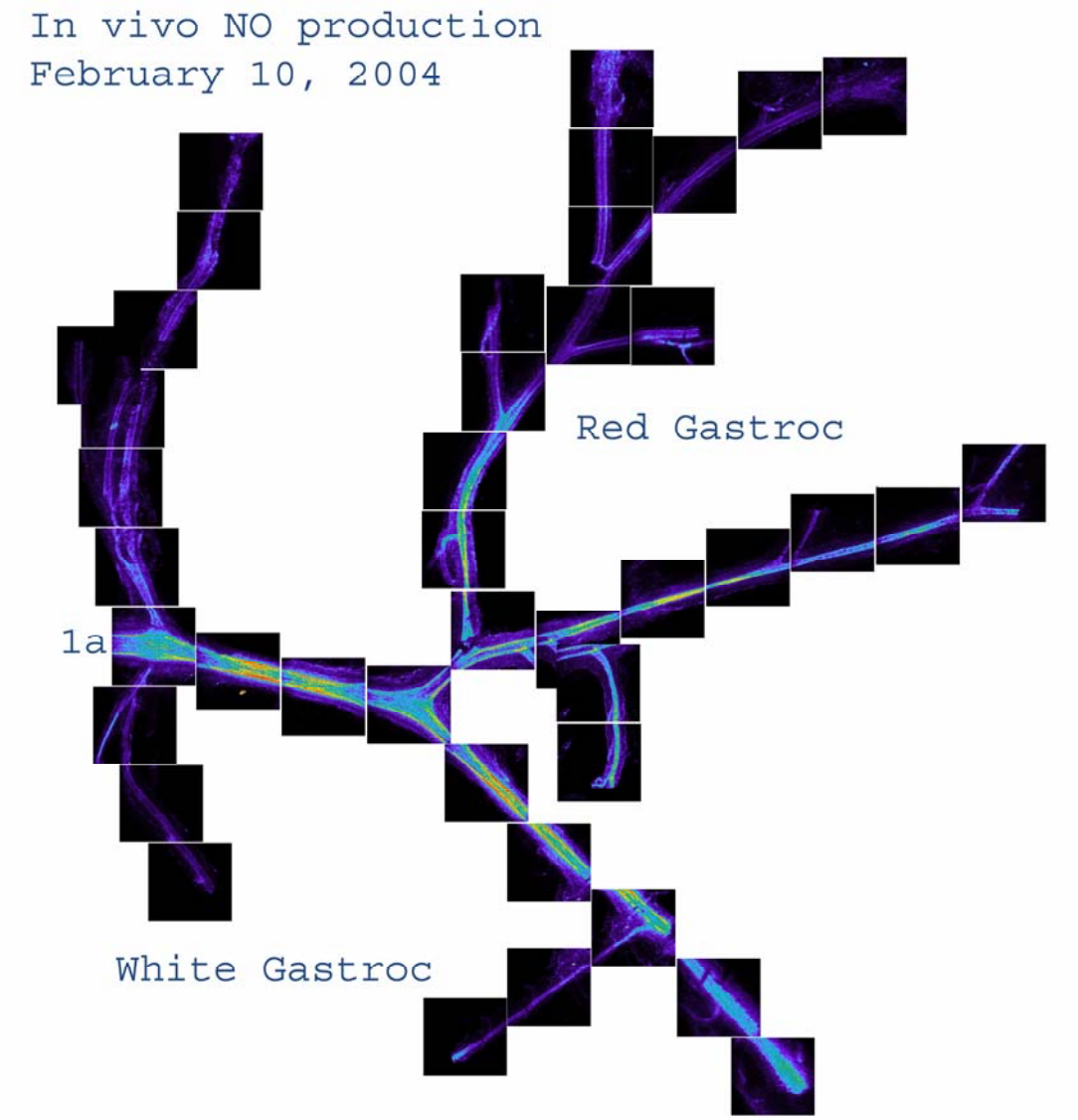
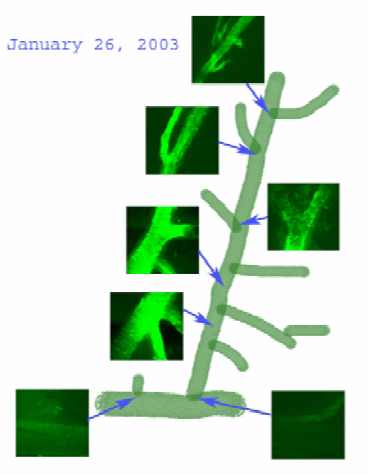


Figure 3: Supraoptic Nucleus
The internal zone of the median eminence stained positive for vasopressin. The external zone of the median eminence stained positive for corticotropin-releasing hormone and vasopressin. IL-10 immunoreactivity was seen in both the internal and external zones of the median eminence and in the ependymal

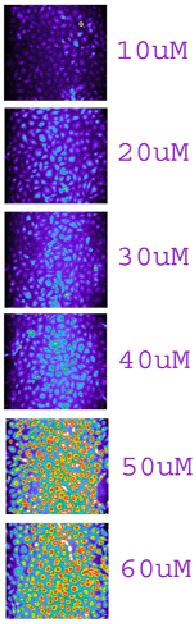
NO production by vessels and endothelial cells



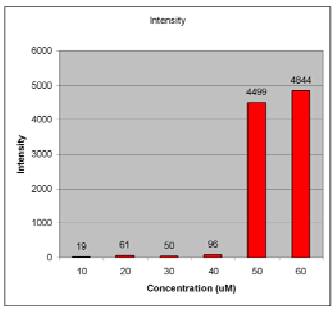
Jan. 26 & With or w/o LNAME



DAF
concentration (uM)



NO production
in cultured
Endothelial Cells
February 18, 2003



June 14

- Image 1: 200x mag
- Image 2-9:600x mag
  - 41 images were captured at .3um increments
  - The attached images are about every 5th image
    - Please note that the images are captured from one side of the vessel wall so that the center and sides of the picture will be at different depths within the tissue