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UNVEILING DIET-INDUCED OBESITY: LEPTIN INSENSITIVITY AND DYSREGULATION OF THE HPA AXIS

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ABSTRACT

UNVEILING DIET-INDUCED OBESITY:
LEPTIN INSENSITIVITY AND DYSREGULATION OF THE HPA AXIS

By

Andrew Changhun Shin

Obesity has reached epidemic proportions in the United States, with more than 32% of adult American population classified as obese. With the national economic burden for treatment of obesity constantly on the rise, the preventive methods and novel therapeutic strategies are in desperate need. Although the etiology of obesity is multifactorial, the most common and probable cause may involve consumption of high-fat (HF) diet in westernized society. Dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis is suggested to be one of the prominent diet-induced neuroendocrine abnormalities associated with obesity, however the underlying mechanisms are not clear.

Current evidence points out that leptin can regulate the HPA axis via suppression of brainstem noradrenergic activity. However, the neuroendocrine dysfunction of the HPA axis in obesity in spite of hyperleptinemia suggests possible leptin insensitivity in brainstem noradrenergic neurons. The goal of this dissertation was first to confirm an obese animal model for presence of dysregulation of the HPA axis, and then to investigate whether the HPA axis perturbation in the diet-induced obese (DIO) rat model is associated with leptin insensitivity in the brainstem noradrenergic neurons. The improvement of the leptin sensitivity may be able to restore the HPA axis regulation and reverse the obese phenotype.

The findings in this research suggest that the chronic HF diet exposure results in dysregulation of the HPA axis in DIO rats as indicated by an increase in both serum leptin levels and the NE levels in the paraventricular nucleus (PVN), and by dissociation between NE and the HPA axis activity. This was associated with increased TH mRNA and reduced leptin signaling in brainstem A1 noradrenergic neurons, suggesting that leptin resistance develops following chronic HF-diet exposure. On the other hand, HF-fed DR animals showed stable leptin levels and intact association between NE and the HPA axis activity without change in the leptin signaling. Acute HF-diet exposure in DIO rats induced a trend of leptin resistance and uncoupling effect between NE and the HPA axis activity, while exogenous leptin injection showed a proper suppression of the PVN NE, suggesting that it is only after HF diet that leptin insensitivity develops. However, leptin injection resulted in no change in the HPA axis in DIO rats, suggesting dissociation between NE and the HPA axis even without HF diet. The final experiment tested whether the improvement of leptin sensitivity by an insulin-sensitizing drug metformin would reduce PVN NE and partially reverse the obese phenotype in HF-fed DIO rats. Metformin treatment resulted in reduction of weight gain, caloric intake, visceral fat depots, and NE levels in the PVN. This was correlated with partially restored association between NE and the HPA axis independent of leptin signaling in the brainstem noradrenergic neurons. Even though the exact mechanisms for the beneficial effects of metformin shown in this dissertation are not clear, it nonetheless opens a possibility for metformin as a low-risk therapeutic approach to correct the neuroendocrine alterations associated with obesity.

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5-HT	5-hydroxytryptamine; serotonin
α-MSH	α-melanocyte-stimulating hormone
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
Ad-36	Adenovirus-36
AgRP	Agouti-related peptide
ANOVA	Analysis of variance
ARC	Arcuate nucleus
AVP	Vasopressin
AW	Adipose weight
BMI	Body mass index
BNST	Bed nucleus of the stria terminalis
BW	Body weight
CART	Cocaine-amphetamine regulated transcript
CCK	Cholecystokinin
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
DEX	Dexamethasone
DIO	Diet-induced obese; diet-induced obesity
DMH	Dorsomedial nucleus of the hypothalamus

DR	Diet-resistant
ELISA	Enzyme-linked immunosorbant assay
FFA	Free fatty acid
GABA	Gamma-amino butyric acid
GI	Gastrointestinal
GR	Glucocorticoid receptor
HF	High-fat
HPA	Hypothalamo-pituitary-adrenal
HPG	Hypothalamo-pituitary-gonadal
HPLC-EC	High performance liquid chromatography with electrochemical detection
i.c.v.	Intracerebroventricular
IL-1β	Interleukin-1β
IL-6	Interleukin-6
i.p.	Intraperitoneal
JAK-2	Janus kinase-2
LC	Locus coeruleus (A6)
LHA	Lateral Hypothalamic area
LR-IR	Leptin receptor immunoreactive
ME	Median eminence
NE	Norepinephrine
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius (A2)
OLETF	Otsuka Long-Evans Tokushima Fatty

PBN	Parabrachial nucleus
PI3K	Phosphatidylinositol-3 kinase
POMC	Proopiomelanocortin
pSTAT-3	Phosphorylated STAT-3
PVN	Paraventricular nucleus of the hypothalamus
qRT-PCR	Quantitative real-time polymerase chain reaction
RIA	Radioimmunoassay
SOCS-3	Suppressor of cytokine signaling-3
STAT-3	Signal transducer and activator of transcription-3
TG	Triglycerides
TH	Tyrosine hydroxylase
UFC	Urinary-free cortisol
VLM	Ventrolateral medulla (A1)
VMH	Ventromedial nucleus of the hypothalamus
WHR	Waist-to-hip ratio

Chapter 1. General Introduction

A. Statement of Purpose

Diet-induced obesity (DIO), probably the most common form of obesity, is a very serious health hazard we face today. It predisposes individuals to chronic diseases such as hypertension, coronary heart disease, Type II diabetes, osteoarthritis, cancers, etc. Over two thirds of the adult American population is considered either obese or overweight with a body mass index (BMI) of 25 or greater. Obese patients typically exhibit central/abdominal obesity, hyperleptinemia, insulin resistance, dyslipidemia, glucose intolerance, and other metabolic and cardiovascular complications (1). In addition, changes in the neuroendocrine system are one of the pathological hallmarks of obesity (2). In particular, basal hypothalamo-pituitary-adrenal (HPA) axis activity and response to various stressful stimuli are altered in obese subjects, suggesting a possible dysregulation of the HPA axis (3-12).

Leptin, a hormone peptide produced predominantly by adipocytes in white adipose tissue, plays a major role in the regulation of energy balance (13). It is also known to cause suppression of the HPA axis (14-17). More recent studies indicate that leptin may suppress the stress axis by decreasing norepinephrine (NE) release in the paraventricular nucleus (PVN) of the hypothalamus, which would then decrease corticotrophin releasing hormone (CRH) secretion from PVN and adrenocorticotropic hormone (ACTH) secretion from anterior pituitary, resulting in reduced glucorcorticoid output (18, 19). The presence of HPA axis

dysregulation in the face of hyperleptinemic state in obese subjects indicates a possibility of leptin insensitivity/resistance at the level of brainstem noradrenergic neurons, which provide noradrenergic innervation to the PVN.

The overall aim of this dissertation research is to investigate the mechanisms by which the HPA axis, or stress axis is affected in diet-induced obesity, and in doing so, elucidate possible leptin signal impairment in noradrenergic neurons in the brainstem. The following research will first characterize the dysregulation of the stress axis in the diet-induced obese (DIO) rat model, and use correlational and mechanismstic approaches to test the hypothesis that leptin signaling is impaired in brainstem noradrenergic neurons, which results in abnormally elevated noradrenergic function that may lead to dysregulation of the HPA axis. The last experiment will test the hypothesis that a method that would restore the leptin sensitivity in brainstem noradrenergic neurons would reverse or partially correct the dysregulation of the HPA axis in DIO rat model. This research could provide a greater understanding of the neuroendocrine alterations of the HPA axis in diet-induced obesity. Because of a strong correlation of abnormal HPA axis activity and metabolic and cardiovascular disorders that stem from obesity, dissecting the mechanisms by which HPA dysregulation is produced will be of importance for the treatment and prevention for such diseases.

B. Obesity

Obesity.... This is a terminology used ubiquitously today. It is a heterogeneous disease in which genetic, environmental, psychological, and other factors are involved. Even though it is not feasible to single out a causative factor for obesity (e.g. stress-induced, genetic-induced, hormone-induced), a large percentage of obese individuals have unhealthy eating habits and a sedentary lifestyle. In simplistic terms, obesity occurs when energy intake exceeds the amount of energy expended over time (20). In many developing countries today, obesity is not a major health concern because a majority of the population in these countries rely on farming, hunting, and fishing for food, and in some cases, there is recurring or continuous famine (21). While increased body weight was a sign of wealth in previous centuries, it is rather considered as a fundamental symptom of chronic disease that can lead to other devastating metabolic and cardiovascular disorders. And this is happening in many developed countries, with the United States leading the world with the highest prevalence of obesity in any age group. Today, obesity has risen to an epidemic level in the United States. Since the late 1980s, adult obesity has steadily increased in this country. Currently, more than 64%, or approximately 127 million adult Americans, are categorized as being obese or overweight (22). Due to a rapid advancement in medicine, we have witnessed lower rates of heart disease-related deaths, longer life expectancy, and healthier aging. However, obesity as a disease now threatens to reverse these hard-won gains we have made during the last 30 years.

Statistics from 2004 National Health and Nutrition Examination Survey (NHANES) surveys show that for children 2-6 years of age, the obesity prevalence increased from 5% to 14% since the mid-seventies. The prevalence has more than tripled in older age groups (6-11 years or 12-19 years) (23). Moreover, the National Institute of Aging recently proclaimed and emphasized the necessity for losing weight as obesity is predicted to cut down life expectancy in the United States by as much as 5 years in the next few decades. This is due to the fact that obesity is one of the leading risk factors for development of Type II diabetes, hypertension, and other cardiovascular diseases. The ever-increasing prevalence of obesity is also heavily affecting the United States economy. In 1998, obesity-related medical expenses accounted for 9.1%, or $78.5 billion, of total U.S. medical expenditures. In 2002, the expenses increased to about 20%, or $92.5 billion, since 1998. NIH has recently launched a national health objective to reduce the prevalence of obesity in adults to less than 15% by the year 2010, however the situation is worsening with obesity-related medical expenses topping $100 billion (24).

Obesity is mainly characterized by morbid weight gain caused by accumulation of fat, hyperphagia, hyperinsulinemia, hyperleptinemia, and a number of peripheral as well as neuroendocrine changes (3). Robust research in the obesity field has been implemented during the past decade to extend our understanding of the risk factors for obesity and the mechanisms underlying obesity development, however researchers still face struggles with gender, age, ethnicity-specific, and other confounding variables. On the other hand, many

researchers focus on the effect of high-fat diet, which is considered the major cause of obesity, on the development and mechanisms of diet-induced obesity (DIO). For the rest of the dissertation below, I will introduce important concepts and pathophysiological changes related to DIO, and demonstrate a novel hypothesis that investigates the mechanisms by which obesity develops. It will be followed by research findings and discussions on interpretation of the data.

C. Leptin

Ob gene

Leptin, the product of the ob gene, is a 167 amino acid long hormone produced predominantly by white adipose tissue. It circulates as a 16 KD protein partially bound to plasma proteins (25). Since the discovery of leptin gene in 1994 by Zhang and colleagues (26), extensive research has been undertaken to uncover the physiological role of leptin. Besides its role in reproduction, regulation of cardiovascular and immune systems, leptin was found to be primarily involved in energy homeostasis. It acts as a signal of nutritional status to the key hypothalamic nuclei that regulate satiety and energy expenditure (13, 27). It is interesting to note that 'leptos' in Greek word is translated as 'thin', which precisely indicates the physiological role of leptin in an organism. Leptin levels increase after feeding and cause suppression of appetite by acting on feeding centers in the hypothalamus. In fact, it has been shown to specifically play a role in food regulation in rodents by acting as a satiety factor. Leptin gene

expression is stimulated after the mice have started eating and remains elevated thereafter for several hours (28). Also, leptin administration to mice acutely reduces food intake(29). On the contrary, fasting decreases serum leptin levels, and refeeding restores the leptin levels in approximately 4 hours (30). Leptin also increases energy expenditure to produce a decrease in body weight (29, 31, 32). Therefore, it is not surprising that a deficiency of, or insensitivity to leptin could lead to increased food intake (33). Moreover, a deficiency of leptin may lead to an increased intake of high fat and carbohydrate food. The role of leptin in energy homeostasis is supported by the fact that leptin-deficient *ob/ob* rodents and long-form leptin receptor (ObRb) defective *db/db* rodents develop obesity and are hyperphagic. Leptin is also produced in low levels in the periphery, including the placenta, skeletal muscle, and stomach (34-37), suggesting different roles of leptin besides controlling energy homeostasis. The importance of leptin production in these tissues and its relevance in regulation of body weight and food intake remains to be ascertained.

Leptin receptors

Leptin elicits its effects via receptor-mediated mechanisms. Leptin receptor belongs to the Class I cytokine superfamily receptors that contain an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic signaling domain. Leptin receptor has similarities to the cytokine receptor homologous domain in the extracellular region (38). Interleukin-6 (IL-6), leukemia inhibitory factor (LIF), and granulocyte-colony stimulating factor

(GCSF) receptors share the highest sequence similarity with the leptin receptor (39). To date, six splice variants of leptin receptors, a – f, have been identified (39). These isoforms have a similar extracellular domain at the amino terminus and most possess transmembrane domains, but only leptin receptor long isoform 'b' (ObRb) has intracellular motifs necessary for signal transduction via JAK-STAT signaling to consequently suppress feeding and control body weight (39). The vital role of ObRb was confirmed in rodents with *db/db* and *fa/fa* mutations. *db/db* mutation induces insertion of premature stop codon at ObRb mRNA transcript, leading to production of receptor isoform 'a' instead. Animals with this mutation are unable to sense circulating leptin. The result is hyperphagia, morbid obesity, sterility, and lack of response to leptin treatment (40, 41). Conversely, *fa/fa* mutation causes substitution of Gln for Pro in the extracellular domain, resulting in diminished expression of all isoforms of leptin receptor. These mutant rats show hyperphagia, hyperlipidemia, weight gain, hyperglycemia, and reduced sensitivity to leptin injection (42-44). Isoform 'e' lacks both intracellular and transmembrane domains, and the role of this specific receptor isoform is not clear, as for other four short isoforms. This will be further discussed in another section below.

Sites of leptin action

Sites of leptin action are almost universal, indicating that the localization of ObRb is widely distributed both centrally and peripherally. At the level of the central nervous system, ObRb mRNA expression has been detected in numerous

sites including the thalamus and Purkinje and granular cell layers of the cerebellum (45-47), however by far the highest expression was detected in the hypothalamus (46). Since the early 1940s and 50s, the hypothalamus has been implicated in the regulation of feeding behavior. Lesions of the ventromedial hypothalamus (VMH) produce hyperphagia and morbid obesity, while lesioning of the lateral hypothalamic area (LHA) resulted in aphagia and even death by starvation (48, 49). The idea of "dual centers" governing feeding behavior emerged as these brain lesioning studies were supported by other groups. VMH was believed to govern 'appetite termination' while LHA regulates 'appetite initiation'. However, more recently this hypothesis of "dual centers" was questioned and pushed to the corner because similar characteristics and phenotypes could not be replicated with those discrete nuclei lesions in other studies (50).

To date, it is well accepted that the mediobasal hypothalamus, which includes nuclei like the dorsomedial hypothalamus (DMH), arcuate nucleus (ARC), paraventricular nucleus (PVN), as well as the VMH and LHA, are all involved in the regulation of energy balance. Leptin's effect of suppressing feeding behaviors is achieved by acting on ObRb in these distinct hypothalamic regions. Immunostaining of signal transducer and activator of signaling-3 (STAT-3), a second messenger critically involved in leptin signal transduction, and c-fos immunoreactivity in these hypothalamic regions after leptin administration confirm the direct action of leptin on these target regions (51, 52). Even though leptin receptors are present in all these areas, the most prominent,

direct leptin-responsive sites are ARC and DMH (46). Other regions receive rich projections from these two, especially from the ARC. This complex neuronal network is believed to mediate the effect of leptin. mRNA expression of short isoforms of leptin receptor are densely expressed in brain microvessels and the choroid plexus, suggesting a possible role for leptin transport across the blood-brain-barrier into the brain (53, 54).

Lower levels of mRNA expression of leptin receptors also have been detected in the brainstem areas (52, 55-57). It is very interesting that the potency of leptin's inhibitory effect of food intake is comparable to that in the hypothalamus. Leptin injection i.c.v. through a lateral ventricular cannula or into the dorsal vagal complex, a critical region that regulates autonomic function, markedly decreases food intake at both acutely and in the long term (56). Hay-Schmidt and colleagues has shown that leptin receptor immunoreactivity is present in noradrenergic and adrenergic neurons in the brainstem by double-labeling with tyrosine hydroxylase (TH), a rate-limiting enzyme for norepinephrine (NE) production (55). Evidence demonstrating the existence of leptin-responsive neurons (POMC and NPY neurons; discussed below) in specific brainstem areas and their dense projections to hypothalamic regions strongly suggest the interaction between not only the forebrain and hindbrain, but also between localized subsets of neurons that can modulate food intake and noradrenergic sympathetic activation, which is important for leptin-induced energy expenditure (58, 59).

Leptin receptor isoform 'a' is also highly expressed in peripheral tissues such as the heart, liver, lungs, skeletal muscle, spleen, white adipose tissue, adrenals, and testes (40, 53, 60-62). Lower levels of functional ObRb mRNA expression are found in the lungs, pancreas, kidneys, adrenals, lymph nodes, as well as in skeletal muscle, brown adipose tissue, and liver (60, 63-65). These findings suggest that leptin is involved in various central and peripheral physiological systems. Indeed, the presence of leptin receptors in gonadal organs suggests that leptin may affect growth and/or function of these organs directly, apart from its indirect central effect via modulating hypothalamo-pituitary-gonadal (HPG) system. Likewise, leptin may play a role in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis by directly affecting glucocorticoid secretion from the adrenal cortex, apart from its central modulating affect via neuropeptides and neurotransmitters in the brain. It can also bind to its receptor on white or brown adipose tissue and mediate lipolytic or thermogenic effects, respectively. Taken together, the widespread distribution of both short and long forms of leptin receptor in the brain as well as in the periphery indicates pleiotrophic functions of leptin.

Leptin signaling mechanisms

Diverse orexigenic and anorexigenic neuropeptides are produced by neurons in the hypothalamus. These comprise a major neural circuitry regulating feeding behavior and body weight. Leptin inhibits the production of orexigenic (appetite-stimulating) peptides and stimulates synthesis of anorexigenic peptides

(appetite-inhibiting). The major orexigenic and anorexigenic neuropeptides are produced by neuropeptide Y (NPY) / agouti-related peptide (AgRP) neurons and proopiomelanocortin (POMC) / cocaine-amphetamine related transcript (CART) neurons, respectively. Leptin stimulates POMC / CART neurons and inhibits NPY / AgRP neurons through a well-characterized intracellular pathway (31).

The fundamental mechanism is the same for leptin's central or peripheral effects. I will focus on leptin signaling in the central nervous system. Activation of ObRb involves leptin binding to the receptor and a rapid activation of Janus-kinase 2 (JAK-2) that is constitutively associated with membrane-proximal regions of ObRb. JAK-2 activation leads to tyrosine phosphorylation of specific sites within ObRb, tyrosine residues 985 and 1138. STAT-3 second messenger is recruited in the cytoplasm, docks at Y1138, and becomes phosphorylated by JAK-2. Phosphorylated STAT-3 (pSTAT-3) at the docking site of intracellular domain of the receptors dissociates and dimerizes in the cytoplasm, and translocates into the nucleus and binds to the DNA binding region to regulate transcription of genes such as POMC and CART in POMC/CART neurons. After a short duration of time, it also induces expression of suppressor of cytokine signaling-3 (SOCS-3) that acts as a negative inhibitor of JAK-2/STAT-3 complex in order to shut down further leptin signaling. On the other hand, leptin binding to its receptors in NPY/AgRP neurons in the arcuate nucleus also activates the JAK2/STAT-3 pathway, but the gene transcription of NPY and/or AgRP is significantly reduced. Moreover, these two neurons have a negative reciprocal relationship in that one can actively inhibit the other and vise versa. Thus, increased levels of circulating

leptin induces elevated leptin signaling, increased production of POMC and its derivative, α-melanocyte-stimulating hormone (α-MSH), in order to elicit suppression in feeding behaviors. Whereas low leptin levels stimulates NPY/AgRP neurons which can downregulate POMC/CART gene expression necessary for the mediation of leptin signaling and cause the animals to become hyperphagic and obese (13, 27, 31, 66).

There are also reports of rapid effects of leptin that cannot be explained by gene expression. Treatment with leptin depolarizes POMC neurons in hypothalamic slice preparations. This is accomplished by inhibiting leptin-responsive NPY neurons in the arcuate nucleus that project to POMC neurons. Because GABA is co-expressed with NPY and is released from axon terminals innervating POMC neurons, leptin treatment can hyperpolarize NPY neurons, thereby reducing release of inhibitory neurotransmitters like GABA from the terminals, finally leading to the disinhibition of POMC neurons (67). Furthermore, glucose-sensitive neurons in the brain and insulin secretion from β-cells in pancreas islets are rapidly regulated by leptin via activation of ATP-sensitive potassium channel or other receptors (68, 69). Taken together, these findings suggest a complexity of mechanisms by which leptin regulates energy balance in the central nervous system.

Leptin resistance in the hypothalamus in diet-induced obesity (DIO)

As aforementioned, leptin is known to act on different, but specific, hypothalamic areas to regulate body weight and food intake. Due to excess

accumulation of fat, obese patients have high serum levels of endogenous leptin; nonetheless, they are unable to regulate their body weight or food consumption. The regulation of energy homeostasis by leptin does not seem to be intact in these obese patients because there is also a lack of effect of exogenous leptin administration to induce weight loss (70), indicating possible leptin insensitivity/resistance at the level of the hypothalamus or impaired leptin transport to the brain. Both have been shown to be the possible reasons why obese patients do not have control on their weight gain and eating habits as demonstated by experimental rodent models. Chronic feeding of HF diet induces leptin resistance at the level of the hypothalamus by decreasing leptin signal transduction as measured by pSTAT-3 immunoreactivity in normal rodents as well as selectively-bred obesity-prone rodents (71-73). Moreover, induction of DIO resulted in impaired transport of leptin across the blood-brain-barrier as measured by transport rate of radioactively iodinated leptin that was intravenously injected (74). However, a more recent study suggests that defective leptin transport into the brain may be an acquired problem associated with age and development of obesity. It has been suggested that a more direct cause of development of obesity may be impaired signaling of leptin in the hypothalamus (73). Similar leptin resistance in extra-hypothalamic areas in the brain may be also present for obesity-related alterations, both peripherally and centrally.

D. HPA axis regulation

HPA axis

Many internal (intrinsic) and environmental (extrinsic) adverse forces, known as 'stressors', lead to enhanced activation of the HPA axis, or stress axis. There are adaptive responses that arise when any type of stressors are encountered. Acute stress response can be deemed helpful to fulfill the body's needs of more focused attention, increased respiration, elevated catabolism, etc., while continuous activation or dysregulation of the stress response can reduce growth hormone secretion, disturb immune-endocrine interactions, and also result in development of chronic diseases such as Cushing's syndrome, central obesity, diabetes mellitus, and cardiovascular diseases. (75).

Activation of the HPA axis involves stimulation of corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus, which would cause adrenocorticotropin (ACTH) release from the anterior pituitary that in turn stimulates corticosterone secretion from the adrenal cortex. Stimulation of the parvicellular neurosecretory cells in the PVN is mediated by innervations from other CRH-immunoreactive brain areas like the perifornical hypothalamic nucleus, the bed nucleus of the stria terminalis (BNST), laterodorsal tegmental nucleus, the parabrachial nucleus (PBN), noradrenergic nuclei (e.g. A1, A2, A6), and the dorsal raphe (76). And this increased CRH release into the PVN has been shown to induce an increase in the CRH mRNA

expression in the PVN (77). Interestingly, PVN CRH neurons have been shown to project and terminate on the PVN parvicellular cell bodies themselves, suggesting the presence of a long and ultra-short positive feedback loop controlling the production and release of CRH (78, 79). PVN CRH neurons also innervate sites other than anterior pituitary including thalamus, amygdala, hippocampus, cerebral cortex, striatum, midbrain, pons, and cerebellum (80-83). It is notable that this group of neurons sends its projections to the brainstem noradrenergic nuclei to increase NE neuronal firing and NE release into the PVN to stimulate the PVN CRH neurons (84-86). The importance of the relationship between central NE and CRH neurons in the PVN will be discussed below. The end product of the HPA axis circuitry, corticosterone, would then bind to glucocorticoid receptors in the PVN, anterior pituitary, and hippocampus in a negative feedback fashion to turn off the stress axis activity (75, 87).

Brainstem noradrenergic nuclei

Among a variety of neurotransmitters and neuropeptides that stimulate the secretion of CRH, norepinephrine (NE) is believed to be of great significance. Retrograde tracer-immunofluorescent studies demonstrated that the noradrenergic inputs originate in three distinct brainstem areas, namely, ventrolateral medulla (VLM; A1), nucleus tractus solitarius (NTS; A2), and locus coeruleus (LC; A6) (88). A1 and A2 noradrenergic groups send ascending projections to the hypothalamus to regulate neuroendocrine and cardiovascular functions (89). However, the largest group of noradrenergic neurons present in A6 region projects extensively to the brain and spinal cord including the cerebral cortex,

thalamus, hypothalamus and cerebellum. The locus coeruleus plays a major role in maintenance of arousal and response to new stimuli (90, 91).

Although they act at different degrees, these three groups of noradrenergic neurons can directly innervate and stimulate parvicellular CRH neurons in the PVN, leading to activation of the HPA axis (92, 93). CRH neurons have been shown to receive rich NE innervations from the brainstem (94). Moreover, direct administration of NE into the PVN can stimulate CRH secretion. Depletion of NE in the PVN by dissecting its noradrenergic innervation from the brainstem can also cause a significant reduction in CRH release (93, 95). This also leads to a marked reduction of corticosterone, hence suppression of the HPA axis (96). Conversely, i.c.v. injection of NE into the PVN induced a marked elevation in circulating corticosterone levels (97). More importantly, the same group showed that compared to other brain regions, the administration of NE into the PVN elicited the highest rise in the circulating corticosterone levels. Taken together, these studies suggest that in spite of widespread distribution of CRH neurons, CRH neurons in the PVN may play a principal role in activation of the stress axis, and that noradrenergic input from the brainstem is a potent stimulator of PVN CRH neurons. This results in elevated circulating corticosterone levels, or activation of the HPA axis.

Role of leptin in regulation of the HPA axis

Other central neurotransmitters like glutamate, serotonin (5-HT), and acetylcholine (ACh) have been shown to elicit stimulatory effects on PVN CRH

neurons to increase corticosterone secretion (98-101). Of course, central endogenous inhibitory neurotransmitters such as POMC-derived peptides, GABA, and substance P exist to suppress CRH release and corticosterone secretion (102-104). On the other hand, peripheral peptides/hormones may also be involved in the regulation of the stress axis. An endogenous peripheral signal that has received much attention in relation to the HPA axis is an adipocyte-derived hormone, leptin. Besides regulation of energy homeostasis, increasing number of studies emphasize leptin's role in neuroendocrine function including the stress axis (16, 105, 106). Earlier studies indicated that leptin might stimulate the stress axis (105, 106). However, more recent studies have shown that leptin administration decreases corticosterone levels in leptin-deficient *ob/ob* mice, indicating that leptin is able to control the HPA axis independently of its role in energy balance (14). Also, our laboratory had observed a decrease in NE levels in the PVN, ARC, and VMH after systemic and peripheral administration of leptin (107). In another study, exogenous administration of leptin normalized the significant increase observed in monoamines in these areas in type-I diabetic rats (19). Furthermore, leptin decreased the efflux of NE from the hypothalamus in vitro (108). More recently, leptin was shown to decrease NE release in the PVN in a dose-dependent manner with a concurrent decrease in serum corticosterone (18). Further, administration of noradrenergic agonists phenylephrine or clonidine prevents leptin-induced decrease in corticosterone in rats (18). Even endogenously produced leptin was shown to behave in the similar manner to decrease NE concentration in PVN and decrease corticosterone production in

virus-induced obesity rat model (109). Taken together, these studies clearly demonstrate the role of leptin on regulation of the HPA axis by suppressing the activity of noradrenergic neurons in the brainstem.

E. HPA axis dysregulation in obesity

At basal state

Daily rhythm of ACTH or cortisol secretion from obese subjects seems to be normal compared to the lean controls (110). However, a majority of studies suggests lower than normal morning samples or lower 24 hours-integrated cortisol levels in obese men (111) Studies of obese women suggest similar cortisol levels at basal state, but increased ACTH pulse frequency and reduced ACTH pulse amplitude in the morning compared to the lean controls (7). Others have measured cortisol in saliva instead of serum due to its non-invasive, stree-free procedure and abundance of the free form of cortisol without salivary flow inteference (112). More variations in the morning salivary cortisol levels were observed in obese male subjects compared to the controls, indicating that the HPA axis dynamics elicit alterations during the peak phase of the daily rhythm. CRH concentrations in the cerebrospinal fluid have also shown to be decreased in obese patients (6). Furthermore, studies investigating urinary-free cortisol (UFC) indicate that 24-hour UFC levels are higher in women higher waist-to-hip ratio (WHR) and abdominal obesity (9, 113, 114). In support of this finding, Pasquali and colleagues showed higher UFC excretion rate during late night to early

morning in obese females compared to normal controls (unpublished). The increased UFC rate showed a significant association with waist circumference or WHR. Alterations in the HPA axis have been shown in polygenically obesity-prone rats in which similar urine corticosterone levels and CRH mRNA expression in the PVN, but decreased glucocorticoid receptor (GR) mRNA in hippocampal CA1 region were detected compared to diet-resistant rats at basal state (115). Taken together, these studies suggest that subtle changes in HPA axis activity exist, particularly in the early morning between obese and normal subjects.

At dynamic states following different stimuli

A majority of dynamic studies where HPA axis was investigated after different challenges with neuropeptides indicate that obese subjects are very likely to have abnormal HPA axis activation. Elevated cortisol secretion following laboratory stress test or increased ACTH and cortisol levels after stimulation with combination of neuropeptides CRH and vasopressin (AVP) have been reported (6, 116). Subjects diagnosed with abdominal obesity also demonstrated elevated cortisol secretion following stimulation with CRH or ACTH analogue (9, 114). Moreover, significant positive correlations between inability to suppress the HPA axis following dexamethasone (DEX) suppression test and BMI, cholesterol, triglycerides, and systolic blood pressure have been observed with salivary cortisol measurements (112). Ljung and colleagues support this finding by demonstrating no change in cortisol levels following DEX suppression test in men

with a WHR greater than 1, indicating perturbation of the HPA axis in obese subjects compared to the lean controls (11).

Similar findings on HPA axis dysregulation following stimulation with neuropeptides or psychological / physical stressful stimuli are documented in rodent studies. Decreased basal morning corticosterone levels were recorded in DIO rats, but their terminal corticosterone levels after stress acquisition were shown to be higher compared to DR rats (117). Pacak and colleageues demonstrated comparable basal and stress-induced ACTH levels, but increased corticosterone in Zucker rats (118). Moreover, increased ACTH levels were detected after restraint stress which was not reproducible following CRH challenge in *fa/fa* rats (119). Collectively, these results in both human and animal models indicate that neuroendocrine changes in the HPA axis of obese subjects exist in the form of hypersensitivity/ hyperresponsiveness, reduced negative feedback mechanisms, or dissociation within the HPA axis loop itself.

Response to diets

A robust interaction between the brain and the GI tract exists since, a number of peptides are activated following food consumption to affect the activity of the HPA axis (120). Obese subjects with BMI greater than 34 have higher cortisol levels after meal ingestion (121). Moreover, women with visceral obesity show rapidly elevated circulating ACTH and cortisol concentrations after mixed meals compared to the control group (7). Different nutrient compositions have been shown to differentially influence the HPA axis activity between obese and

normal control subjects. Vicennati and colleagues demonstrated that women with visceral obese phenotype following a meal rich in carbohydrates responded with higher cortisol levels compared to subcutaneously obese or normal weight controls (122). On the other hand, the postprandial cortisol levels in the viscerally obese women in the same study were reduced significantly compared to the normal weight controls following a meal rich in fat and protein. These results indicate that HPA axis response is altered in obese subjects following meal ingestion, but more importantly, specific types of nutrient compositions. This sort of altered HPA axis activity is also observed in rodent models following either acute or chronic HF diet. Male rats fed HF diet for either 7 or 21 days had elevated plasma corticosterone levels with a trend of increase in plasma ACTH levels compared to the control animals (123). This increase in HPA axis indices was augmented in HF-fed rats when both groups were treated with acute restraint stress, suggesting that different dietary nutrients can induce alterations in the HPA axis activity at basal as well as at stressful state in HF-induced obese rats.

Can HPA axis perturbation cause obesity?

Although most evidence is from epidemiological and clinical studies that show a strong association between HPA axis dysregulation and abdominal obesity as shown above, studies by Shively and colleagues (124) had shown that dysfunctional HPA axis leads to development of obesity and other associated metabolic and cardiovascular disorders in non-human primates. In the studies, chronic physical and psychological stressful challenges exposed to *cynomolgus*

macaques monkeys for two years resulted in increased body weight and visceral fat deposition, insulin resistance, glucose intolerance, adrenal hypertrophy, enhanced cortisol response to ACTH stimulation, hypertriglyceridemia, and increased incidence of coronary atherosclerosis. These findings indicate that obesity and other associated disorders can be resulted from chronic maladaptation to environmental stressors, whether they are physical, emotional, infection, or nutrition-induced. This establishes a stronger association between neuroendocrine changes in the HPA axis and the central / abdominal obesity.

F. Metformin

Metformin is an oral biguanide insulin-sensitizing agent that has been most widely used to treat Type II diabetic patients due to its beneficial effects on hepatic glucose production, serum lipid profile, and body weight (125-128). Treatment with metfomin has been shown to reduce body weight in obese patients (129, 130), and this is supported by similar findings in obese animal studies (131-133). Metformin treatment showed anorectic effect and reduced food intake and body weight in Zucker male rats. Besides its well-known role in enhancing insulin sensitivity, recently metformin has been suggested to restore leptin sensitivity in HF-fed obese rats that are leptin resistant (134). Moreover, the same group has demonstrated similar anorectic and weight-reducing effects of metformin in Otsuka Long-Evans Tokushima Fatty (OLETF) rats that lack cholecystokinin (CCK) receptors. This indicates that metformin may mediate regulation of food intake and body weight by both leptin and insulin. However,

the recently proposed mechanism of metformin action needs to be further investigated, both in its selectivity of brain regions and even on better obese animal models that truly have a polygenetic propensity to develop obesity, just like humans.

G. Obese animal model (DIO & DR)

The DIO model in rodents is widely used to study development of obesity after high-fat (HF) diet exposure as the physiological and phenotypical alterations mimic those in obese humans. Unlike *fa/fa* rats in which obesity is inherited as an autosomal recessive gene, the bimodal pattern of inheritance of DIO and DR traits is deemed polygenic (135). In general, people in their daily lives consume high-fat, high-carbohydrate diets in westernized society. Even so, a group of people is prone to gain weight more easily than the other group, given the same diet (136). Another extreme group of people may not gain weight at all even with higher consumption of dietary fat. This distinction indicates the fundamental polygenic difference between DIO-prone and DR-prone people. Along the same line, selectively outbred male Spargue-Dawley rats provide a great model to study obesity in that it generates two phenotypically different types – hyperphagic and obese DIO animal group vs. relatively lean diet-resistant (DR) animal group (135). DIO and DR rats can be differentiated by the final body weight after chronic exposure to high-fat (HF) diet. The F_1 generation classifies males and females of the lowest quartile in weight measurement after HF exposure as DR group, whereas rats with the highest body weight are classified as DIO group

(135). The males and females within its phenotypical classification are mated, and the offspring of each phenotype are placed back to chow diet, mated within the group, and this goes for over 20 generations to finally produce the true phenotype with 100% penetrance (135). With these two substrains of different phenotypes, we can investigate the mechanisms involved in the pathogenesis of obesity, such as differential regulation of neuromodulators like catecholamines on various brain areas related to energy homeostasis on either chow or HF diet, and more globally speaking, the influence of maternal obesity on the neuroendocrine mechanisms of the offspring or any correlative central alterations to aging process.

H. Thesis Objective

Since neuroendocrine alterations of the HPA axis are known to occur in diet-induced obese (DIO) subjects, the studies described in the following chapters were designed to test the hypothesis that the DIO rats develop dysregulation of the HPA axis via leptin signal impairment in the brainstem noradrenergic neurons. The experiments were designed to test the following hypotheses: 1) Selectively bred DIO rats that have polygenetic propensity to become obese develop dissociation between brainstem NE and the HPA axis following chronic HF diet, 2) The dissociation in HF-fed DIO rats is associated with the leptin insensitivity at the level of brainstem noradrenergic neurons, and 3) Treatment with metformin will normalize the HPA axis regulation of chronically HF-fed DIO rats by restoring or at least partially correcting the leptin signal impairment in the noradrenergic neurons in the brainstem. A greater understanding of the mechanisms involved in the neuroendocrine dysregulation of the HPA axis in diet-induced obesity could lead to the development of alternative treatment or prevention of metabolic and associated cardiovascular disorders that stem from obesity.

Chapter 2. Materials and Methods

A. Animals

DIO and DR rats

Breeding pairs of DIO and DR rats were obtained from Charles River Laboratories, Inc., Wilimington, MA. Animals were bred in our colony and were weaned after 1 month. After weaning, the animals were fed regular chow diet (Teklad 8640 diet; 3.11 kcal/g, 5% fat; Harlan, Indianapolis, IN) until they were 9 weeks old. Animals were divided into groups and were fed regular rat chow or high fat (HF) diet *ad libitum* as described below, and housed on a 12:12-h light-dark schedule with the ambient temperature maintained at 23 ± 2°C. Animals were used in the experiments in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals,* and the protocol was approved by the Institutional Animal Care and Use Committee at MSU.

B. Treatments

Dietary treatment

DIO and DR groups were placed on regular chow diet or high-fat (HF) pellet diet. Chow diet contained 23% protein, 72% carbohydrate, and 5% calories as fat with an energy density of 3.11 kcal/g (Teklad 8640; Harlan, Indianapolis, IN). The HF diet contained 20% protein, 35% carbohydrate, and 45% calories as

fat with an energy density of 4.73 kcal/g (D12451; Research Diets Inc., New Brunswick, NJ). The treatment lasted 1, 6, or 7 weeks, depending on each experiment protocol. The initial body weight of animals was recorded. Food intake was measured on a daily or weekly basis until the end of treatment. At the end of the 1 or 6-week period, the rats were sacrificed by decapitation, and their brains were collected, frozen in dry ice, and stored at -70°C. Trunk blood was collected, the serum was separated, and stored at -20°C until RIA and ELISA analyses. Visceral fat pads including retroperitoneal, peri-renal, and epididymal fat were removed from carcasses and weighed.

Leptin treatment

One group of DIO and DR rats was injected with 500 μg of rat recombinant leptin (R&D systems, Minneapolis, MN) i.p. in 250 μl of saline. Leptin was prepared in citrate buffer (pH 4.5). Rats were sacrificed 5 h later and the trunk blood was collected. Brains were removed and frozen immediately in dry ice and stored at -70°C for processing as described below.

Metformin treatment

DIO animals received oral administration of metformin (Spectrum Chemicals & Laboratory Products, Inc.; New Brunswick, NJ) in either low or high dose dissolved in drinking water. On average, the animals in the low-dose groups (LD-Met) were given 60 mg/kg of BW per daily. The animals in the high-

dose groups (HD-Met) were given 300 mg/kg of BW daily. The metformin concentrations were readjusted to the body weight once a week.

C. Brain / brainstem microdissection

After sacrifice and brain removal, serial sections (300 μm thick) of the brain and brainstem were obtained using a cryostat maintained at -10°C. The sections were transferred to cover slips, which were placed on a cold stage set at -10°C. Palkovits microdissection was used to isolate the paraventricular nucleus (PVN) and median eminence (ME) from brain sections with the help of a stereotaxic atlas (137). Care was taken to include all subdivisions of the nucleus from multiple serial sections. The ME was stored at -70°C until analysis for CRH concentrations using ELISA. The PVN was stored in 0.1 M $HClO_4$ at -70°C and analyzed for norepinephrine (NE) concentrations using HPLC-EC. Brainstem areas A1, A2, and A6 were microdissected in the similar manner and stored at -70°C until analysis for pSTAT-3 and SOCS-3 protein expressions by western blot. Another set of microdissected brainstem tissues were subjected to detection of β-actin, TH, and ObRb mRNA gene expressions by quantitative Real Time – PCR. For this set of brainstems, cover slips and other apparatus were wiped with RNAseZap (Sigma-Aldrich Co., St. Louis, MO) prior to mounting sections to prevent degradation of RNA by potential RNAase.

D. HPLC-EC

The HPLC system consisted of a Shimadzu LC-10 AT/VP pump, a phase II, 5 μm ODS reverse-phase, C-18 column (Phenomenex, Torrance, CA), a glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN) placed inside a Shimadzu CTO-10 AT/VP column oven at 37°C, and a LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN) connected to a computer with the class VP chromatopac software (Shimadzu, Columbia, MD). The mobile phase was composed of monochloroacetic acid (14.14 g/L), sodium hydroxide (4.675 g/L), octanesulfonic acid disodium salt (0.3 g/L), ethylenediaminetetraacetic acid (0.25 g/L), acetonitrile (3.5%), and tetrahydrofuran (1.4%). The mobile phase was made in pyrogen-free water and then filtered and degassed through a Milli-Q purification system (Millipore, Bedford, MA, USA) and pumped at a flow rate of 1.8 ml/min. The range of the detector was 1.0 nA full scale, and the potential of the working electrode was 0.65 V. At the time of HPLC analysis, microdissected PVN tissue samples were thawed and homogenized in 150 μl of 0.1 M $HClO_4$ using a micro-ultrasonic cell disruptor (Kontes, Vineland, NJ) and centrifuged at 10,000 x *g* for 10 min. 120 μl of the supernatant was mixed with 30 μl of the internal standard (0.05 M dihydroxybenzylamine) and 125 μl of this mixture was injected into the HPLC system. The sensitivity of the system was < 1 pg.

E. Western blot

Microdissected brainstem tissues were homogenized in 45 μl bicin lysis buffer (pH 7.6) with addition of protease inhibitors sodium orthovanadate (NaOV) and Phenylmethanesulfonyl fluoride (PMSF). 10μl of loading buffer was added to the same amount of homogenized samples and mixed before incubation in water medium at 95°C for 5 minutes. The samples were then kept in ice until loading into the wells. Samples were separated by SDS polyacrylamide gel electrophoresis using precast gels and transferred to nitrocellulose membrane by Semi-dry blot apparatus. Membranes were blocked for 1 hour with Odyssey Blocking Buffer (Li-Cor, Lincoln, NE), washed with 1X TBS 4 times for 5 minutes each, and then cut in two for separate detection of proteins. Membranes were then incubated in pSTAT-3 or SOCS-3 rabbit polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution overnight at 4°C. Membranes were washed with 1X TBS 4 times for 5 minutes each, and then incubated in goat anti-rabbit polyclonal infra-red secondary antibody at 1:10000 dilution for 1 hour at room temperature (Rockland, Gilbertsville, PA). Membranes are washed 4 times with 1X TBS for 5 minutes each, and the pSTAT-3 and SOCS-3 bands were detected using Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE).

F. ELISA

Serum leptin levels were measured in duplicate using a commercial ELISA kit (TiterZyme Kits, Assay Design, Ann Arbor, MI). According to the manufacturer's specifications, the plates were read at 450 nm using an ELISA reader (ELx800, BioTek Instruments, Inc., Winooski, VT). The sensitivity of the kit was 46.7 pg/ml. CRF EIA kits (Phoenix Pharmaceuticals, Inc., Belmont, CA) were used to determine CRH protein levels in the ME of the hypothalamus. This competitive binding assay had a minimum sensitivity of 0.30 ng/ml. Protein concentrations in the tissue were measured and CRH levels were expressed as ng/μg protein.

G. Protein assay

Tissue homogenate samples (10 μl) were used in duplicate in the protein assay. Protein levels were determined using a micro BCA assay (Pierce, Rockford, IL). Absorbance of each sample was obtained at 562 nm using an ELX 800 microplate reader (Biotek Instruments, Winooski, VT). NE and CRH concentrations were expressed as pg/μg protein.

H. Quantitative RT-PCR

RNA extraction

The RNA was extracted from the brainstem punches using MELT Total Nucleic Acid Isolation System (Ambion Inc, Austin, TX) according to the manufacturer's protocol. Using the Multi-Enzymatic Liquefaction of Tissue (MELT) mix provided in the kit, the tissue was digested. After on-bead Turbo DNAse digestion (Ambion), the RNA was eluted in a volume of 500 µl. First strand cDNA was synthesized by reverse transcribing 250 ng of total RNA using Superscript III First strand synthesis Supermix for qRT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

qRT-PCR

The real-time quantitative PCR mix consisted of 1x Platinum SYBR Green PCR Masterr Mix-(Applied Biosystems, Foster city, CA) and 300 nM of forward and reverse primers. 2.5 µl (equivalent of 50ng of single-stranded cDNA) of the RT reaction was used for quantization of β-actin mRNA, TH mRNA and ObRb mRNA.

An 115-bp product for rat tyrosine hydroxylase (Gen Bank accession no. NM 012740) was amplified using the following primers: forward, 5'-CTACCAGCCTGTGTACTTTGTGTC-3'; reverse, 5'-CAGTGTGTACGGGTCAAACTTC-3' and 121-bp product for rat ObRb mRNA (Gen Bank accession no. D 84550) using the primer set: forward, 5'-

GAGAGGCTGCTGAAATCGTC-3'; reverse, 5'-CTCCTGAGCCATCGAGTCTC-3'. Similarly, a 146-bp product of rat ß-actin (GenBank accession no. NM 031144) was amplified using the following primer set: forward, 5'-ATCATGAAGTGTGACGTTGACAT-3'; reverse, 5'-ATGATCTTGATCTTCATGGTGCTA-3'. The reactions were performed in The Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) with the following settings: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 C for 15 sec, 60 °C for 60 sec, and 72 °C for 35 sec. At the end of amplification, a melting curve analysis was done by heating the PCR products to 65–95 °C and held for 15 sec at increments of 0.2 °C, and the fluorescence was detected to confirm the presence of a single amplification product. Each sample was run in duplicate to obtain average C_T values for TH mRNA, ObRb mRNA and ß-actin mRNA. For negative controls, No-RT controls were used as template in place of single-stranded cDNA in the real-time quantitative PCR. TH mRNA, ObRb mRNA quantity were expressed as a proportion of ß-actin mRNA quantity following the standard curve method for converting log-linear C_T values to RNA values. The relative amount of TH mRNA and ObRb mRNA in various brain stem areas were then compared.

I. Radioimmunoassay

A double antibody RIA for corticosterone was performed using a tracer and standards from Diagnostic Products Corp. (Los Angeles, CA) and indigenous

primary and secondary antibodies. The samples (50 µl) were assayed in duplicate as described previously. The sensitivity of the assay was 0.2 ng/ml.

J. Statistics

Changes in NE and CRH concentrations and serum hormone levels after different treatments were analyzed by ANOVA followed by post hoc Fisher's least significant difference (LSD) test. Weekly or daily caloric intake differences in DIO & DR animals after regular chow and HF diet exposure were analyzed by repeated measures ANOVA followed by post hoc Fisher's LSD test. Average caloric intake and feed efficiency, body weight (BW) at the end of treatment, BW gain/week during the treatment period, total white adipose tissue weight, and the fat/BW ratio between DIO and DR animals within each dietary treatment besides leptin injection group were analyzed by ANOVA followed by Fisher's LSD test. Type II regression analyses were performed with Prism 4 software to explore the relationships between serum leptin and HPA axis indices, namely, NE levels in the PVN, CRH concentrations in the ME and serum CORT. Leptin values are plotted on the x-axis due to the causal effect of leptin on NE and other HPA axis indices to test how well leptin levels can predict the levels of NE, CRH, and CORT. For the same rationale, NE values are plotted on the x-axis against CRH and CORT values. In addition, we tested whether the linear slopes of DIO and DR groups are significantly different from each other.

Chapter 3. Dysregulation of the HPA axis in diet-induced obesity (DIO)

A. Introduction

Regulation of the HPA axis, or stress axis, is critical for adapting to acute or chronic stressors. As an adaptive stress response, HPA axis activation leads to changes in overall body metabolism in an effort to utilize available energy resources to protect the organism against stressors. These include promotion of catabolic effects in the liver, muscle, and adiposue tissue, and inhibition of anabolic effects in growth and reproduction (138). Because of this, a careful and well-orchestrated regulation of the HPA axis is very important in maintaining a balance in the overall energy metabolism. Even a subtle HPA axis dysregulation can shift the energy balance and may contribute to development of obesity, as supported by many epidemiological, clinical, and empirical animal studies (3-12), as well as failure or retardation of growth and reproduction (139).

Neurotransmitters in the brain are known to regulate many centrally mediated functions such as neuroendocrine, sleep cycle, respiratory, energy homeostasis, and reproductive systems. Among a variety of these neurotransmitters, norepinephrine (NE) plays a significant role in stimulating the HPA axis. CRH neurons receive rich innervation from noradrenergic nuclei in the brainstem, and they are stimulated following direct injection of NE into the PVN (94, 95). Previous studies in our lab have shown that leptin is capable of decreasing the noradrenergic activity in the hypothalamus using different paradigms. Exogenous administration of leptin normalized the significant

increase of NE concentrations in the arcuate nucleus (ARC), PVN, and ventromedial hypothalamus (VMH) in streptozotocin-induced type I diabetes (19). In addition, leptin decreased the efflux of NE from the hypothalamus in an *in vitro* setting (108). Moreover, leptin was shown to decrease NE release in the PVN in a dose-dependent manner with a concurrent decrease in serum corticosterone in an *in vivo* rat model (140). More recently, increased serum leptin levels induced by Ad-36 viral infection were correlated with a reduction in NE concentrations in the PVN of Wistar male rats (109). These studies indicate that leptin is capable of decreasing noradrenergic activity that targets PVN CRH neurons in the hypothalamus. This in turn could play a major role in causing a suppression of the stress axis.

Neuroendocrine changes have been observed in genetically obese rodent models, however, the status of HPA axis function and noradrenergic activity in the PVN of polygenetically susceptible DIO rats has not been investigated before. Hence, the aim of this study was to characterize the neuroendocrine abnormality in the HPA axis and central noradrenergic function, and investigate potential mechanisms that contribute to this phenomenon. Therefore, I hypothesized that after prolonged feeding of HF diet, DIO rats will show dissociation between the stress axis and brainstem NE, possibly by decreased functional leptin receptor (ObRb) gene expression in the brainstem noradrenergic neurons, while DR rats will show normal functions. Also, the gene expressions of tyrosine hydroxylase (TH), the rate-limiting enzyme for production of NE, may be upregulated in the noaradrenergic neurons associated with changes in ObRb gene expressions. If

this hypothesis is correct, then: A) the DIO animals will have increased weight gain and serum leptin levels, B) DIO animals will have increased noradrenergic functions in terms of NE concentrations in the PVN of the hypothalamus, C) they will elicit a type of dissociation between the HPA axis and NE, as measured by CRH in the ME and serum corticosterone, and D) this could be possibly mediated by decreased ObRb or elevated TH gene expressions in the noradrenergic neurons. This will provide an idea about the neuroendocrine changes in the HPA axis in DIO animals compared to DR animals, and the critical relationship between serum leptin, central noradrenergic activity, and the HPA axis.

B. Experimental Design

To determine if dissociation between the HPA axis and the brainstem noradrenergic function occur in DIO rats, 9 week-old DIO and DR male rats were treated with 45% HF diet (D12451; Research Diets Inc., New Brunswick, NJ) or regular laboratory chow diet for duration of 6 weeks. The following groups were used (n=6-8/group):

Group 1: DIO-Chow

Group 2: DR-Chow

Group 3: DIO-HF

Group 4: DR-HF

Initital and final body weight were recorded. At the end of treatment (i.e. when the animals were 15 weeks old), all groups were sacrificed by decapitation, the trunk blood was collected, and brains were removed and snap-frozen in liquid nitrogen. Brains were sectioned in 300 μm, and PVN of the hypothalamus was microdissected. Microdissected tissues were homogenized and analyzed for NE concentrations via HPLC-EC. ME was also homogenized in lysis buffer. Both serum leptin and ME CRH protein levels were analyzed by commercially available ELISA kits. To test if the increased noradrenergic function is associated with leptin, brainstems were also sectioned in 300 μm, and noradrenergic areas A1, A2, and A6 were microdissected. These samples were subjected to quantitative RT-PCR to measure TH, β-actin, and ObRb gene expressions. Serum corticosterone levels were analyzed by Radioimmunoassay.

C. Results

Body weight gain

Body weight gain (mean±SE; g) in DIO and DR rats fed with regular chow or HF diet is shown in Fig. 3-1. Body weight gain was calculated by subtracting the intitial body weight from the final body weight in grams. All groups were significantly different from each other ($p<0.05$). When DIO and DR rats were given chow for six weeks, DIO rats had higher body weight gain compared to the chow-fed DR counterparts (165.5±4.8 vs. 96.6±5.9; $p<0.05$). A similar significant increase was seen for other DIO and DR groups following HF diet for 6 weeks (185.5±8.4 vs. 135.1±4.8; $p<0.05$). Moreover, HF-fed animals showed a significant increase in their body weight gain compared to chow-fed animals ($p<0.05$).

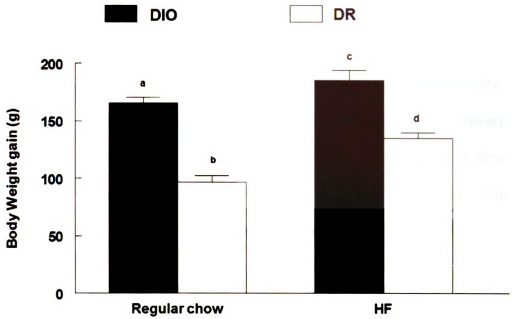


Fig. 3-1. Body weight gain (g) of DIO and DR rats after 6 weeks of chow or HF diet (n=6-8 per group). Weight gain is calculated by subtracting the initital body weight from the final body weight. Note the significant difference between all groups, with DIO rats having increased weight gain compared to DR rats. Also note the signficiant increase between HF-fed and chow-fed animals. Alphabetical notations indicate significant differences between groups $p<0.05$.

Serum leptin levels

Leptin levels (Mean±SE; ng/ml) of DIO and DR rats following 6 weeks of chow or HF diet are shown in Fig. 3-2. There was no difference in serum leptin levels between chow-fed DIO and DR animals (3.0±0.2 vs. 3.4±0.4 respectively). However, HF-fed DIO animals had significantly higher leptin levels compared to HF-fed DR animals (8.9±0.7 vs. 3.7±0.4 respectively; $p<0.05$). Comparison between dietary treatments revealed that there was no stastistical difference between chow-fed DR and HF-fed DR animals. However, HF-fed DIO animals had significantly elevated leptin levels compared to chow-fed DIO animals ($p<0.05$).

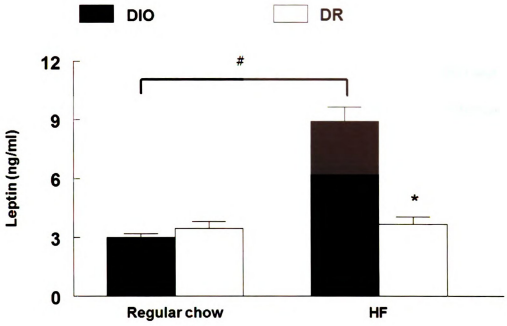


Fig. 3-2. Serum leptin levels following chow or HF diet for 6 weeks in DIO and DR animals (n=6-8 per group). No difference was seen in chow-fed animals, but HF-fed DIO animals increased their serum leptin levels compared to DR counterparts. Also note the significant increase in leptin levels of HF-fed DIO rats compared to chow-fed DIO rats. *indicates significant differences ($p<0.05$) compared to HF-fed DIO animals; # indicates significant difference ($p<0.05$) between HF-fed DIO and chow-fed DIO rats.

NE concentrations in the PVN of the hypothalamus

PVN NE concentrations (Mean±SE; pg/μg protein) of DIO and DR rats following 6 weeks of chow or HF diet are shown in Fig. 3-3. There was no difference whether both phenotypes were fed chow (17.4±2.3 and 18.9±2.7 in DIO and DR respectively) or HF diet (81.2±15.9 and 69.7±22.2 in DIO and DR groups respectively). However, HF-fed animals had higher NE concentrations in the PVN when compared to chow-fed animals ($p<0.05$).

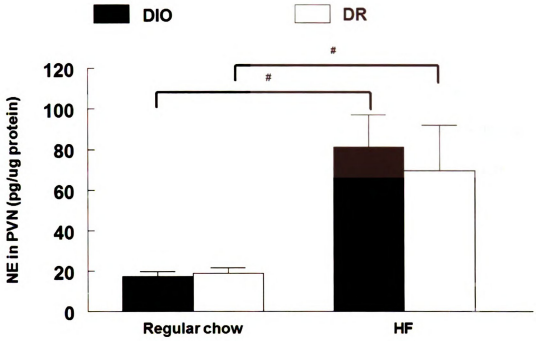


Fig. 3-3. NE concentrations in the PVN of DIO and DR rats after 6 weeks of chow or HF diet (n=6-8 per group). It was analyzed by ANOVA followed by post-hoc LSD test. The effect of diet was apparent regardless of phenotypes. Difference in phenotype, however, was not significant. # indicates significant ($p<0.05$) differences between HF-fed and chow-fed animals.

CRH protein levels in ME of the hypothalamus

CRH protein concentrations in DIO and DR rats after 6 weeks of chow or HF diet are shown in Fig. 3-4. There was no statistical difference between chow-fed DIO and DR rats (2.0±0.4 vs. 1.4±0.3 respectively). However, there was a statistically significant increase in CRH levels of HF-fed DR rats (4.8±0.5) compared to HF-fed DIO rats (2.7±1.0; $p<0.05$). CRH levels in HF-fed and chow-fed DIO animals were not significantly different from each other. However, HF-fed DR rats had higher CRH levels compared to chow-fed DR rats ($p<0.05$).

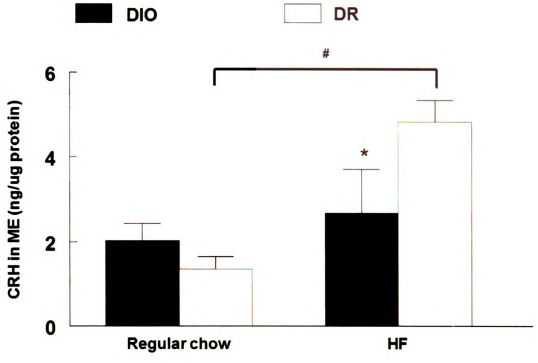


Fig. 3-4. CRH protein levels in ME of the hypothalamus after 6 weeks of chow or HF diet exposure on DIO or DR rats (n=6-8 per group). HF-fed DR animals had significantly increased CRH levels compared to HF-fed DIO animals. Also note the significant increase in CRH levels in HF-fed DR rats compared to chow-fed DR rats. * indicates significant difference ($p<0.05$) compared to HF-fed DR group; # indicates significant ($p<0.05$) between HF and chow-fed DR rats.

Serum corticosterone levels

Serum corticosterone after 6 weeks of HF or chow diet in DIO and DR rats is shown in Fig. 3-5. In line with ME CRH data, there was no difference between DIO and DR groups after chow diet (64.2±9.8 and 104.3±21.0 in DIO and DR respectively). HF diet exposure, however, significantly increased the serum corticosterone levels in the DR group compared to the DIO group (351.3±80.4 vs. 131.7±17.8 in DR and DIO groups respectively; $p<0.05$). HF-fed DR group also had significantly higher levels of serum corticosterone compared to chow-fed DR group ($p<0.05$). This difference was not observed between HF and chow-fed DIO animals.

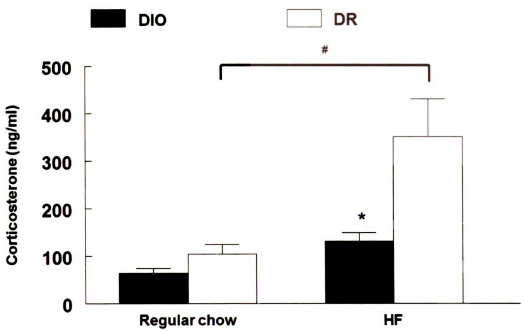


Fig. 3-5. Serum corticosterone levels in DIO and DR rats after 6 weeks of chow or HF diet exposure (n=6-8 per group). HF-fed DR group had higher levels of serum corticosterone compared to HF-fed DIO rats and chow-fed DR rats. *$p<0.05$ compared to HF-fed DR group; # indicates significant ($p<0.05$) difference between HF and chow-fed DR groups.

Quantitative RT-PCR analysis of TH gene expression in the brainstem

TH mRNA expression in A1: TH mRNA values are expressed as the ratio of TH mRNA:β-actin mRNA in A1 (VLM) noradrenergic region in the brainstesm (Fig. 3-6A). HF-fed DIO group had significantly higher expression of TH mRNA compared to chow-fed DIO group (0.16±0.03 vs. 0.09±0.02 in HF fed vs. chow fed groups respectively; $p<0.05$). Also, HF-fed DIO group had significantly higher TH expression compared to either chow-fed DR (0.09±0.02) or HF-fed DR groups (0.10±0.02; $p<0.05$). There was no statistical difference between chow-fed DIO, chow-fed DR, and HF-fed DR groups.

TH mRNA expression in A2: Gene expression of TH in A2 (NTS) noradrenergic region in the brainstem is shown in Fig. 3-6B. There were no effects of dietary treatment or phenotype on A2 TH gene expression. There was no significant difference between the groups, as analyzed by post-hoc LSD test following ANOVA.

TH mRNA expression in A6: TH gene expression in the A6 (LC) noradrenergic region in the brainstem is shown in Fig. 3-6C. As in A2, there was no significant difference between the groups. DIO animals tended to have higher TH mRNA expression compared to DR animals, but it was not statistically significant.

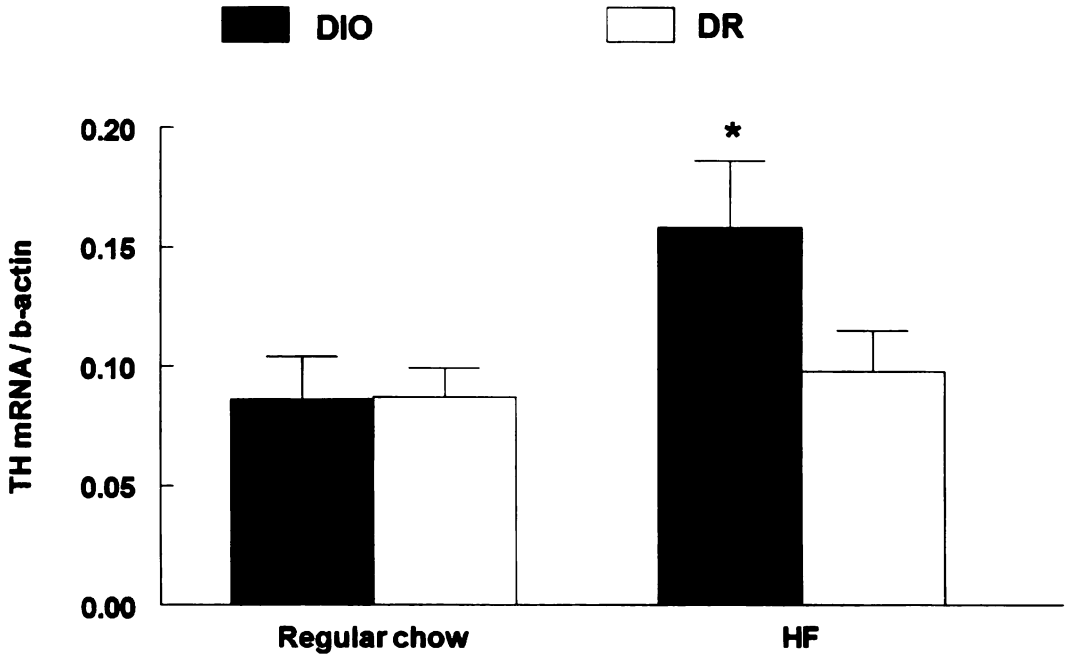


Fig. 3-6A: TH mRNA expression in the A1 noradrenergic region in the brainstem (n=6-8 per group). The values were normalized to β-actin values. Note the significant increase of the TH expression in HF-fed DIO group. *$p<0.05$ compared to other groups.

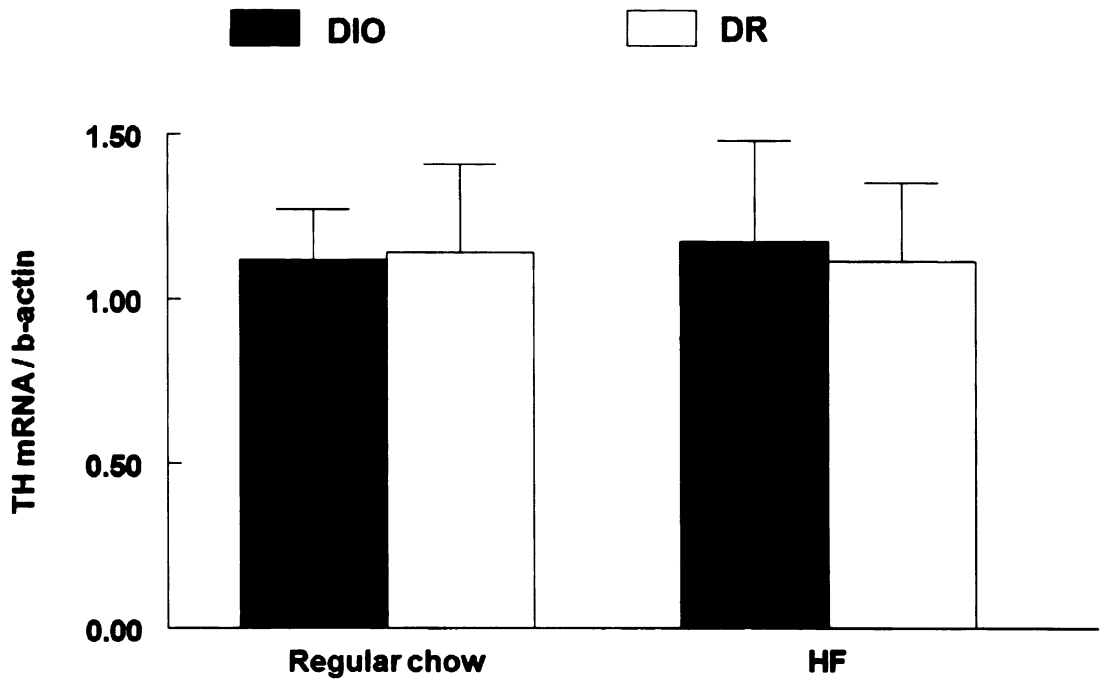


Fig. 3-6B: TH mRNA expression in the A2 noradrenergic region in the brainstem (n=6-8 per group). The values were normalized to β-actin values. Note no significant difference between groups.

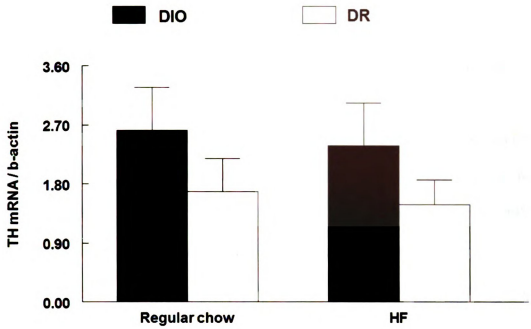


Fig. 3-6C: TH mRNA expression in A6 noradrenergic region in the brainstem (n=6-8 per group). The values were normalized to β-actin values. Note: Even though DIO groups tended to have higher TH gene expression compared to DR groups, the difference was not statistically significant.

Quantitative RT-PCR analysis of ObRb gene expression in the brainstem

ObRb mRNA expression in A1: Gene expression of the long-form of the functional leptin receptor (ObRb) in A1 (VLM) brainstem noradrenergic region is shown in Fig. 3-7A. No statistical difference was observed, and there was no difference between any of the groups.

ObRb mRNA expression in A2: ObRb gene expression in A2 (NTS) noradrenergic region in the brainstem is shown in Fig. 3-7B. As in A1 region, no intergroup difference was found by ANOVA. There was an increase in ObRb expression in the HF-fed DIO group (1.6 ± 0.3) compared to the chow-fed DIO group (1.3 ± 0.3), HF-fed DR group (1.3 ± 0.1), and the chow-fed DR group (1.3 ± 0.2), but the difference was not statistically significant.

ObRb mRNA expression in A6: Fig. 3-7C shows quantitative values of ObRb gene expression indicated as a ratio of ObRb mRNA to β-actin. There was no stastistical difference between any of the groups.

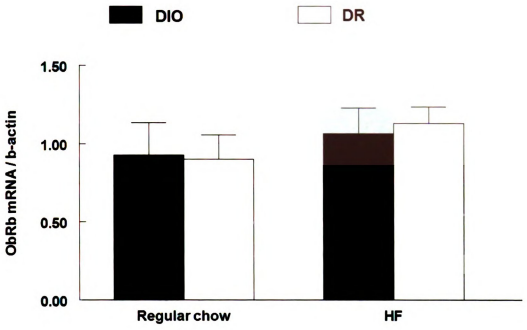


Fig. 3-7A: Long-form leptin receptor (ObRb) mRNA expression in the A1 noradrenergic region in the brainstem (n=6-8 per group). The values were normalized to β-actin values. Note no significant difference between any of the groups.

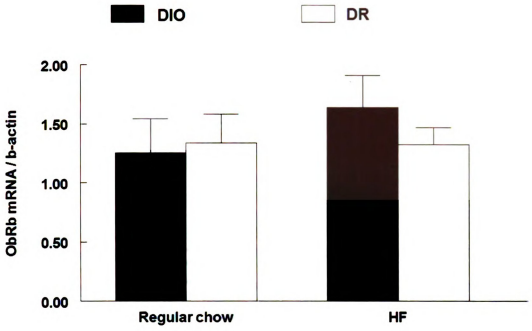


Fig. 3-7B: Long-form leptin receptor (ObRb) mRNA expression in A2 noradrenergic region in the brainstem (n=6-8 per group). The values were normalized to β-actin values. There was an increase in the gene expression in HF-fed DIO group compared to rest of the groups, but there was no statistical difference.

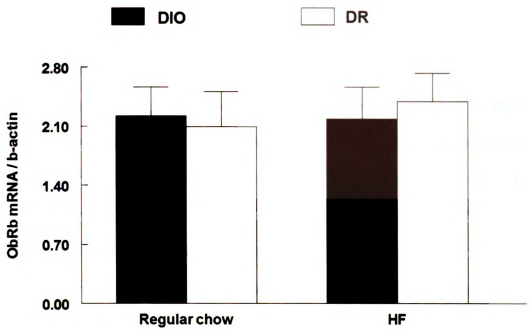


Fig. 3-7C: Long-form leptin receptor (ObRb) mRNA expression in the A6 noradrenergic region in the brainstem (n=6-8 per group). The values were normalized to β-actin values. As in A1 and A2 regions, no statistical difference between any of the groups was found.

D. Discussion

The purpose of this study was to investigate the changes in stress axis activity in DIO and DR rats after chronic exposure to a HF diet, and the possible mechanisms that are involved in precipitating these changes. DIO and DR rats, through polygenic mode inheritance (135), demonstrate a myriad of diverging physiological characteristics. Although both DIO and DR rats gained weight after being placed on a HF diet, only DIO animals had elevated leptin levels after a HF diet. However, DR animals had an activated stress axis in response to the HF diet as indicated by an increase in NE levels in the PVN, CRH levels in the ME, and serum corticosterone. DIO rats, on the other hand, had elevated levels of NE in the PVN, possibly due to the increased TH gene expression in A1 brainstem noradrenergic neurons, but neither CRH nor serum corticosterone levels were increased after a HF diet, indicating possible lack of the leptin sensitivity and dysregulation at multiple levels of the stress axis.

In the present study, I observed a significant increase in body weight gain in DIO rats compared to DR rats after they were placed on a HF diet for 6 weeks (Fig. 3-1). This is in accordance with other studies that showed marked differences in weight gain in DIO and DR rats even after one week, or two weeks of HF diet (135, 141). This finding is of importance particularly because the animals were housed and bred by my laboratory after the purchase of the first breeding pairs. Unlike the gene knockout obese models, one of the concerns was whether the animals would behave the way they are supposed to and maintain their polygenic differences and the propensity to become obese, with and without

HF diet exposure. This can be reflected by the resulting phenotypes or gross parameters such as body weight change, food intake, or amount of visceral fat pads. After breeding several generations and testing for the body weight gain differences between DIO and DR animals following chronic chow or HF diet treatments, I have confirmed that the animals conserved their polygenic inheritance of susceptibility through generations, and that significant genetic drift has not occurred (Fig. 3-1).

Leptin, a hormone produced from adipocytes in white adipose tissue, plays a major role on regulation of energy balance (13). It signals nutritional status to key regulatory centers of the hypothalamus and controls long-term body weight regulation and food intake (142, 143). A recent study by Schwartz et al. (144) suggests that forebrain leptin signaling is also actively involved in short-term regulation of food intake, or induction of satiety, by regulating hindbrain responses to peripheral satiety signals. However, subjects that are DIO-prone gain a substantial body weight in spite of high circulating leptin levels, indicative of leptin resistance in the brain, specifically at the level of the hypothalamus (73, 145-147). Our findings on serum leptin agree with other studies that have observed an increase in leptin levels in DIO rats, but not in DR rats after exposure to a HF diet (141, 148). In general, serum leptin levels correlate well with the amount of fat depots in the body (149, 150). The stable serum leptin levels in spite of a significant body weight gain after HF diet exposure in DR rats suggests that there could be differences in body fat deposition or inherent differences between DIO and DR rats. In light of this, the stable serum leptin levels can be

interpreted as a defense mechanism toward metabolic challenges under HF diet. DIO rats, on the other hand, exhibit hyperleptinemia which by itself is known to disrupt leptin signaling in the hypothalamus (72). The ability of DR rats to regulate the secretion of leptin into the circulation in spite of increased weight gain, and most likely adipose mass, may play a critical role in maintaining proper leptin signaling.

Besides regulation of energy homeostasis, leptin is also involved in the modulation of the hypothalamo-pituitary-adrenal (HPA) axis (14-17). Since 1997, a substantial body of evidence suggests that leptin suppresses the stress axis (14-17, 106, 151). More recent studies indicate that this may be mediated via central noradrenergic systems (19, 107). Noradrenergic neurons in the brainstem innervate the PVN in the hypothalamus which contains a large number of CRH neurons. Upon NE action, CRH neurons are stimulated, resulting in activation of the stress axis (152). Central and systemic administration of leptin can decrease NE concentrations in the PVN and this is accompanied by a significant decrease in corticosterone (107). Moreover, increases in endogenous serum leptin levels were correlated with a significant decrease in NE concentrations in the PVN along with reductions in serum corticosterone in a virus-induced obesity model (153). However, in the present study we observed exactly the opposite; markedly increased serum leptin levels in DIO rats after HF diet failed to suppress NE levels in the PVN, and the hyperleptinemia increased NE concentrations in the PVN by at least four-fold (Fig. 3-3). This indicates that DIO rats may become insensitive to circulating leptin upon exposure to a HF diet.

With supporting evidence demonstrating a rather quick response of noradrenergic activity to leptin (140), the suppressive action of leptin is most probably mediated in a direct manner. Insensitivity of noradrenergic neurons to leptin observed in this study may be localized either at terminals in the PVN or at the level of the cell bodies in the brainstem. This is very important since brainstem consists of many areas that are related to food intake and body weight regulation, including ventrolateral medulla (A1), area postrema, nucleus tractus solitarius (A2), locus ceoruleus (A6), and parabrachial nucleus (154), thus the interaction with metabolic signals like leptin is likely to generate important physiological responses. It is possible that leptin can directly downregulate noradrenergic activity via acting on functional leptin receptors in noradrenergic neurons in A1, A2, and A6 regions. In fact, Hosoi et al. (57) have shown that noradrenergic fibers arising from the brainstem are rich in functional leptin receptors. Therefore, a logical way of exploring the mechanism of the noradrenergic insensitivity in HF-fed DIO rats is that these animals could have reduced production of the functional leptin receptors (ObRb) in the brainstem noradrenergic neurons. If less number of receptors is generated, there would be less binding sites for leptin to elicit its effect, which in turn would translate into diminished inhibitory effect of leptin on the noradrenergic activity.

What is observed in the gene expression analysis in the current study is very interesting, because there were no differences between chow-fed and HF-fed animals in the gene expression of the ObRb (Fig. 3-7). No difference was observed between different phenotypes as well. However, the gene expression of

tyrosine hydroxylase (TH), the rate-limiting enzyme for the generation of dopamine and norepinephrine (NE), was significantly upregulated in A1 noradrenergic region of HF-fed DIO rats compared to the chow-fed DIO rats (Fig. 3-6). This reveals two unprecedented findings and interpretations; first, lack of change in ObRb gene expression in noradrenergic regions between chow and HF-fed DIO rats suggests that the insensitivity to leptin is probably not a result of reduced functional leptin receptors. This would leave two other options that can lead to the observed leptin insensitivity: defective leptin receptor signaling, and impairment of leptin transport. The latter is not a likely mechanism however, especially in this polygenic rat model, because defective transport was indicated only after the animals reached old age (73). On the other hand, impaired leptin signaling has been thought to be the primary reason for leptin insensitivity in various obese animal models (28), and stands as a likely candidate mechanism for the observed leptin insensitivity in noradrenergic neurons. One of leptin's major downstream signal pathways involves regulation of phosphorylated signal transducer and activator of transcription-3 (pSTAT-3) and the negative inhibitor suppressor of cytokine signaling-3 (SOCS-3). It is possible that leptin insensitivity observed in DIO rats could involve one or both of these molecules. In support of this, TH mRNA expression was markedly upregulated in A1 noradrenergic neurons in HF-fed DIO rats. Secondly, considering the ability of leptin to regulate both TH synthesis and its phosphorylation state (155), it is quite possible that the defective leptin signaling in noradrenergic neurons can bring about changes in the production of TH at the transcription level and result in loss

of leptin inhibition, that will lead to increased noradrenergic activity as shown by elevated NE concentrations in the PVN in the current study. Leptin signaling in these rats will be investigated in the following chapter.

Besides leptin insensitivity, the present study indicates that there is dysregulation of the stress axis in DIO rats after a HF diet, but not in DR rats (Fig. 3-3, 3-4, 3-5). In HF-fed DIO rats, even though NE levels in the PVN were significantly increased, both CRH levels in the ME and serum corticosterone concentrations were not different from chow-fed DIO rats. NE is a potent CRH stimulator and the role of NE in stress axis activation has been well documented (94, 95). The clear elevation of NE levels in the PVN but lack of any change in CRH and serum corticosterone observed in the present study suggest dysregulation of the stress axis. This is supported by other studies in which glucose-induced sympathetic activation is associated with failure of CRH neuronal activation in the PVN in DIO rats (156, 157). Taken together, these studies support our findings of stress axis dysregulation in DIO rats. This could probably contribute to the development of obesity in these rats (118).

In contrast to DIO rats on the HF diet, stress axis functioning was unaffected in DR rats after HF diet exposure. However, it is not clear why NE levels were elevated in these animals in spite of the stable serum leptin levels. The increased stress axis activity after HF diet exposure indicates that these animals perceive the HF diet as a stressor. Other mediators as a consequence of the diet could have activated noradrenergic neurons, causing stimulation of the stress axis. This warrants further investigation.

Of course, I cannot rule out the possibility that leptin can bring about the suppressive effects by acting directly at the level of adrenal gland. Indeed, there is evidence showing presence of both short and long forms of leptin receptors in rat adrenocortical cells, and that leptin can directly affect the growth of cells as well as modulate the glucocorticoid secretion (158-160). Also, there is evidence suggesting that leptin can directly inhibit glucocorticoid secretion from adrenal gland (161, 162). However, in light of studies suggesting that both acute and chronic systemic administration of leptin can suppress CRH secretion from PVN of the hypothalamus, ACTH from anterior pituitary, and reduce glucocorticoid secretion from adrenal gland (15, 19, 106-108, 151), it is very possible that the suppression of the glucocorticoid output by leptin is most likely mediated centrally.

In summary, the results from this study indicate that the increase in serum leptin levels in HF-fed DIO rats fail to suppress noradrenergic activity, indicating possible leptin resistance in noradrenergic neurons. No change in ObRb, but increased TH mRNA expression in A1 noradrenergic neurons in HF-DIO rats only support the possibility of leptin signal impairment in these rats. This lack of sensitivity to leptin and increased noradrenergic activity in the PVN are associated with dysregulation of the stress axis in DIO rats. These noradrenergic and HPA axis abnormalities may be responsible for development and/or promotion of obesity. Further studies are needed to understand the integrity of leptin signaling in the brainstem after chronic exposure to HF diet in this animal model.

E. Summary

The experiment described in this chapter tested the hypothesis that there is a neuroendocrine dissociation between the HPA axis and the brainstem noradrenergic function in HF-fed DIO rats, possibly mediated through decreased long-form leptin receptor gene expressions in the noradrenergic neurons. I have found that the prolonged HF diet exposure resulted in increased noradrenergic activity, but no change in the status of the HPA axis in DIO rats, indicating dysregulation of the HPA axis. Also, the increased noradrenergic activity in HF-fed DIO rats in spite of the hyperleptinemic state suggests possible lack of leptin signaling due to downregulation of functional leptin receptors (ObRb). Gene expression analysis by quantitative RT-PCR confirmed that there is indeed an increase in TH mRNA expression in A1 region of HF-fed DIO rats, which may explain the increased noradrenergic activity in the PVN of the hypothalamus. On the other hand, no difference in ObRb expression was found in any of the noradrenergic regions, suggesting that the leptin insensitivity observed in brainstem noradrenergic neurons are not attributed to downregulation of the receptors. This indicates that other mechanisms such as leptin signal impairment in the brainstem noradrenergic neurons may contribute to the overall disinhibition/activation of the noradrenergic function. Taken together, HF-fed DIO rats have uncoupling between the HPA axis and the brainstem noradrenergic activity in the PVN, for which post-receptor leptin signal impairment is a likely mechanism.

Chapter 4. Responsiveness of the HPA axis to leptin is impaired in diet-induced obese (DIO) rats

A. Introduction

Diet-induced obesity (DIO), possibly the most common form of obesity, is a serious health hazard that is characterized by excessive caloric intake and fat accumulation. One of the endogenous factors that regulates food intake and body weight is leptin (26). Leptin is a hormone that is predominantly secreted by white adipose tissue. It increases with feeding and adiposity, and acts on hypothalamic centers to decrease food intake and increase energy expenditure, thereby controlling body weight (13, 27).

Changes in the neuroendocrine system are one of the pathological hallmarks of obesity (2). In particular, basal stress axis activity and response to various stressful stimuli are altered in obese subjects, suggesting a possible dysregulation of the stress axis (3-12). The stress axis, or the hypothalamo-pituitary-adrenal (HPA) axis, comprises corticotropin-releasing hormone (CRH) neurons located in the paraventricular nucleus (PVN) in the hypothalamus, corticotrophs in the anterior pituitary that secrete adrenocorticotropic hormone (ACTH), and the adrenal cortex that secretes corticosterone (CORT) (75). Norepinephrine (NE) serves as an important stimulator of CRH neurons, and the PVN receives strong noradrenergic innervation from the brainstem (92, 163, 164). On the other hand, leptin is known to inhibit the HPA axis, and this effect is believed to be mediated through reductions in hypothalamic NE (15, 19, 106-108,

151). This relationship between leptin, NE, and the HPA axis may be altered in hyperleptinemic obese patients that manifest abnormal HPA axis activity.

A number of epidemiological and empirical studies demonstrate a strong correlation between HPA dysregulation and abdominal obesity, however, it is far from clear where and how this dysregulation occurs (2). This is of importance because HPA dysregulation is also associated with insulin resistance, indicating that it may be responsible for the development of a number of metabolic and cardiovascular disorders. Thus, identifying the underlying hormonal alterations and possible central noradrenergic imbalance is critical for understanding the mechanisms that lead to development of obesity and associated disorders.

As supported by other clinical and animal obesity models, in the previous chapter, I have validated a type of dysregulation of the HPA axis in polygenically susceptible DIO animals after chronic HF diet exposure. Further, elevated noradrenergic functions induced by possible leptin insensitivity in the brainstem noradrenergic neurons have been witnessed. However, currently two questions remain unanswered: Do these DIO animals have intact noradrenergic tone and proper regulation of the HPA axis prior to HF diet exposure? What is the mechanism by which these central anomalies occur after HF diet exposure?

A better characterization of the HPA axis perturation and noradrenergic dysfunction in detail is needed. Moreover, investigating the leptin signal impairment as one of the underlying mechanisms is necessary before implementing any therapeutic approach. To address these concerns, exogenous leptin was utilized to observe the responsiveness of noradrenergic neurons as well

as the HPA axis. I hypothesized that the responsiveness of the HPA axis and the central noradrenergic tone would remain intact in leptin-injected chow-fed DIO rats. Acute exposure to HF diet will be added to dissect the HPA axis regulation further and test if the axis is intact. I hypothesized that chronic HF-fed DIO rats would show dissociation between the brainstem noradrenergic system and HPA axis in association to leptin signal impairment in brainstem noradrenergic neurons. If these hypotheses are correct, then: A) the noradrenergic activity in the PVN and the HPA axis activity would decrease following injection of an exogenous leptin, B) there will be dissociations between leptin, NE, and the HPA axis in HF-fed DIO rats, C) pSTAT-3, an important leptin signaling molecule, will be decreased after chronic HF diet, and D) SOCS-3, a critical negative feedback inhibitor of leptin signaling, will stay the same, or be increased after chronic HF diet.

B. Experimental Design

The study described in this chapter was designed to investigate the responsiveness of noradrenergic neurons and the HPA axis to exogenous leptin, and to further look into the possibility of leptin resistance in the brainstem noradrenergic neurons after HF diet exposure. To test this, 9 week-old adult male DIO and DR rats were divided randomly into groups of 6-8 animals each (4 DIO and 4 DR groups; total 8 groups). One chow-fed DIO and one DR group were injected with with 500 μg of rat recombinant leptin (R&D systems, Minneapolis, MN) in 250 μl of saline. Rats were sacrificed 5 hours later and the trunk blood was collected. Brains were removed and frozen immediately in dry ice and stored at -70°C for processing as described below. The rest of the groups were placed on regular chow diet for six weeks or HF pellet diet for duration of either 1 week or 6 weeks. The animal groups used in this study are as follows:

Group 1: DIO- Chow with leptin	Group 5: DIO-HF 1 week
Group 2: DR-Chow with leptin	Group 6: DR-HF 1 week
Group 3: DIO-Chow	Group 7: DIO- HF 6 weeks
Group 4: DR-Chow	Group 8: DR-HF 6 weeks

The HF diet contained 20% protein, 35% carbohydrate, and 45% calories as fat with an energy density of 4.73 kcal/g (D12451; Research Diets Inc., New Brunswick, NJ). The initial body weight of animals was recorded. Food intake was measured on a daily or weekly basis until the end of treatment. At the end of

the 1 or 6-week period, the rats were sacrificed, and their brains were collected, frozen in dry ice, and stored at -70°C. Trunk blood was collected, the serum was separated, and stored at -20°C. The abdominal fat pads were removed from the carcass and weighed. Brains were sectioned in 300 μm, and the PVN of the hypothalamus was microdissected. The PVN tissues were homogenized and analyzed for NE concentrations via HPLC-EC. ME was also microdissected and homogenized in lysis buffer. Both serum leptin and ME CRH protein levels were analyzed by commercially available ELISA kits. To test if there is leptin signal impairment in noradrenergic neurons, brainstems were also sectioned in 300 μm, and noradrenergic areas A1, A2, and A6 were microdissected. These samples homogenized in lysis buffer and subjected to western blot to detect pSTAT-3 and SOCS-3 protein expressions. Serum corticosterone levels were analyzed by Radioimmunoassay. To portray the neuroendocrine perturbations in DIO animals, earlier data of Sprague-Dawley rats from Dr. Kimberly A. Clark's published article in 2006 were used to do Type II regression analyses to explore the relationships between leptin and HPA axis indices including NE, CRH, and corticosterone (CORT). Similar analyses of DIO and DR animals were also conducted for comparisons between each other and from the Sprague-Dawley rats.

C. Results

Final body weight (BW, mean±SE; g), BW gain/week (mean±SE; g), total white adipose tissue weight (AW, mean±SE; g) and AW to BW percent ratio in DIO and DR rats are shown in Table 4-1. The final body weight of chow-fed DIO rats (391.9±7.7) was significantly higher than that of chow-fed DR rats (310.0±5.0; $p<0.0001$). The same was true for DIO and DR animals exposed to either 1 week (403.0±2.7 vs. 315.6±3.6) or 6 weeks of HF (525.9±13.3 vs. 421.0±9.1; $p<0.0001$). HF-fed phenotypes for 6 weeks had significantly higher BW compared to the other groups ($p<0.05$).

BW gain

BW gain is expressed per week to eliminate confusion between different duration of treatments. Even with chow diet, DIO rats gained significantly more body weight (mean±SE; g) per week (21.1±0.6) than DR rats (15.8±0.7; $p<0.001$). 1 week of HF exposure did not produce any significant change in the BW gain between the two phenotypes ($p<0.08$). However, prolonging the duration of HF diet treatment to 6 weeks significantly increased the BW gain in DIO (35.9±1.3) compared to DR animals (29.7±1.2; $p<0.05$). Chow-fed animals had significantly decreased BW gain compared to animals treated with 1 week or 6 weeks of HF diet ($p<0.05$).

Total adipose tissue weight

Upon sacrifice, visceral fat depots such as perirenal, epididymal and retroperitoneal fat pads were removed and weighed as a whole for each animal.

Chow-fed DIO animals had more adipose weight (AW; mean±SE; g; 13.5±1.1) compared to DR animals (5.2±0.2; $p<0.0001$). Exposure to HF diet resulted in significantly higher AW in DIO rats either after 1 week (10.5±0.6 vs. 6.4±0.3) or 6 weeks of HF exposure (22.0±1.6 vs. 10.2±0.7; $p<0.0001$). Also, animals treated with 6 weeks of HF diet had significantly higher adipose weight compared to animals under other treatments ($p<0.05$).

AW to BW ratio

In order to correct for the body weight differences, AW was normalized to the body weight in percentage (Mean±SE; %), and this was compared between DIO and DR rats within each treatment. Similar to AW, there was a statistically significant difference in AW to BW ratio between chow-fed DIO and DR rats (3.4±0.2 and 1.7±0.1 respectively; $p<0.0001$). The same was true for HF-fed animals whether they were on 1 week (2.6±0.2 vs. 2.0±0.1) or 6 weeks of HF diet (4.2±0.3 vs. 2.4±0.2; $p<0.001$). Chow-fed DIO group had significantly higher AW to BW ratio compared to 1 week HF DIO goup, but this was significantly less compared to 6 weeks DIO HF group ($p<0.05$). On the other hand, 6 weeks HF-fed DR group had significantly higher AW to BW ratio compared to the chow-fed DR group ($p<0.05$).

Treatment	Parameters	*DIO*	*DR*
Chow	Final body weight (BW; g)	391.9±7.7*	310.0±5.0
	BW gain/week (g)	21.1±0.6*	15.8±0.7
	Total adipose tissue weight (AW; g)	13.5±1.1*	5.2±0.2
	AW to BW ratio (%)	3.4±0.2*	1.7±0.1
HF 1 wk	Final body weight (BW; g)	403.0±2.7*	315.6±3.6
	BW gain/week (g)	36.0±1.6[a]	28.9±2.6[a]
	Total adipose tissue weight (AW; g)	10.5±0.6*	6.4±0.3
	AW to BW ratio (%)	2.6±0.2*[c]	2.0±0.1
HF 6 wks	Final body weight (BW; g)	525.9±13.3*δ	421.0±9.1δ
	BW gain/week (g)	35.9±1.3*[a]	29.7±1.2[a]
	Total adipose tissue weight (AW; g)	22.0±1.6*[b]	10.2±0.7[b]
	AW to BW ratio (%)	4.2±0.3*[c]	2.4±0.2[d]

Table 4-1. Body weight and total adipose tissue weight of DIO and DR rats on various treatments. Nine week-old DIO and DR rats (n=6-8/group) were fed 1 week or 6 weeks of chow or HF diets. Weekly body weight was recorded until sacrifice. At the time of sacrifice, total white adipose tissue weight (visceral and retroperitoneal) was measured. * $p<0.05$ compared to DR animals in the same group; **Final body weight:** δ $p<0.05$ compared to the rest of the groups in the same phenotype; **BW gain/week:** [a] $p<0.05$ compared to chow group in the same phenotype; **Total adipose tissue weight:** [b] $p<0.05$ compared to the rest of the groups in the same phenotype; **AW to BW ratio:** [c] $p<0.05$ compared to rest of the groups in the same phenotype; [d] $p<0.05$ compared to chow group in the same phenotype.

Caloric intake

The daily and weekly caloric intake and average weekly caloric intake (Mean±SE; kcal) in DIO and DR rats for different treatments are shown in Fig. 4-1. The average weekly caloric intake was significantly higher in DIO rats after 1 week of HF diet compared to DR rats and even when they were on a chow diet or 6 weeks of HF diet (Fig. 4-1C). Exposure to HF diet for 1 week resulted in a consistent increase in caloric intake in DIO rats compared to DR rats ($p<0.01$) throughout the observation period (Fig. 4-1A). However, caloric intake was highest on day 1 and tended to decline in both groups as the week progressed. A similar trend was observed with 6 weeks of HF (Fig. 4-1B). In this group, caloric consumption was higher during the first week in HF-fed DIO and DR animals, but it was dramatically reduced from 767.9±16.4 to 667.1±14.8 in the first week to 653.5±20 and 579.9±22.5 by the second week in DIO and DR rats, respectively. This reduction in calorie intake from the second week to the sixth week is reflected in the average calorie intake over the 6 week period (Fig. 4-2C). HF-fed DIO groups for either 1 week (885.3±14.6) or 6 weeks (691.9±16.7) had significantly increased caloric intake compared to chow-fed DIO group (621.6±2.8; $p<0.05$). The same was true for HF-fed DR groups for either 1 week (704.1±24.5) or 6 weeks (583.0±16.8) compared to chow-fed DR group (503.1±6.6; $p<0.05$).

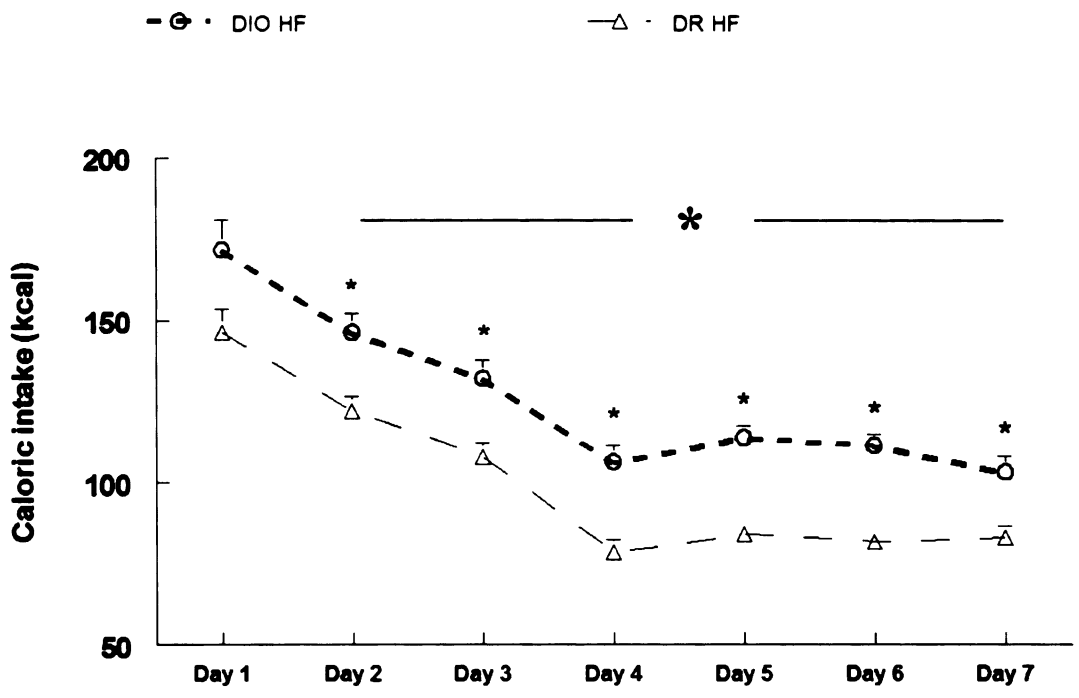


Fig. 4-1A. Caloric intake in DIO and DR rats during 1 week of HF diet exposure (n=6-8 each group). Daily caloric intake was recorded until sacrifice. Note the consistent increase of daily caloric intake in DIO group. Both groups showed a progressive decline in caloric consumption after 1st day. *$p < 0.05$ compared to DR animals.

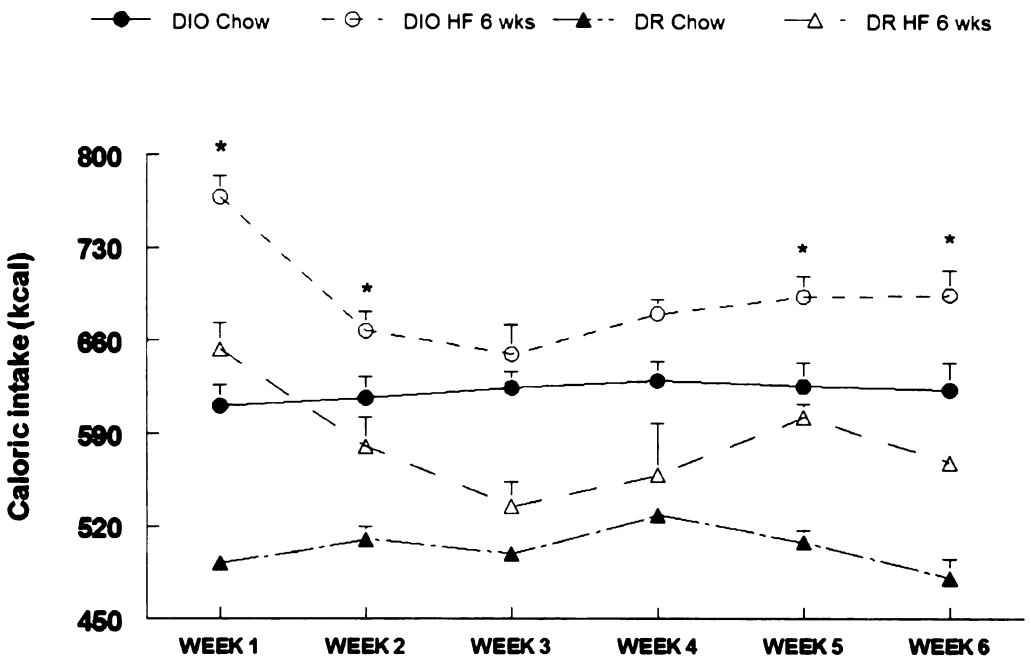


Fig. 4-1B. Caloric intake in DIO and DR rats during 6 weeks of HF diet exposure (n=6-8 each group). Weekly caloric intake was recorded until sacrifice. Note the HF-fed animals kept consistant increase in weekly caloric intake compared to chow-fed animals during the treatment period. HF-fed DIO group had greater caloric consumption compared to HF-fed DR group, but both groups showed a rapid decline of caloric consumption after 1st week of HF diet exposure. *$p<0.05$ between HF-fed and chow-fed groups.

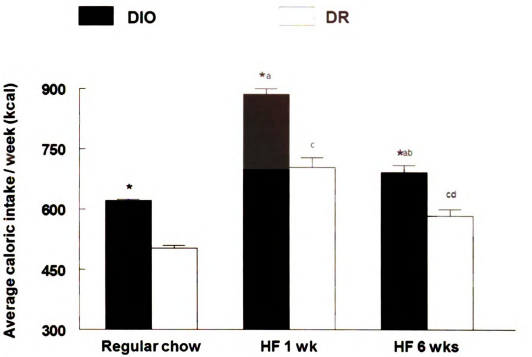


Fig. 4-1C. Average caloric intake/week in DIO and DR rats that are exposed to either 1 week of HF diet, or 6 weeks of chow or HF diet (n=6-8 each group). Average caloroic intake was calculated from the sum of total calories consumed divided by treatment days or weeks. DIO groups had higher average caloric consumption regardless of dietary treatments compared to DR groups. Note the increase in 1 week HF-fed DIO rats compared to 6 weeks HF-fed DIO rats are influenced by the dramatic drop of caloric consumption following 1st week of HF diet. *$p<0.05$ compared to DR animals within group; $^{a}p<0.05$ compared to the chow group of the same phenotype; $^{b}p<0.05$ compared to HF 1 week group of the same phenotype; $^{c}p<0.05$ compared to chow group of the same phenotype; $^{d}p<0.05$ compared to HF 1 week group of the same phenotype.

Feed efficiency

Feed efficiency (Mean±SE; g/kcal*10^3) was calculated as body weight gain in grams divided by the energy consumption in kcal over the observation period (Fig. 4-2). There were no significant differences in feed efficiency between DIO and DR rats whether they were on 1 week or 6 weeks of HF or chow. However, HF-fed animals had a higher feed efficiency compared to chow-fed animals such that 1 week HF-fed DIO and DR animals (40.7±1.8; 40.9±3.0 in DIO and DR groups respectively) or 6 week HF-fed DIO and DR animals (52.0±2.2; 49.8±2.3) had significantly higher feed efficiency compared to their chow-fed counterparts (34.2±1.4; 30.5±1.3 in DIO and DR groups respectively; $p<0.05$). Moreover, DIO and DR groups fed HF diet for 6 weeks had significantly higher feed efficiency compared to 1 week HF-fed animals ($p<0.05$).

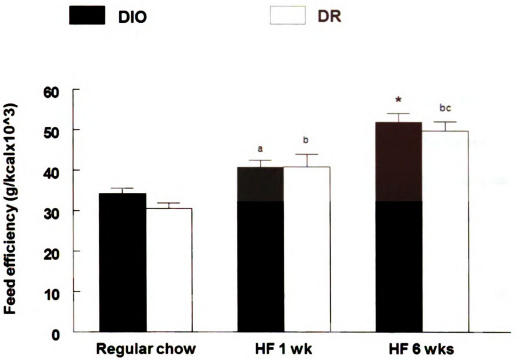


Fig. 4-2. Feed efficiency of DIO and DR animals following 1 week of HF or 6 weeks of chow or HF diet. Note no significant difference between phenotypes within each dietary treatment. Prolonged duration of HF diet, however, significantly increased the feed efficiency. * $p<0.05$ compared to the chow group of the same phenotype; [a] $p<0.05$ compared to HF 6 weeks group of the same phenotype; [b] $p<0.05$ compared to the chow group of the same phenotype; [c] $p<0.05$ compared to 1 week HF group of the same phenotype.

Serum leptin levels

Serum leptin levels in DIO and DR rats that were subjected to the different treatment regimens are shown in Fig. 4-3. Exogenous administration of leptin increased serum leptin levels both in DIO and DR rats. There was a 4-fold increase in leptin levels (ng/ml; Mean±SE) in both DIO (8.3±1.9) and DR (4.2±0.5) rats compared to their chow-fed counterparts ($p<0.05$). Moreover, leptin levels in DIO rats were twice as that seen in DR rats after leptin treatment. Exposure to 1 week of HF diet tended to increase serum leptin levels in DIO rats (3.9±0.2) compared to chow-fed DIO rats (2.6±0.2), but it was not statistically significant. Increasing the duration of HF exposure to 6 weeks increased serum leptin levels further by more than two-fold in DIO rats (6.4±0.3) compared to chow-fed DIO rats ($p<0.05$). These levels were also higher than that seen in DIO rats after 1 week of HF diet ($p<0.05$). In contrast, serum leptin levels remained low in DR rats whether they were exposed to 1 week (1.6±0.24) or 6 weeks (1.9 ±0.1) of the HF diet, and were not significantly different from chow-fed DR rats. Taken together, DIO animals in all treatment groups had higher levels of leptin compared to their DR counterparts.

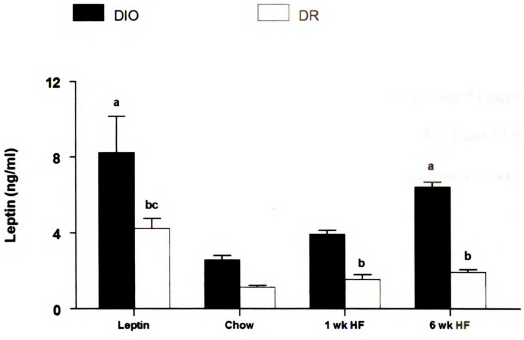


Fig. 4-3. Serum leptin levels in DIO & DR rats after various treatments. Adult male DIO & DR rats (n=6-8 each group) were treated with 500μg rat recombinant leptin, one week of HF diet, or 6 weeks of chow or HF diet Serum was collected from trunk blood after sacrifice. Leptin levels were measured by ELISA and analyzed by ANOVA followed by post-hoc LSD test. [a] $p<0.05$ compared to DIO animals on chow & 1 week HF group; [b] $p<0.05$ compared to corresponding DIO animals within group; [c] $p<0.05$ compared to DR animals in the rest of the groups.

NE concentrations in the PVN of the hypothalamus

Changes in NE concentrations in the PVN of DIO and DR rats after the various treatments are shown in Fig. 4-4A. NE levels (Mean±SE; pg/μg protein) in the PVN were not different between chow-fed DIO and DR rats. Exogenous administration of leptin decreased NE levels by 35% in DIO (13.4±0.5) and by 42% in DR (10.4±0.3) rats compared to the chow-fed groups (20.3±1.1 and 17.5± 1.3 in DIO and DR rats respectively, $p<0.05$). In contrast, exposure to 1 wk of HF produced an increase in NE levels in the PVN in DR, but not in DIO animals. Exposure to 6 weeks of HF diet produced a further increase (80%) in NE levels in DIO (35.7±3.9) and an 88% increase in DR rats (32.0±2.4) compared to chow-fed animals ($p<0.05$). Overall, NE levels increased gradually and significantly in DR animals with duration of HF exposure. While NE levels increased with HF exposure in DIO animals also, it was significant only after 6 weeks of HF diet. It must be noted that NE levels were elevated in DIO rats in spite of high leptin levels in these animals.

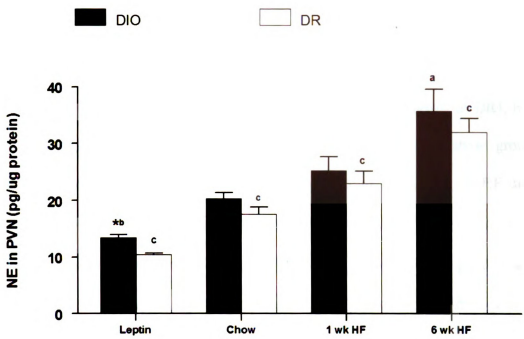


Fig. 4-4A. NE concentrations in the PVN of DIO & DR animals after various treatment regimens. Adult male DIO/DR rats were given i.p. leptin, or exposed to 1 week or 6 weeks of HF/chow. [a] $p<0.05$ compared to other DIO animals; [b] $p<0.05$ compared to chow group; [c] $p<0.05$ across treatments; * $p<0.05$ compared to respective DR animals. There is a consistant elevation of PVN NE in DR groups. Note the same is observed in DIO groups in spite of consistant increase in serum leptin levels (Fig. 4-2).

CRH levels in the ME of the hypothalamus

Fig. 4-4B shows CRH protein levels (Mean ± SE; ng/μg protein) in the ME of DIO and DR rats after different treatments. As with NE levels in the PVN, there were no significant differences in CRH concentrations between chow-fed DIO and DR rats. Leptin administration did not affect CRH levels in DIO, but significantly reduced CRH levels in DR rats compared to the chow group (20.5±5.0 vs. 11.7±2.1; $p<0.05$). In contrast, exposure to 1 week of HF diet produced a marked increase in CRH concentration in DIO rats (45.8 ± 11.5) compared to the chow-fed DIO animals (18.6±7.1), but returned to basal levels (18.5±3) after 6 weeks of HF diet. On the other hand, 1 week of HF exposure tended to increase CRH levels in DR rats (31.8±6.3) compared to in chow-fed DR group (20.5±5.0), but this was not statistically significant. Exposure to 6 weeks of HF diet produced a robust increase in CRH levels only in DR rats (45.1±5.2; $p<0.05$). Altogether, CRH levels increased in DR rats parallelling the rise in NE levels in the PVN. Although CRH levels still responded to elevations in NE after 1 week of HF diet in DIO rats, this link was lost after 6 weeks of HF diet.

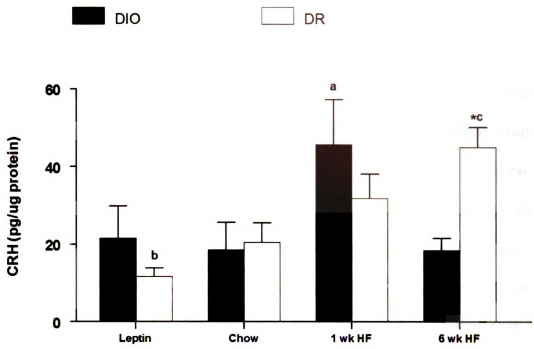


Fig. 4-4B. CRH in the ME in DIO & DR animals after various treatment regimens. Adult male DIO/DR rats were given i.p. leptin, or exposed to 1 week or 6 weeks of HF/chow. CRH levels were measured by ELISA from microdissected ME. [a] $p<0.05$ compared to other DIO groups; [b] $p<0.05$ compared to DR animals in 1 week HF & 6 wk HF groups; [c] $p<0.05$ compared to DR animals in chow group; * $p<0.05$ compared to respective DIO animals. There is a consistant elevation in ME CRH protein levels in DR animals with longer exposure of HF diet. Note a dramatic increase in CRH levels in 1 week HF-fed DIO animals, but normalization to the levels of chow-fed DIO animals after 6 weeks of HF diet exposure.

Serum corticosterone (CORT)

Consistent with CRH concentrations, serum CORT levels (Mean±SE; ng/ml; Fig. 4-4C) were not different between DIO and DR rats when fed chow diet. Leptin injection decreased serum CORT levels significantly only in DR rats (178.0±14.1) compared to chow-fed DR rats (273.9±46.3; $p<0.05$). This effect was not observed in DIO rats. Exposure to 1 week of HF diet increased serum CORT levels significantly both in DIO (409.7±41.3) and DR (384.5±12.6) rats compared to their chow-fed counterparts (214.5±16 and 273.9±46.3 in DIO and DR rats, respectively; $p<0.05$). In contrast, 6 weeks of HF exposure significantly increased CORT levels only in DR (488.7±36.7; $p<0.05$) and not in DIO rats (270.5±49.2) compared to their respective chow group. Taken together, CORT levels followed the pattern observed in CRH concentration in DIO and DR rats. While CORT levels increased gradually but significantly in DR with duration of HF exposure, it increased briefly after 1 week of HF but declined to basal levels after 6 weeks of HF in DIO rats.

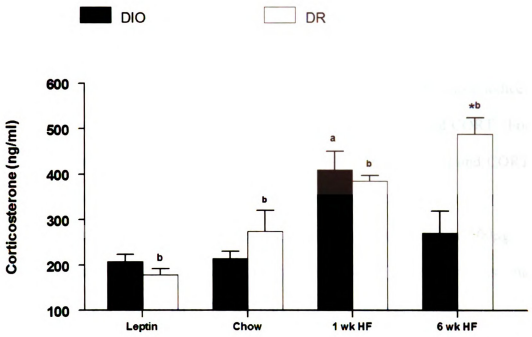


Fig. 4-4C. Serum CORT levels in DIO & DR animals after various treatment regimens. Adult male DIO/DR rats were given i.p. leptin, or exposed to 1 week or 6 weeks of HF/chow. Serum from trunck blood was used to measure CORT by RIA. $^{a}p<0.05$ compared to other DIO animals; $^{b}p<0.05$ compared to DR animals across treatments; $*p<0.05$ compared to respective DIO animals. Note the similarity of serum CORT levels with ME CRH levels of both phenotypes shown in Fig. 4-4B.

Relationship between leptin and HPA indices in Sprague-Dawley rats

Type II regression analyses were performed to explore the relationship between serum leptin and HPA axis indices, namely, NE levels in the PVN, CRH concentrations in the ME and serum CORT (Fig. 4-5). Leptin values are plotted on the x-axis due to the causal effect of leptin on NE and other HPA axis indices to test how well leptin levels can predict the levels of NE, CRH, and CORT. For the same rationale, NE values are plotted on the x-axis against CRH and CORT values.

Sprague-Dawley male rats were treated with saline, 100μg, or 500μg of rat recombinant leptin i.p. and serum leptin levels, PVN NE concentrations, and serum CORT were measured in the previous study by Clark et al (2006).

The relationship between leptin and NE (Fig. 4-5A) or CORT (Fig. 4-5B) of the Sprague-Dawley male rats showed an inverse association. On the other hand, the relationship between PVN NE and CORT (Fig. 4-5C) showed a positive association. These associations were not statistically significant, however.

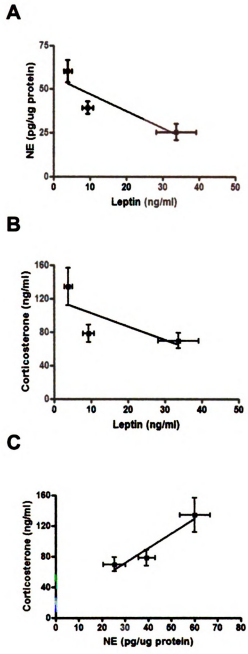


Fig. 4-5. Type II regression analyses of the relationships between leptin and HPA indices. Values of leptin and HPA axis indices (NE, CORT) in Sprague-Dawley rats were borrowed from Clark et al. (2006) for regression analyses. Young Sprague-Dawley male rats received saline, 100μg, or 500μg of rat recombinant leptin i.p., and serum leptin, PVN NE, and serum CORT were measured. Three relationships – leptin vs. NE, leptin vs. CORT, and NE vs. CORT – are shown for normal Sprague-Dawley rats. Note the obvious negative or positive associations between variables.

Relationships between leptin and HPA indices in DIO and DR rats

Leptin - NE: Regression analysis depicting the association between leptin and NE levels in the PVN in all four treatments for both DIO and DR groups are shown in Fig. 4-6A. For comparison purposes, please refer to Fig. 4-5 for analyses in Sprague-Dawley rats. A clear trend of an inverse relationship between leptin and NE was apparent in Sprague-Dawley rats. A similar relationship was evident in DR animals, where elevated leptin levels reduced NE concentrations in the PVN, however the association was not statistically significant (r^2=0.34, F=1.02, p=0.42). On the contrary, no such relationship was evident in DIO rats. However, the slope comparison between DIO and DR animals (F=0.47, p=0.53) indicates that the effect of leptin on NE in the DIO group is not different from that in the DR group.

Leptin - HPA Axis: Relationships between leptin and CRH and leptin and CORT in DIO and DR animals are shown in Fig. 4-6D and 4-6B. Please refer to Leptin-CORT relationship in Sprague-Dawley rats depicted in Fig. 4-5 for comparison purpose. There were no significant differences in relationship between leptin and CRH or CORT (r^2=0.11, F=0.24, p=0.67; r^2=0.10, F=0.22, p=0.68; leptin:CRH and leptin:CORT, respectively) in DIO animals. In spite of rather obvious inverse associations between the variables, the findings were not significant for DR animals either (r^2=0.29, F=0.80, p=0.47; r^2=0.34, F=1.05, p=0.41; leptin:CRH and leptin:corticosterone, respectively). As a result, the slopes between DIO and DR animals were not statistically different from each other (F=0.27, p=0.63).

NE - HPA Axis: We also explored the relationship between NE levels in the PVN and the stress axis indices – CRH and CORT – in DIO and DR animals (Fig. 4-6E, 4-6C). Please refer to NE-CORT relationship in Sprague-Dawley rats depicted in Fig. 4-5 for comparison purpose. There was a strong, but not significant positive relationship between NE and CORT in Sprague-Dawley rats. A similar relationship was observed in DR animals (r^2=0.99, F=272.4, p=0.0037; r^2=0.99, F=178.4, p=0.0056 for NE:CRH and NE:CORT, respectively) where increased PVN NE concentrations were statistically strongly associated with both elevated CRH and CORT. On the other hand, there was no association between NE and CRH or CORT in DIO rats (r^2=0.0017, F=0.0035, p=0.96; r^2=0.18, F=0.44, p=0.58; NE:CRH and NE:CORT, respectively). However, the slopes between DIO and DR were not significantly different for either association (F=2.33, p=0.20; F=2.25, p=0.21; NE: CRH and NE:CORT, respectively).

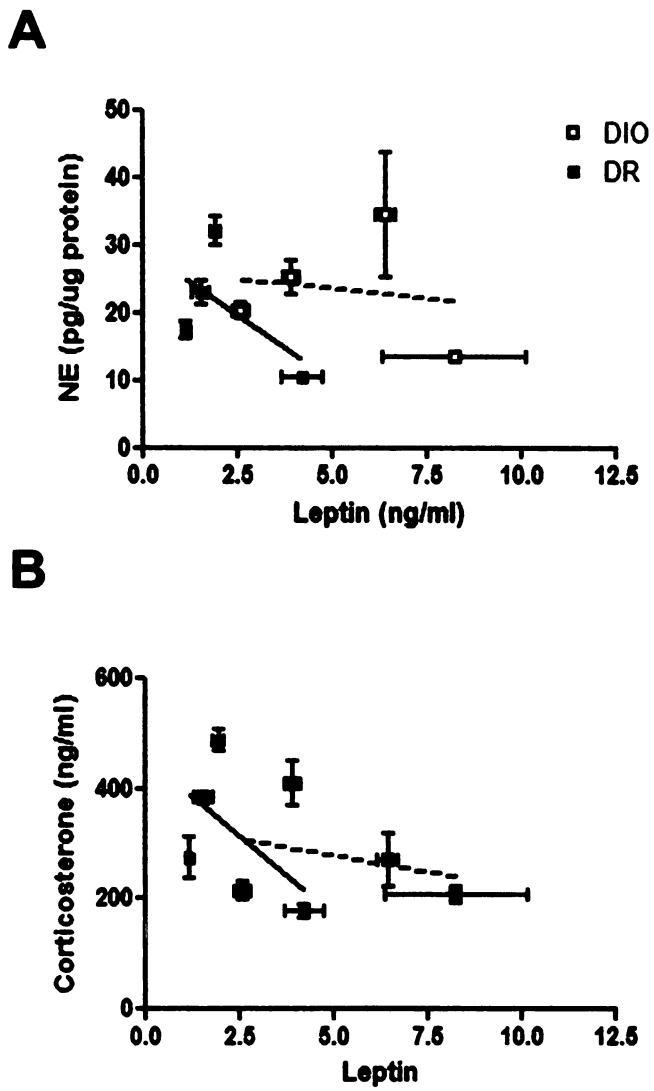
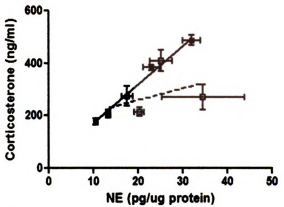


Fig. 4-6. Type II regression analyses depicting associations between leptin and HPA axis indices in DIO and DR rats. Relationships between leptin and HPA axis indices (NE, CRH, CORT) in DIO and DR rats are shown. Either 500μg rat recombinant leptin, one week of HF diet, or 6 weeks of chow or HF diets was given to DIO & DR rats (n = 6-8 each group). Relationships are shown in slopes for both DIO (dashed line) and DR (solid line) groups. Deviation of the slopes was not significantly different from zero for all but in NE vs. CORT and NE vs. CRH regressions in DR rats.

Fig. 4-6 continued

C



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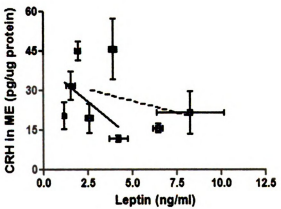

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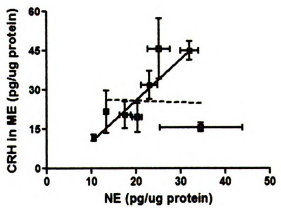


Protein expression analysis of pSTAT-3 in the brainstem

pSTAT-3 protein expression in A1: Protein expression of pSTAT-3 in the A1 (VLM) noradrenergic region in the brainstem is shown in Fig. 4-7A. It is expressed as values of optical density after chow-DIO group value was normalized to 1.0. No difference was found between chow-fed DIO and DR animals. However, there was a significant decrease in pSTAT-3 protein expression in DIO group after HF diet (0.3 ± 0.06) compared to HF-fed DR group (1.1 ± 0.1) or chow-fed DIO (1.0 ± 0.1) or DR (1.0 ± 0.1; $p<0.05$) animals.

pSTAT-3 protein expression in A2: Fig. 4-7B shows pSTAT-3 protein expression in A2 (NTS) region between chow or HF-fed DIO and DR animals analyzed by densitometry. Chow-fed DIO rats had significantly lower protein expression compared to their counterparts (1.0 ± 0.3 vs. 3.3 ± 0.6; $p<0.05$). HF-fed DIO group (1.1 ± 0.3) also had significantly reduced pSTAT-3 expression compared to HF-fed DR group (3.0 ± 0.6; $p<0.05$). No differences were found within the same phenotype.

pSTAT-3 protein expression in A6: The expression in A6 (LC) noradrenergic region of the braintem between DIO and DR animals is shown in Fig. 4-7C. No statistical difference was found between any of the groups.

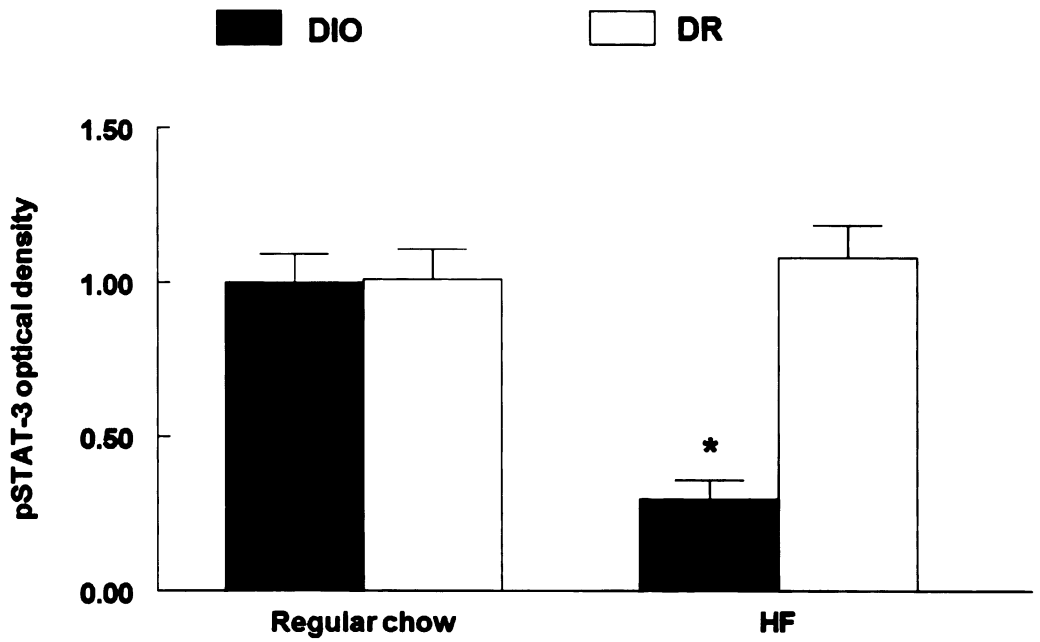


Fig. 4-7A: pSTAT-3 protein expression in the A1 region of the brainstem in DIO and DR groups (n=4-6 per group). The protein was detected by western blot and analayzed by densitometry. Note the significant reduction of pSTAT-3 expression after HF diet exposure only in DIO group. *$p<0.05$ compared to the rest of the groups.

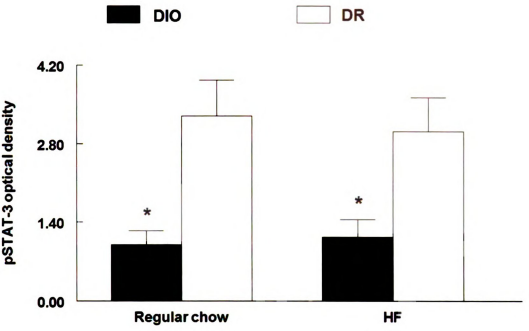


Fig. 4-7B: pSTAT-3 protein expression in the A2 region of the brainstem in DIO and DR groups (n=4-6 per group). The protein was detected by western blot and analayzed by densitometry. Note the significant reduction of pSTAT-3 expression in DIO groups regardless of the dietary treatments. *$p<0.05$ compared to the respective DR group.

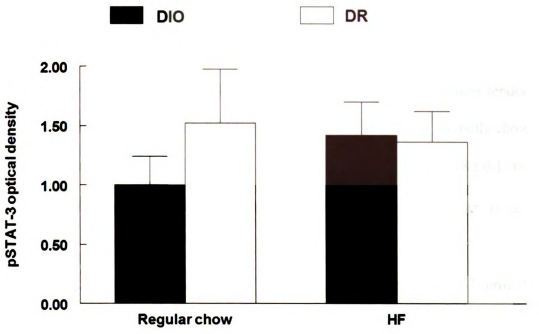


Fig. 4-7C: pSTAT-3 protein expression in the A6 region of the brainstem in DIO and DR groups (n=4-6 per group). The protein was detected by western blot and analayzed by densitometry. Note the significant reduction of pSTAT-3 expression after HF diet exposure only in DIO group. * $p<0.05$ compared to the rest of the groups.

Protein expression analysis of SOCS-3 in the brainstem

SOCS-3 protein expression in A1: Protein expression of negative feedback inhibitor of leptin signaling, SOCS-3, in the A1 (VLM) noradrenergic region of the brainstem is shown in Fig. 4-8A. It is expressed as values of optical density after chow-DIO group value was normalized to 1.0. DIO groups tended to have higher expression of SOCS-3 compared to DR groups in both chow (1.0 ± 0.05 vs. 0.8 ± 0.1 DIO vs. DR respectively) and HF diet (0.8 ± 0.1 vs. 0.5 ± 0.1, DIO vs. DR respectively), but the differences were not statistically significant.

SOCS-3 protein expression in A2: Fig. 4-8B shows SOCS-3 protein expression in the A2 (NTS) region between chow or HF-fed DIO and DR animals analyzed by densitometry. The chow-fed DIO group had significantly higher expression of SOCS-3 compared to the chow-fed DR group (1.0 ± 0.1 vs. 0.3 ± 0.06; $p<0.05$). HF-fed DIO animals also had higher SOCS-3 expression compared to the HF-fed DR group (0.9 ± 0.2 vs. 0.5 ± 0.15), but the difference was not statistically significant.

SOCS-3 protein expression in A6: The expression in A6 (LC) noradrenergic region of the braintem between DIO and DR animals is shown in Fig. 4-8C. No statistical difference was found between any of the groups.

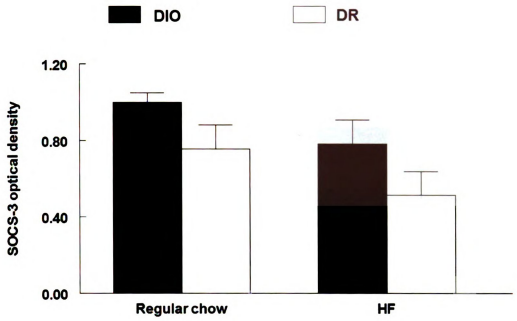


Fig. 4-8A: SOCS-3 protein expression in A1 region of the brainstem in DIO and DR groups (n=5-8 per group). The protein was detected by western blot and analayzed by densitometry. No difference was found between groups.

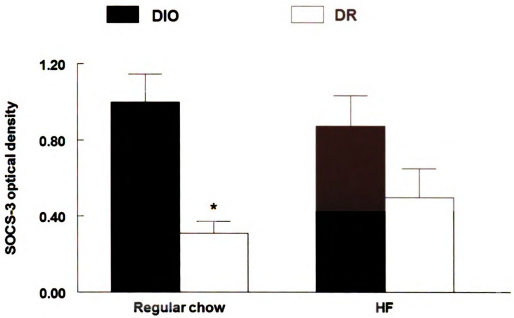


Fig. 4-8B: SOCS-3 protein expression in A2 region of the brainstem in DIO and DR groups (n=5-8 per group). The protein was detected by western blot and analyzed by densitometry. Note the significant reduction of SOCS-3 expression in the chow-fed DR group compared to its counterpart. The same trend was observed for HF-fed groups, but it was not stastistically significant. * $p<0.05$ compared to the respective DIO group.

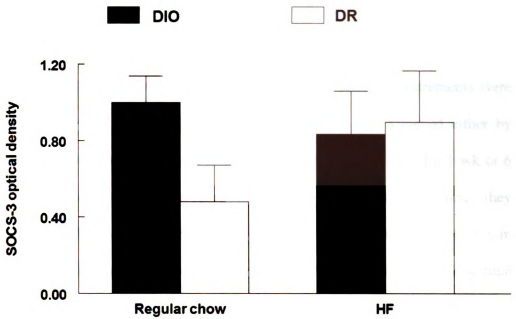


Fig. 4-8C: SOCS-3 protein expression in A6 region of the brainstem in DIO and DR groups (n=5-8 per group). The protein was detected by western blot and analayzed by densitometry. There was a reduction in SOCS-3 expression in chow-fed DR group compared to its counterpart, but it was not statistically significant.

D. Discussion

This chapter further explored the neuroendocrine HPA axis alterations in DIO rats in greater detail. As in Chapter 3, the present study investigated changes in three different parameters, 1) NE concentrations in the PVN, 2) CRH levels in the ME and 3) serum CORT in DIO and DR rats. These measurements were made in response to changing levels of leptin, which was achieved either by treating these animals with leptin or by placing them on a HF diet for 1 wk or 6 wks. DIO and DR rats differed significantly in the amount of calories they consumed, body and adipose tissue weight they gained, but more importantly, in their stress axis responsiveness to the various treatments. This is the first time that the differences in HPA function have been dissected in these polygenic DIO and DR rats in depth. The following paragraphs provide possible reasons for the differences that we observed in stress axis responses in DIO and DR animals in this study and relate to findings from the previous chapter.

The fact that DIO animals consume more calories than DR animals is well established (165) and is supported by the current findings (Fig. 4-1). The higher caloric intake in DIO rats is clearly reflected in an increase in body weight, higher body weight gain per week, and adipose tissue weight (Table 4-1). The increase in adipose tissue weight most probably accounts for the higher serum leptin levels seen in DIO animals (Fig. 4-3). When placed on a HF diet, DIO rats gained weight more rapidly than chow-fed animals, indicating that they were becoming more energy efficient as evident in Fig. 4-2. On the contrary, DR animals only had a moderate increase in adipose tissue weight and that was evident only after 6

weeks of HF diet. However, leptin levels in DR animals were unaffected by the increased amount of abdominal fat depots (Fig. 4-3). These results are supported by other studies in which HF exposure increased serum leptin levels and pulse amplitude in DIO rats but not in DR rats (141, 148, 166). This suggests inherent differences between DIO and DR rats in their ability to store fat and secrete leptin.

We considered the possibility that the differences in serum leptin levels caused by the HF diet could contribute to the differences in the stress axis response in DIO and DR animals. Leptin is involved in HPA regulation and can suppress the stress axis (14-17, 106, 151, 167), but the mechanisms involved are unclear. Recent studies indicate that this may be mediated via central noradrenergic systems (19, 107). NE is one of the most important regulators of CRH and the HPA axis (95, 168, 169). Noradrenergic neurons in the brainstem innervate the PVN (88, 170) and when noradrenergic activity increases in the PVN, CRH neurons are stimulated resulting in activation of the stress axis (95, 152, 168). Since leptin suppresses the stress axis activity, it is quite possible that this effect is mediated by a reduction in NE levels. In fact, central and systemic administration of leptin, or treatment with an adipogenic Ad-36 virus that increases endogenous leptin levels, have all been shown to decrease NE concentrations and/or release in the PVN, and this was accompanied by a significant reduction in serum CORT (107, 153, 171). However, in the present study, this inverse relationship between leptin, NE and CORT was lost in DIO, but not in DR rats. This was most pronounced when the animals were placed on a

HF diet. Since HF diet elevates serum leptin levels, we performed experiments to determine if DIO and DR animals responded differently to leptin *per se*. A single systemic injection of leptin resulted in a significant decrease in NE concentrations in the PVN in both DIO and DR rats (Fig. 4-4A). However, exposure to 1 week or 6 weeks of HF diet increased NE levels in the PVN in both DIO and DR rats. It should be noted that NE levels increased in DIO rats despite the higher circulating leptin levels in these animals. This clearly indicates a loss of leptin sensitivity in DIO animals, most probably in noradrenergic neurons located in the brainstem. They are closely linked to food intake and body weight regulation (154), and rich in leptin receptors (57). Defective leptin receptor signaling has been identified to be important for leptin insensitivity (172). Any of leptin's downstream signal pathway mediators such as phosphorylated signal transducer and activator of transcription-3 (pSTAT-3) or suppressor of cytokine signaling-3 (SOCS-3), could play a role in this phenomenon (172). Thus, I measured the protein expression of these mediators from chow-fed and 6 week HF-fed groups. Leptin-injected groups were not evaluated because PVN NE levels from both DIO and DR groups were properly reduced following the exogenous leptin treatment. Likewise, acutely HF-treated groups were not assessed further because the main focus of the current study and the dissertation was to investigate whether leptin signal impairment is present after chronic exposure to HF diet.

As expected, there was a dramatic reduction in pSTAT-3 protein expression in noradrenergic neurons in the brainstem of 6 week HF-fed DIO rats. The decrease was site-specific that it was observed only in brainstem A1, but not

A2 or A6 noradrenergic regions. The reduction in pSTAT-3 protein expression is associated with the elevated expression of TH mRNA in A1 region as shown in Chapter 3. Clearly, A1 has been shown to send out the most-dense projections to parvicellular dorsomedial part of the PVN, which contains a robust immunoreactivity to CRH (88), indicating the critical role of A1 noradrenergic neurons in stimulating CRH neurons in the PVN upon exposure to stressful stimuli. It is quite possible that leptin can directly downregulate noradrenergic functions by decreasing the production of TH mRNA, and this may be mediated by increasing the suppressive leptin signaling in the noradrenergic neurons in the brainstem. In fact, leptin has been shown to affect both TH gene transcription as well as post-transcriptional status by phosphorylation in adrenal medulla (155, 173), so it is very possible that leptin can affect TH activity or production in noradrenergic neurons. The leptin signal impairment and increased noradrenergic function as evidenced by TH mRNA upregulation in A1 region after chronic HF diet in previous and current study strongly supports the notion of direct inhibitory action of leptin on noradrenergic neurons at normal state, which is lost in chronically HF-fed DIO rats.

In contrast to DIO rats, NE concentration increased in DR animals despite stable circulating leptin levels. One possible explanation for this increase is that the HF diet or some factor produced as a result of HF diet exposure triggers a stress response in DR animals. This possibility can also explain the increase in NE levels in DIO rats. In DR rats, elevated NE levels in the PVN were followed by increases in ME CRH and serum CORT in a duration-dependent manner.

Moreover, systemic leptin injection was able to suppress all arms of the HPA axis in these animals, suggesting that the regulation of the HPA axis is intact in DR rats (Figs. 4-4). This response was reversed in DIO rats. Although leptin injection decreased NE levels in the PVN in DIO rats, it did not result in a corresponding decrease in either ME CRH or serum CORT, indicating dissociation between PVN NE and the rest of the HPA axis. This finding challenges my fundamental hypothesis that leptin insensitivity or resistance in the brainstem noradrenergic neurons may contribute to development of dysfunctional HPA axis regulation. The finding rather indicates that the HPA axis perturbation is probably independent of leptin sensitivity in the brainstem and the consequent abnormal increase in the noradrenergic activity. While this may be the case, the HF-fed DIO animals manifest all signs of diet-induced obesity including the obese phenotype, increased ingestive behavior, and the HPA axis dysregulation. While abnormal HPA axis activity is well associated with development of obesity, an alternative explanation for obesity development may involve the observed increase in noradrenergic activity in the PVN. Besides its role in the regulation of the HPA axis, NE levels in the PVN are also associated with feeding behavior (174). NE injections into the PVN or treatment with α-2 adrenergic agonists such as clonidine are known to stimulate feeding (175-177). Moreover, lesioning of noradrenergic fibers that innervate the PVN have suppressed feeding behavior, suggesting that NE levels in the PVN are critical for feeding (178). This may explain the increased caloric intake, visceral fat depots, and the weight gain in HF-fed DIO rats. The same increase in HF-fed DR rats may explain similar

increase in weight gain, fat depots, and caloric consumption compared to the chow-fed DR rats.

On the other hand, when NE levels increased in the PVN in DIO rats after 6 weeks of HF exposure, both CRH levels and serum CORT remained unchanged compared to the chow group, suggesting a similar dissociation between PVN NE and the rest of the HPA axis. This suggests a possible mechanism for the observed dysregulation of the stress axis in DIO rats. This is supported by other studies in which CRH neurons were unaffected by glucose-induced sympathetic activation in DIO rats (156, 157). This can attributed to reduced α_2-adrenoceptor binding in the PVN, suggesting a possible postsynaptic, noradrenergic deficit in the PVN in DIO rats (156). Taken together, these studies support our findings of stress axis dysregulation in DIO rats.

As indicated by the regression analyses of the relationships between leptin, PVN NE and serum CORT from an earlier study in Sprague-Dawley rats, it is clear that serum leptin is inversely related to PVN NE and serum CORT. The results from the present study demonstrate that while this relationship is intact in DR rats, it is altered in DIO rats (Fig. 4-5; 4-6). There are several possible reasons for the difference in HPA responsiveness in DIO and DR rats. Leptin insensitivity by signal impairment in noradrenergic neurons could be one factor as shown above. The suppressed inhibition of noradrenergic neurons by leptin resistance can bring about changes in modulation of the HPA axis activation. However, this does not directly account for the dissociation between PVN NE and CRH. Since the lack of responsiveness of CRH neurons is seen in DIO rats both

with exogenous leptin and HF diet, it could be an inherent failure in noradrenergic function in these obese animals. This needs further investigation.

In summary, the results from this study indicate that there is a clear dissociation between PVN NE and CRH in DIO rats. While acute hyperleptinemia is capable of decreasing NE levels in DIO rats, HF diet increases NE levels in the PVN. This occurs despite high circulating levels of leptin, and is correlated with leptin signal impairment in the selective brainstem noradrenergic neurons with chronic exposure. On the other hand, since HF diet exposure increases NE levels in the PVN in both DIO and DR rats, the possible involvement of stress-inducing factors related to the HF treatment should be considered. Leptin resistance in the brainstem may be the mechanism by which noradrenergic function is abnormally upregulated that is associated with subsequent alterations in the HPA axis activity that can promote obesity development. Dysregulation of the HPA axis may be independent of the observed brainstem leptin insensitivity. Correction of leptin resistance at the level of the brainstem noradrenergic neurons seems to be a possible approach to normalize the HF diet-induced noradrenergic activity in the PVN to reduce food intake and consequent development of obesity.

E. Summary

This chapter was proposed to dissect the neuroendcorine changes in polygenically susceptible DIO rats more thoroughly by subjecting them to various treatments, including administration of exogenous leptin and different durations of exposure to a high fat diet. An experiment was designed to test the hypothesis that the brainstem noradrenergic activity and the HPA axis activity in chow-fed DIO rats would remain intact and decrease following administration of exogenous leptin. One group of animals exposed to 1 week of HF diet was included to shed light on the acute cental and hormonal changes that occur in the course of obesity development.

As shown in Chapter 3, DIO animals had increased body weight gain following HF diet compared to DR animals. Parameters regarding caloric intake and amount of fat pads support and explain the resulting phenotypes and changes in the neuroendocrine system. A single injection of leptin significantly increased serum leptin levels in both DIO and DR rats and decreased NE levels in the PVN compared to chow-fed animals. This produced the expected decrease in CRH concentrations in the ME and serum CORT in DR rats. However, it did not affect either CRH or serum CORT levels in DIO rats, indicating dissociation between CRH and NE in these animals. HF feeding did not increase leptin levels and produced a duration-dependent elevation in HPA axis activity in DR rats. On the contrary, HF feeding produced a duration-dependent increase in serum leptin levels in DIO animals which failed to suppress PVN NE levels, suggesting possible leptin insensitivity in noradrenergic neurons in the brainstem. Moreover,

HF feeding for longer periods resulted in dissociation between NE and CRH in DIO rats. Investigation of leptin signaling in the brainstem noradrenergic areas revealed reduced pSTAT-3 protein expression in A1 noradrenergic region. This reduction of pSTAT-3 expression in HF-fed DIO rats can explain the elevated TH mRNA expression in A1 region shown in Chapter 3, providing evidence for the first time the direct suppressive effect of leptin on noradrenergic neurons, which is lost in HF-fed DIO rats. Moreover, increased PVN NE in both phenotypes following HF diet strongly suggests the role of HF diet as a stressor. Taken together, the perturbation of the HPA axis with leptin insensitivity in the brainstem noradrenergic neurons observed in DIO rats after chronic HF diet exposure may play an important role in the promotion of obesity in these animals.

Chapter 5. Chronic metformin treatment reduces PVN NE and partially restores the regulation of the HPA axis in HF-fed DIO rats

A. Introduction

Perturbation of the HPA axis is strongly correlated with abdominal obesity. The hyperleptinemia observed in obese subjects is unable to suppress the HPA axis, indicating lack of sensitivity to leptin. The inhibitory action of leptin is most probably mediated via directly affecting the noradrenergic neurons in the brainstem. This has been supported by results from previous chapters where HF-fed DIO rats had dysregulation of the stress axis and increased NE concentrations in the PVN in spite of hyperleptinemia. This can be attributed to the observed increase in TH mRNA expression and corresponding reduction in pSTAT-3 protein expression in brainstem A1 noradrenergic neurons in these rats. This indicates that DIO rats under HF diet develop leptin signal impairment in selective brainstem noradrenergic neurons that leads to abnormally increased noradrenergic activity, which is associated with dysregulation of the HPA axis and subsequent propensity to develop/promote obesity. In such cases, restoration of proper leptin signaling in the brainstem may be an ideal strategy to normalize noradrenergic activity and possibly reduce excess caloric consumption. As a result, restoration of the noradrenergic balance may at least partially correct the obese phenotype and eating behavior. Whether this will be associated with restoration of the HPA axis regulation is unknown.

Previous studies investigating ways to avoid and prevent leptin resistance in the hypothalamus have mostly employed knockout rodent models that are

deficient in negative regulators of leptin sensitivity such as SOCS-3 (179, 180). Indeed, these findings confirmed the significance of SOCS-3 as the key regulator of hypothalamic leptin resistance in obese rats, and offered suppression of SOCS-3 as a novel therapeutic approach for obesity, diabetes, and associated disorders. However, engineering gene deletion for the treatment of human obesity raises a number of concerns, including ethical and consequential health issues. Rather, an approach that has less potential health risks and uses non-invasive methods with comparable correction of metabolic/neuroendocrine abnormalities would be ideal for both research and clinical settings.

Metformin is an oral biguanide insulin-sensitizing agent that has been most widely used to treat Type II diabetic patients due to its beneficial effects on hepatic glucose production, serum lipid profile, and body weight (125-128). Treatment with metfomin has been shown to reduce body weight in obese patients (129, 130), and this is supported by similar findings in obese animal (131-133). Metformin treatment produced an anorectic effect and reduced food intake and body weight in Zucker male rats. Besides it's well-known role in enhancing insulin sensitivity, recently metformin has been suggested to restore leptin sensitivity in HF-fed obese rats that are leptin resistant (134). Moreover, the same group has demonstated similar anorectic and weight-reducing effects of metformin in Otsuka Long-Evans Tokushima Fatty (OLETF) rats that lack cholecystokinin (CCK) receptors. These restoring effects were attributed to increased pSTAT-3 protein expression in the mediobasal hypothalamus in response to leptin injection. Also, POMC protein expression was elevated in the

hypothalamus after stimulation with leptin in HF-fed metformin-treated rats compared to saline-treated HF-fed rats, suggesting increased or restored leptin sensitivity. This indicates that metformin may mediate regulation of food intake and body weight by both leptin and insulin. However, it is not clear if metformin will be able to restore the leptin sensitivity in the brainstem areas in chronically HF-fed DIO rats. Hence, I hypothesized that treatment with metformin in DIO rats under prolonged HF diet will bring back the leptin sensitivity in brainstem noradrenergic neurons and reduce feeding. Even though the HPA axis perturbation after HF diet exposure is probably not due to the leptin resistance in the brainstem as indicated in the previous chapter, considering its beneficial effects in many physiological systems, there is a possibility that metformin may also be able to restore the HPA axis regulation and correct the dissociation between NE and the HPA axis. The improvement of the leptin sensitivity in the brainstem noradrenegic neurons and possible restoration of the HPA axis regulation may affect the DIO animals to gain less weight and eat less. If the hypothesis is correct, then: A) the body weight gain, caloric intake, and amount of abdominal fat pads will be reduced, B) not only the normalization of PVN NE concentrations and the HPA axis activity, but also the connection between the noradrenergic activity and the HPA axis will be restored, and C) leptin signaling will be elevated as measured by pSTAT-3 and SOCS-3 in A1, A2, and A6 noradrenergic regions in the brainstem. These expected findings can greatly enhance our knowledge of the action and mechanism of metformin in diet-

induced obesity, and offer a relatively safe therapeutic approach for a number of metabolic disorders associated with obesity.

B. Experimental Design

The study described in this chapter was designed to investigate whether metformin can restore the HPA axis regulation and normalize the obese phenotype of DIO rats by increasing the leptin signaling in the brainstem noradrenergic neurons and reducing PVN NE levels. To test this hypothesis, only DIO animals were used because DR animals showed intact leptin signaling in the brainstem as well as intact connection between noradrenergic activity and the HPA axis, as shown in the previous chapters. 9-week-old DIO male rats were given 7 weeks of chow or HF diet (6 groups; n=9-10 each). At the end of 3rd week of chow or HF diet, DIO animals received oral administration of metformin (Spectrum Chemicals & Laboratory Products, Inc.; New Brunswick, NJ) in either low or high dose dissolved in drinking water. On average, animals in the low-dose groups (LD-Met) were given 60 mg/kg of BW per day. Animals in the high-dose groups (HD-Met) were given 300 mg/kg of BW daily. The metformin concentrations were readjusted to the body weight once a week. A diagram showing groups and treatments are shown in Fig. 5-1. After treatment with metformin for 4 weeks, the animals were sacrificed by decapitation. Their brains were collected, frozen in dry ice, and stored at -70°C. Trunk blood was collected, the serum was separated, and stored at -20°C. The abdominal fat pads were removed from the carcass and weighed. Brains were sectioned in 300 μm, and the PVN of the hypothalamus was microdissected. The PVN tissues were homogenized and analyzed for NE concentrations via HPLC-EC. ME was also microdissected and homogenized in lysis buffer. Both serum leptin and ME CRH

protein levels were analyzed by commercially available ELISA kits. To test if metformin had any effect on the leptin signaling in the noradrenergic neurons, brainstems were also sectioned in 300 μm, and noradrenergic areas A1, A2, and A6 were microdissected. These samples were homogenized in lysis buffer and subjected to western blot to detect pSTAT-3 and SOCS-3 protein expressions. Serum corticosterone levels were analyzed by radioimmunoassay. To test if the circuitry within the HPA axis is restored in HF-fed DIO rats following metformin treatment, Type II regression analyses were performed to explore the relationships between NE, CRH, and corticosterone (CORT). This was compared to both HF-fed DIO rats (from Chapter 4) and normal Sprague-Dawley rats (Clark et al. 2006; Chaper 4).

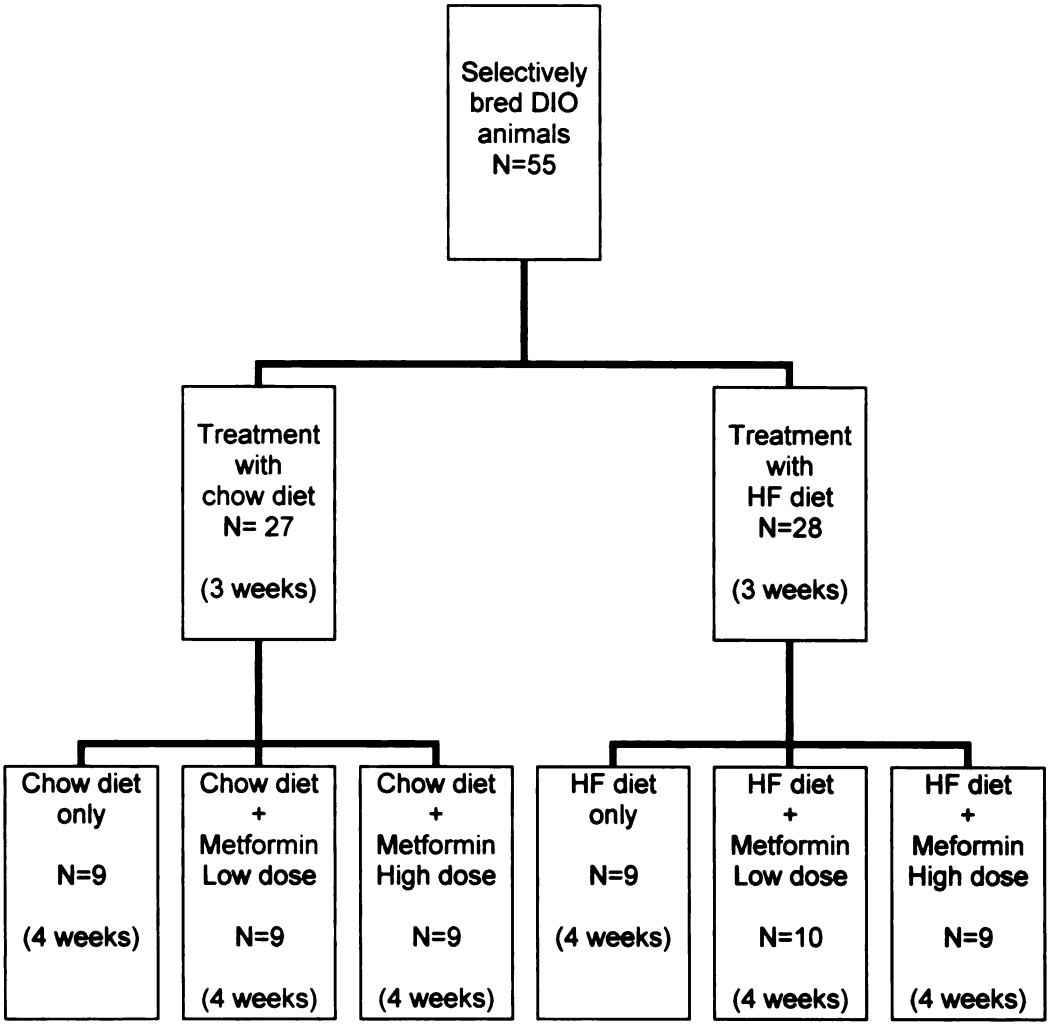


Fig. 5-1. Diagram illustrating the experimental design. Treatments include diets and different doses of metformin. Metformin was dissolved in drinking water and administered orally. The concentrations of metformin were readjusted to the change in body weight once a week. Animals without metformin treatment received normal fresh drinking water.

C. RESULTS

Body weight

Percent changes in body weight (Mean±SE; %) during the observation period are shown in Fig. 5-2A-C. This figure illustrates percent changes for chow-fed animals only, while Fig. 5-2B shows percent changes in body weight for HF-fed animals. There is a significant reduction in body weight in metformin-treated animals compared to the control animals starting at the 4^{th} week (Fig. 5-2A). Only HF-fed HD-Met animals showed a significant decline in body weight compared to control animals starting at the 4^{th} week (Fig. 5-2B).

Fig. 5-2D shows final body weight (Mean±SE; g) in all six groups. Chow-fed animals receiving LD-Met (472.8±11.6) and HD-Met (470.6±12.8) weighed significantly less than the corresponding control chow group (514.7±10.0; $p<0.05$), and the same was true for HF-fed animals ($p<0.05$). HF-fed control group was also significantly heavier (545.1±12.4) compared to chow-fed animals ($p<0.05$).

Body weight gain (Mean±SE; g) of animals after 7 weeks of treatment is shown in Fig. 5-2E. As in final body weight (Fig. 5-2D), treatment of chow-fed animals with LD-Met (146.1±5.6) or HD-Met (120.7±9.4) significantly reduced their body weight gain compared to their chow-fed controls (172.8±7.3; $p<0.05$). Similar significant differences were observed in HF-fed animals ($p<0.05$). Also, the HF-fed control group (208.1±8.1) had significantly higher weight gain compared to the chow-fed control group ($p<0.05$). DIO-chow+HD-Met group

had significantly reduced body weight gain compared to the rest of the groups ($p<0.05$). Likewise, DIO-chow+LD-Met group had a significant reduction of weight gain compared to dietary control groups and DIO-HF+LD-Met group ($p<0.05$). There was a significant decrease in body weight gain in HF-fed+HD-Met group compared to the HF-fed LD-Met group ($p<0.05$).

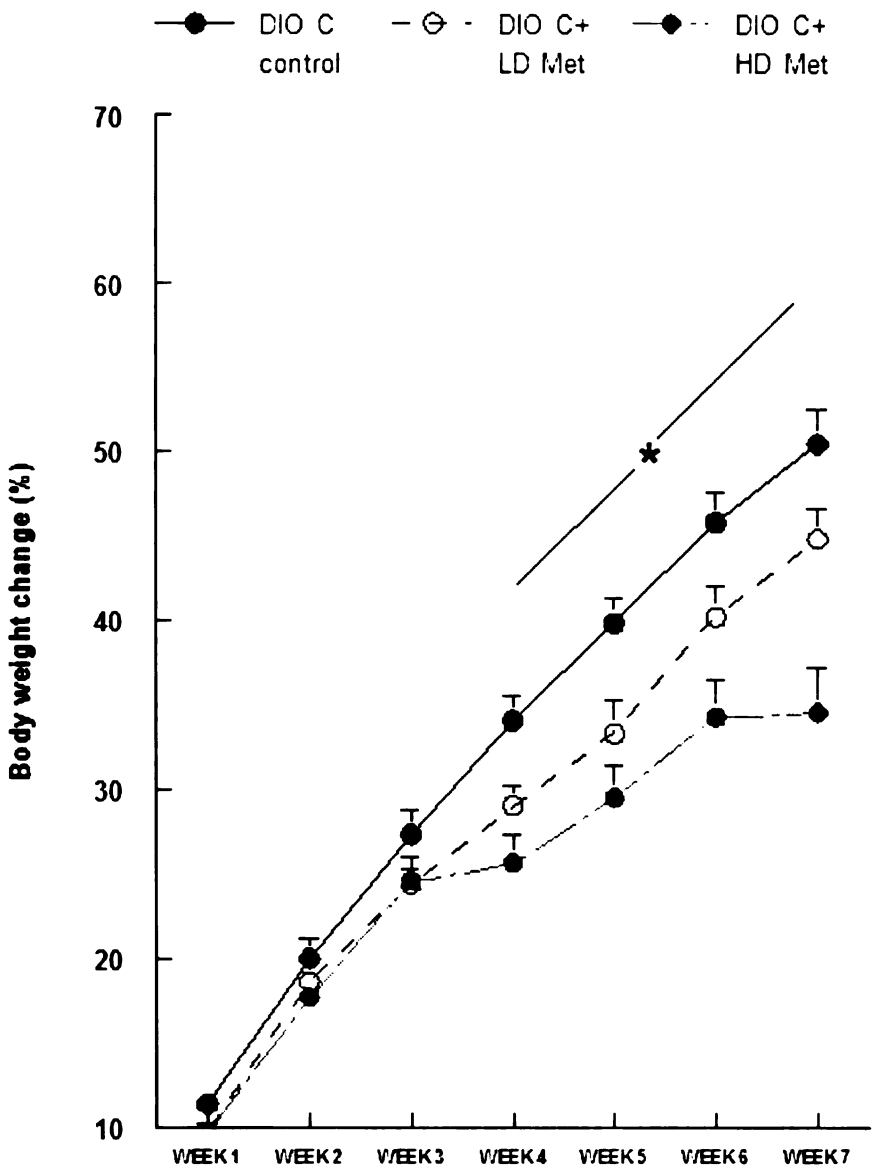


Fig. 5-2A. Body weight change in percent (Mean±SE; %) between chow-fed animals. Repeated measures ANOVA followed by post-hoc LSD test shows a significant reduction in body weight changes in metformin-treated animals compared to the control group starting at the 4th week. Note that this is a week after the beginning of metformin treatment, indicating that the changes observed are indeed mediated by metformin. Greater reduction in body weight change is observed in HD-Met animals compared to the controls. * $p<0.05$ compared to control group.

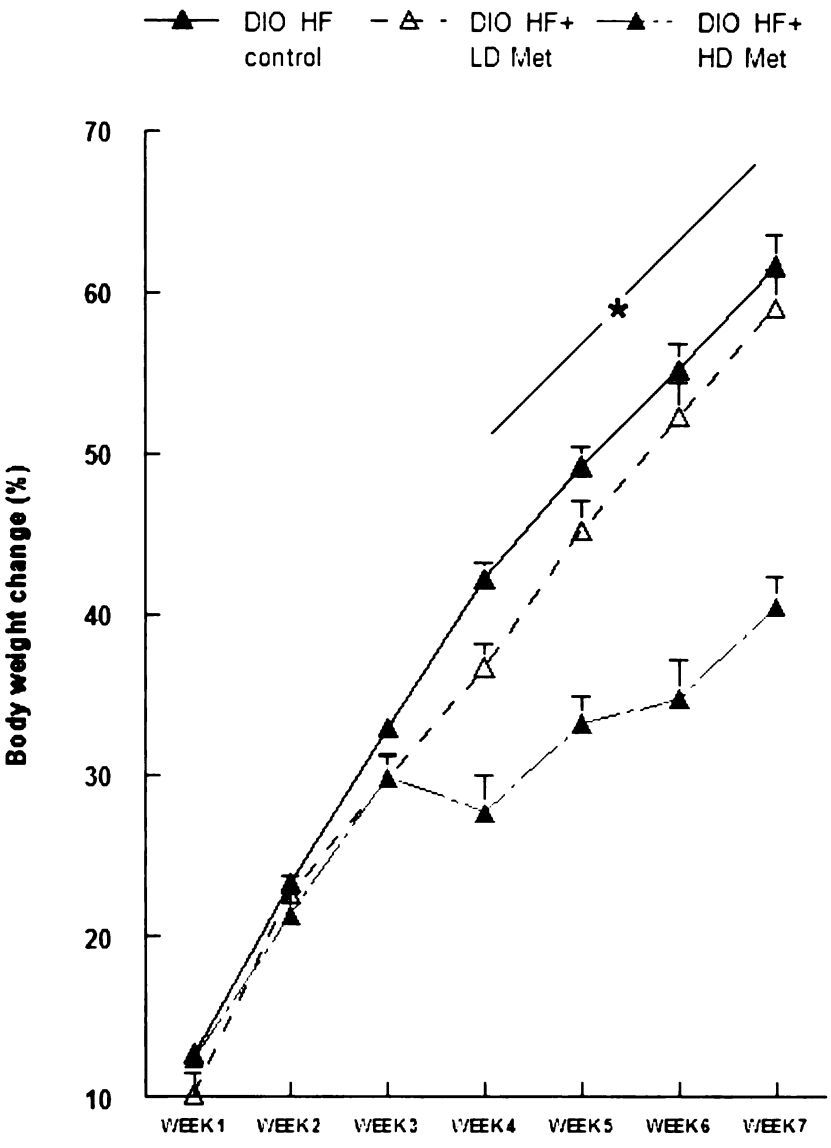


Fig. 5-2B. Body weight change in percent (Mean±SE; %) in HF-fed animals. A significant decline in weight was observed in HD-Met animals compared to the HF-fed control group starting at week 4. No statistically significant difference was observed between the LD-Met animals and the control group. * $p<0.05$ between HD-Met animals and the control group.

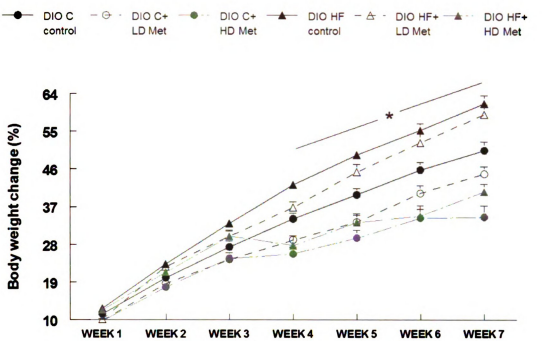


Fig. 5-2C. Body weight change in percent (Mean±SE; %) in all six groups combined. Statistical difference was observed starting at week 4 (a week after beginning metformin treatment). Chow-fed metformin-treated animals had significantly lower weight changes compared to the chow-fed control group. Only the HF-fed HD-Met animals had significantly lower weight changes compared to the HF control group. * $p<0.05$ between metformin-treated and respective control animals with the exception of HF-fed LD-Met animals.

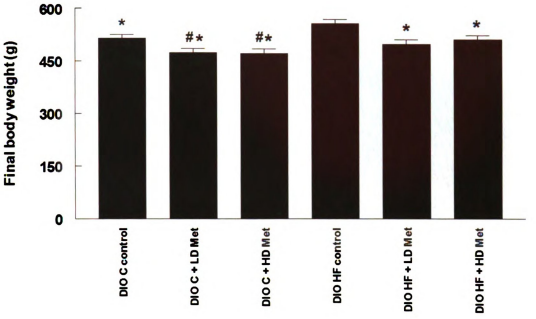


Fig. 5-2D. Final body weight (Mean±SE; g) in all six groups after dietary and metformin treatments. Metformin-treated groups had significantly lower final body weight compared to their respective controls. Moreover, HF-fed control group had significantly higher final body weight compared to chow-fed animals. * $p<0.05$ compared to DIO HF control; # $p<0.05$ compared to DIO chow control.

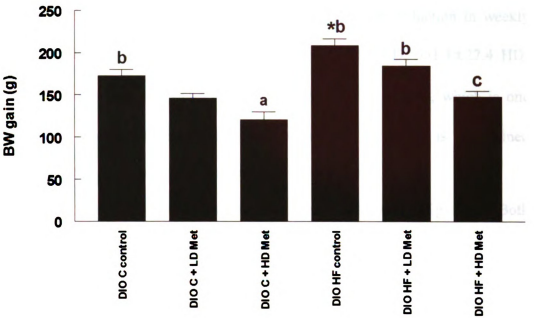


Fig. 5-2E. Body weight gain (Mean±SE; g) in all groups after dietary and metformin treatments. HF-fed DIO contol group had significantly higher body weight gain compared to the rest of the groups. Likewise, DIO C+HD Met group had significantly lower weight gain compared to the rest of the groups. Chow and HF-fed metformin-treated groups had significantly reduced weight gain compared to their respective control group. * $p<0.05$ compared to rest of the groups; [a] $p<0.05$ compared to rest of the groups; [b] $p<0.05$ compared to DIO C+LD Met group; [c] $p<0.05$ compared to DIO HF+LD Met group.

Caloric Intake

Weekly and total caloric intake for chow-fed groups (Mean±SE; Kcal) are shown in Fig. 5-3 A and B. There was a significant reduction in weekly caloric intake in metformin-treated groups (566.3±21.2 LD; 551.3±22.4 HD) compared to the control group (647.0±22.0; $p<0.05$) at week 4, which is one week after metformin exposure. This significant reduction is maintained throughout the metformin treatment period (Fig. 5-3A; $p<0.05$).

The total caloric intake for chow-fed groups is shown in Fig. 5-3B. Both LD (4086.2±88.5) and HD (4028.9±115.9) metformin-treated groups had significantly lower total caloric intake compared to the control group (4378.1±83.7; $p<0.05$). There was no difference between metformin-treated groups.

Weekly and total caloric intake for HF-fed groups (Mean±SE; Kcal) are shown in Fig. 5-4A and B. As in the chow groups, at week 4 and onwards, there was a significant reduction in weekly caloric intake in both LD and HD metformin-treated animals compared to the HF control animals ($p<0.05$). The consistent reduction in caloric intake was greater in the HD-Met animals, but it was not statistically significant (Fig. 5-4A).

Fig. 5-4B shows the total caloric intake for HF-fed groups. Treatment with metformin, both LD (4541.2±117.7) and HD (4616.9±76.3) significantly lowered total caloric intake compared to HF-fed controls (5141.3±123.7; $p<0.05$).

There was no statistical difference between LD and HD metformin-treated animals.

Fig. 5-5A and B shows weekly caloric intake and total caloric intake (Mean±SE; Kcal) in both chow and HF-fed groups combined. Significant differences were observed between metformin-treated animals and their respective control group starting at week 4 (Fig. 5-5A; $p<0.05$). HF-fed control group (5141.3±123.7) had higher total caloric intake compared to the chow-fed control group (4378.1±83.7; $p<0.05$), and the same applies to HF-fed metformin-treated groups ($p<0.05$). Chow-fed metformin-treated groups had significantly lower total caloric intake compared to the rest of the groups ($p<0.05$).

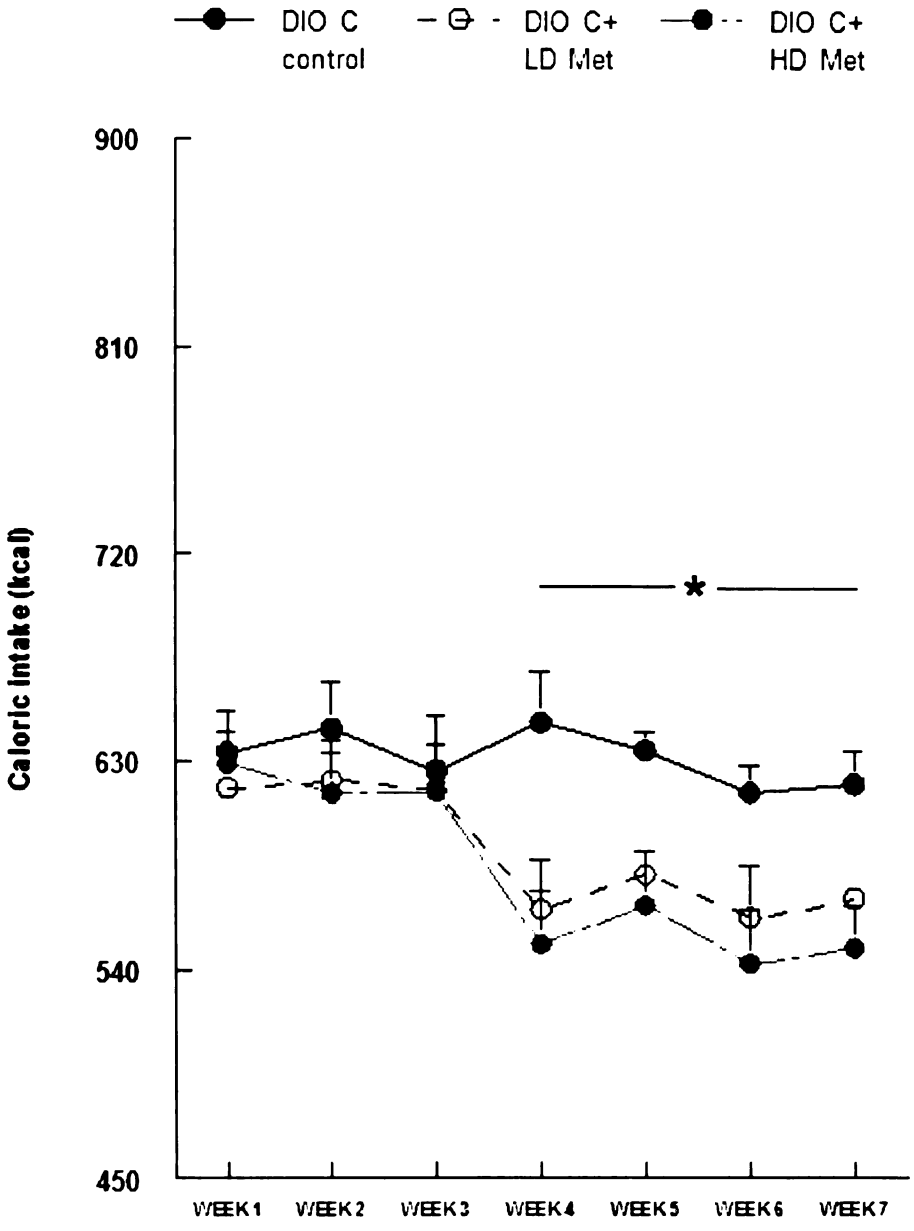


Fig. 5-3A. Weekly caloric intake (Mean±SE; Kcal) in chow-fed groups. Note the significant reduction in caloric intake between metformin-treated animals and the control group starting at week 4. HD metformin tended to decrease the caloric intake more, but it was not statistically significant. * $p<0.05$ between metformin-treated animals and control animals.

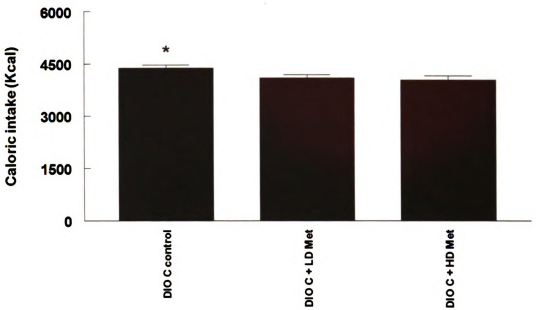


Fig. 5-3B. Total caloric intake (Mean±SE; Kcal) in chow-fed DIO groups. A significant reduction of total caloric intake was observed in metformin-treated animals compared to the control group. No difference was found between low-dose and high-dose metformin-treated animals. * $p<0.05$ compared to metformin-treated groups.

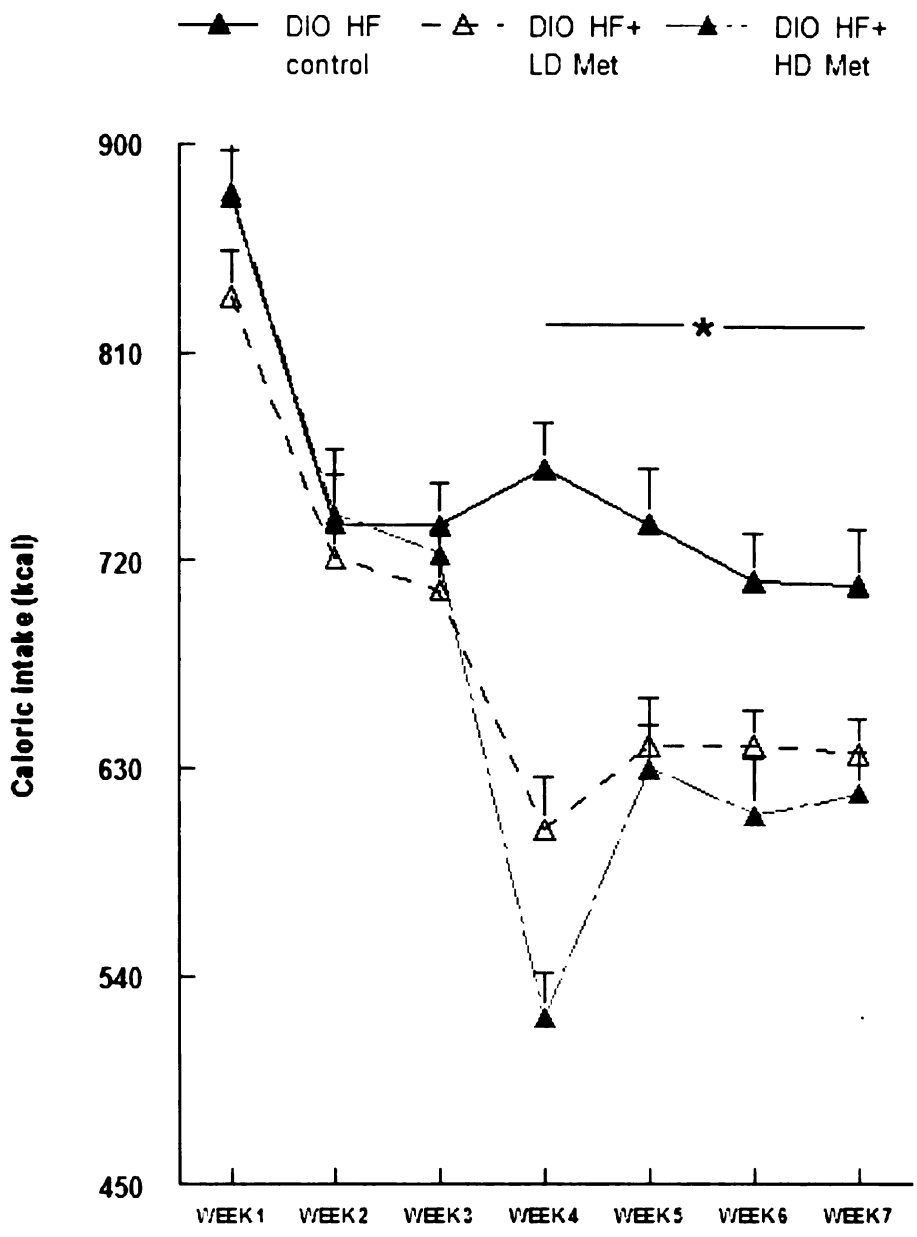


Fig. 5-4A. Weekly caloric intake (Mean±SE; Kcal) of HF-fed groups. Note the significant reduction of caloric intake between metformin-treated animals and control group starting at week 4. High-dose of metformin tended to reduce the caloric intake to a greater degree, but it was not statistically significant. * $p<0.05$ between metformin-treated animals and control animals.

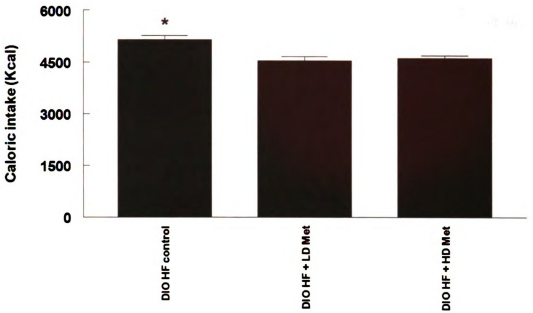


Fig. 5-4B. Total caloric intake (Mean±SE; Kcal) in HF-fed DIO groups. A significant decrease in total caloric intake was observed in metformin-treated animals compared to the control group. No difference was found between LD and HD metformin-treated animals. * $p<0.05$ compared to metformin-treated groups.

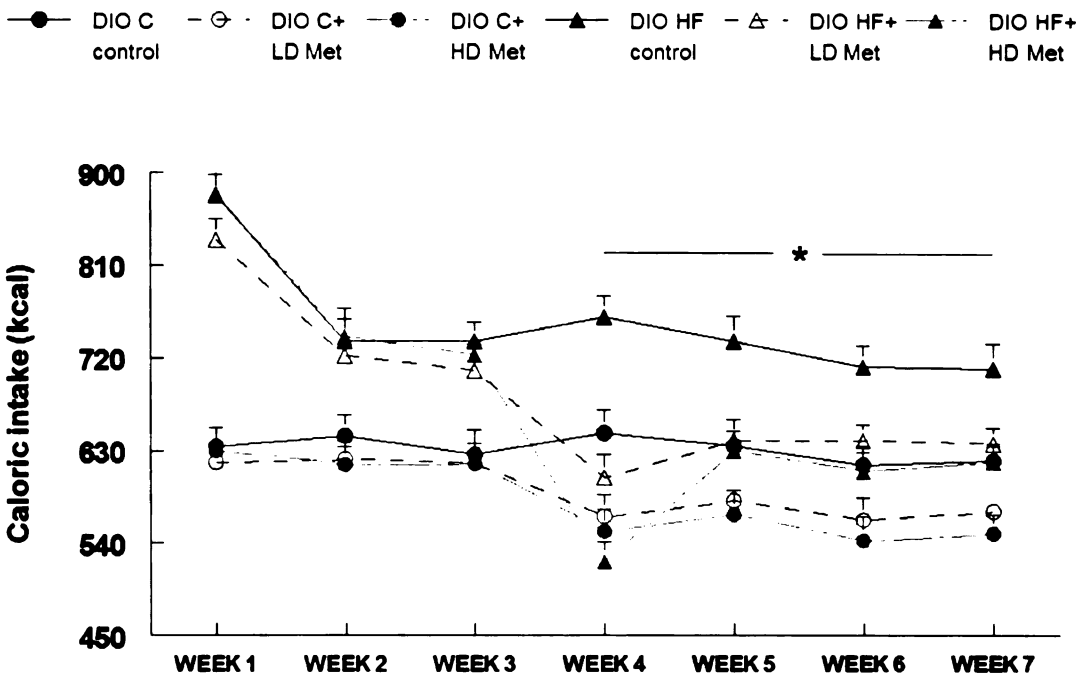


Fig. 5-5A. Weekly caloric intake (Mean±SE; Kcal) of both chow and HF-fed groups. Note the significant reduction of caloric intake between metformin-treated animals and their respective control group starting at week 4. HD metformin tended to reduce the caloric intake to a greater degree, but it was not statistically significant. * $p<0.05$ between metformin-treated animals and their respective control animals.

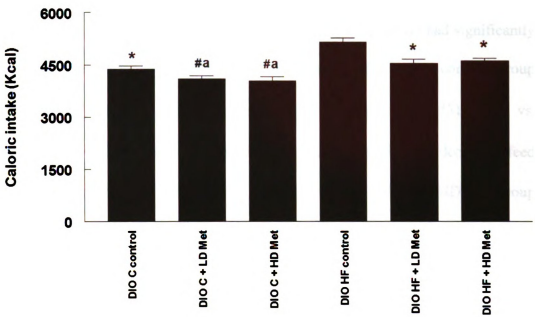


Fig. 5-5B. Total caloric intake (Mean±SE; Kcal) in both chow and HF-fed DIO groups. Chow-fed control group and HF-fed metformin-treated groups had significantly lower total caloric intake compared to HF-fed control group. Likewise, chow-fed metformin-treated groups had significantly decreased caloric intake compared to all HF-fed groups. No difference was found between the respective LD and HD metformin-treated animals. * $p<0.05$ compared to DIO HF control; # $p<0.05$ compared to DIO C contol; [a] $p<0.05$ compared to all HF-fed groups.

Feed efficiency

Feed efficiency (Mean±SE; g/kcal*10^3) was calculated as body weight gain in grams divided by the energy consumption in kcal over the observation period (Fig. 5-6). Chow-fed high-dose metformin-treated group had significantly reduced feed efficiency (29.4±1.9) compared to the chow control group (37.4±1.0; $p<0.05$). The same was true for HF-fed animals (31.1±1.0 vs. 41.5±0.9; $p<0.05$). Chow-fed control group also had significantly decreased feed efficiency compared to HF-fed control group ($p<0.05$). DIO HF+HD Met group showed reduced feed efficiency compard to the HF-fed low-dose group (38.9±0.7; $p<0.05$).

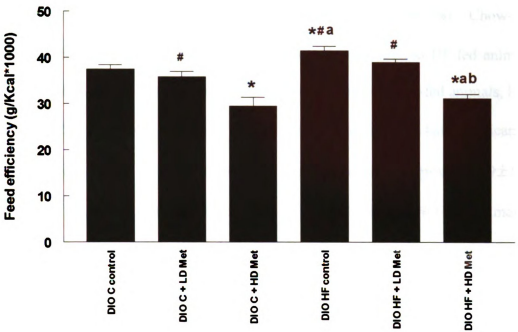


Fig. 5-6. Feed efficiency (Mean±SE; g/kcal*10^3) was calculated as body weight gain in grams divided by the energy consumption in kcal over the observation period. Chow-fed controls had significantly reduced feed efficiency compared to HF-fed control group. DIO HF+HD Met group showed significant decrease compared to the corresponding control group. DIO C+HD Met group had the lowest feed efficiency among the groups. * $p<0.05$ compared to DIO C control; # $p<0.05$ compared to DIO C+HD Met group; $^{a}p<0.05$ compared to DIO C+LD Met group; $^{b}p<0.05$ compared to rest of DIO HF groups.

Adipose tissue

Fig. 5-7A and B shows total visceral fat tissue (Mean±SE; g) and adipose tissue (AW) to body weight (BW) ratio in percent (Mean±SE; %). Chow-fed animals had significantly reduced visceral fat pads compared to HF-fed animals ($p<0.05$; Fig. 5-7A). While there was no difference within chow-fed animals, HF-fed LD (25.9±1.8) or HD (27.6±1.6) metformin-treated animals had significantly reduced visceral fat depots compared to the HF-fed control group (33.9±1.7; $p<0.05$). There was no difference between HF-fed LD-Met and HD-Met animals.

The same pattern was observed when the amount of visceral fat was normalized to body weight (AW/BW; Fig. 5-7B). Chow-fed animals had significantly reduced visceral fat pads compared to the HF-fed animals ($p<0.05$). HF-fed low-dose (5.2±0.3) or high-dose (5.5±0.2) metformin-treated animals had significantly reduced ratio compared to the HF-fed control group (6.2±0.2; $p<0.05$). Low-dose HF-fed animals were not statistically different from high-dose HF-fed animals.

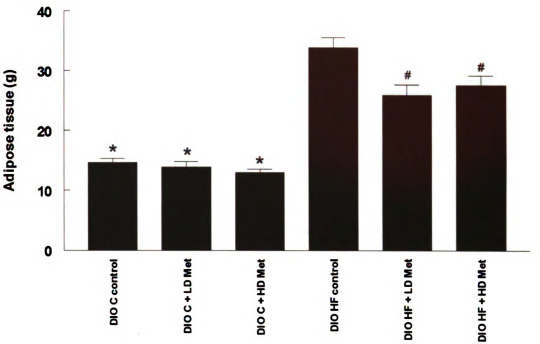


Fig. 5-7A. Adipose tissue (Mean±SE; g) in chow and HF-fed groups. Visceral adipose tissue including perirenal, epididymal, and retroperitoneal fat depots were collected after sacrifice and weighed as a whole. Note the significant reduction of fat pads in chow-fed groups compared to HF-fed groups. HF-fed metformin-treated animals had significantly decreased visceral adipose tissue compared to the control group. * $p<0.05$ compared to HF-fed groups; # $p<0.05$ compared to DIO HF control.

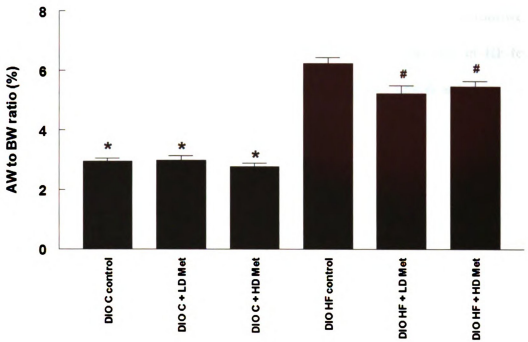


Fig. 5-7B. Amount of visceral adipose tissue (AW) normalized to final body weight (BW; Mean±SE; %). The ratio significantly decreased in chow-fed groups compared to the HF-fed groups. Likewise, HF-fed metformin-treated groups had significantly reduced ratio compared to the HF-fed control group. * $p<0.05$ compared to HF-fed groups; # $p<0.05$ compared to DIO HF control.

Serum Glucose

Serum glucose (Mean±SE; mg/dl) is shown in Fig. 5-8. No significant difference was observed between any groups after analysis by ANOVA followed by post-hoc LSD test. Although glucose levels tended to increase in HF-fed groups compared to chow-fed groups, the difference was not significant.

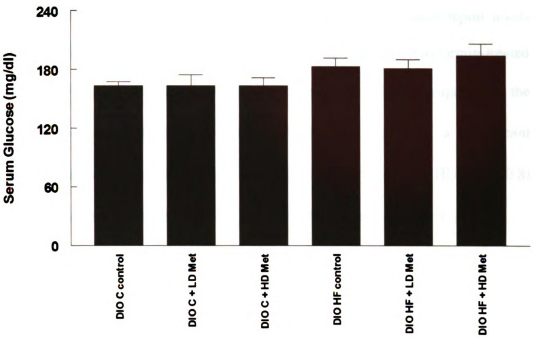


Fig. 5-8. Serum glucose levels (Mean±SE; mg/dl) in chow and HF-fed groups. There was no statistically significant difference between any of the groups. Metformin did not produce any significant effect on serum glucose levels.

Leptin

Serum leptin levels (Mean±SE; ng/ml) in chow and HF-fed animals are shown in Fig. 5-9. Chow-fed animals had significantly reduced leptin levels compared to the HF-fed groups ($p<0.05$). Chow-fed high-dose metformin-treated group had significantly lower serum leptin levels (2.7±0.4) compared to the chow-fed controls (4.0±0.4; $p<0.05$). Moreover, there was a significant reduction in serum leptin levels in HF-fed LD (5.6±0.4) and HD (6.4±0.8) metformin-treated animals compared to the HF-fed controls (9.3±0.4; $p<0.05$).

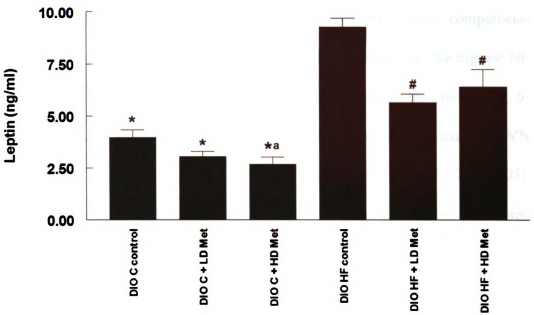


Fig. 5-9. Serum leptin levels (Mean±SE; ng/ml) in chow and HF-fed animals. Chow-fed animals had significantly lower leptin levels compared to the HF-fed groups. Note there was a significant reduction in serum leptin levels in HF-fed metformin-treated animals compared to the HF-fed controls. * $p<0.05$ compared to HF-fed groups; # $p<0.05$ compared to DIO HF control; [a]$p<0.05$ compared to DIO C control.

HPA indices – PVN NE, ME CRH, and serum CORT

Fig. 5-10 A to C shows PVN NE (Mean±SE; pg/ug protein), ME CRH (Mean±SE; pg/ug protein), and serum CORT (Mean±SE; ng/ml) comparisons between chow-fed and HF-fed groups. HF-fed control group had the highest NE concentrations in the PVN (28.4±2.4) compared to the rest of the groups (Fig. 5-10A; $p<0.05$). DIO chow+HD Met group showed a significant reduction in PVN NE (14.3±1.8) compared to the chow-fed control group (20.2±2.2; $p<0.05$). Also, there was a significant reduction in PVN NE concentrations in HF-fed groups following low-dose metformin (19.8±1.2) compared to HF-fed control group ($p<0.05$). Increased dose of metformin significantly reduced the PVN NE levels in HF-fed groups (12.4±1.1; $p<0.05$).

ME CRH protein levels are shown in Fig. 5-10B. Chow animals exposed to metformin had reduced CRH levels (22.6±1.3 for low-dose; 21.3±2.3 for high-dose) compared to the control group (32.0±2.4; $p<0.05$). DIO HF+HD Met group had significantly reduced CRH levels (20.7±3.0) in the ME compared to both chow and HF-fed controls (32.0±2.4 for chow controls; 30.0±5.2 for HF controls; $p<0.05$). There was no difference between chow and HF-fed control groups.

Serum CORT levels are shown in Fig. 5-10C. High-dose metformin in both chow and HF-fed groups induced a significant decrease in serum CORT levels (180.0±20.4 and 198.9±10.0) compared to the respective control groups (271.7±17.0 and 301.5±32.6, respectively; $p<0.05$). However, there was no

difference between animals with different doses. Also, no difference was found between chow and HF-fed control groups.

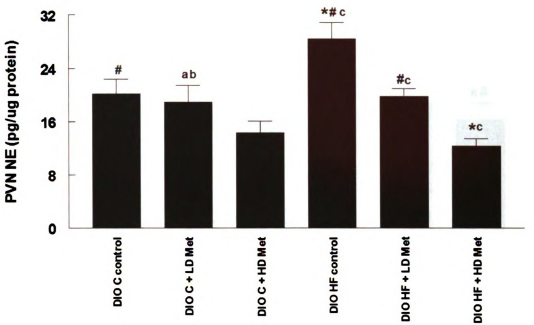


Fig. 5-10A. PVN NE levels (Mean±SE; pg/ug protein) in both chow and HF-fed groups. HF-fed control group had the highest NE concentrations in the PVN compared to the rest of the groups. Note there was a significant reduction in PVN NE concentrations in HF-fed groups following metformin exposure compared to HF-fed control group. Increased dose of metformin significantly reduced the PVN NE levels in HF-fed groups. * $p<0.05$ compared to DIO C control; # $p<0.05$ compared to DIO C+HD Met; $^{a}p<0.05$ compared to DIO HF control; $^{b}p<0.05$ compared to DIO HF+HD Met; $^{c}p<0.05$ compared to each other.

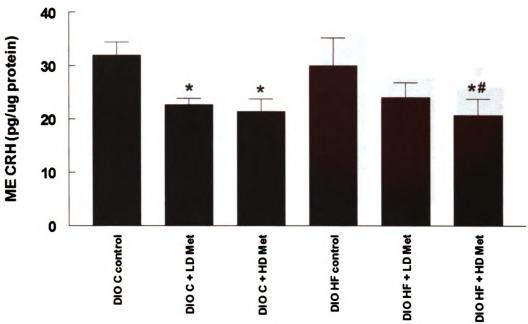


Fig. 5-10B. ME CRH protein levels (Mean±SE; pg/ug protein) in chow and HF-fed groups. CRH was measured by ELISA and analyzed by ANOVA followed by post-hoc LSD test. Chow-fed animals exposed to metformin had reduced CRH levels compared to the control group. DIO HF+HD Met group had significantly lower CRH levels in the ME compared to both chow and HF-fed controls. As in previous Chapters, there was no difference between chow and HF-fed control groups. *$p<0.05$ compared to DIO C control; # $p<0.05$ compared to DIO HF control.

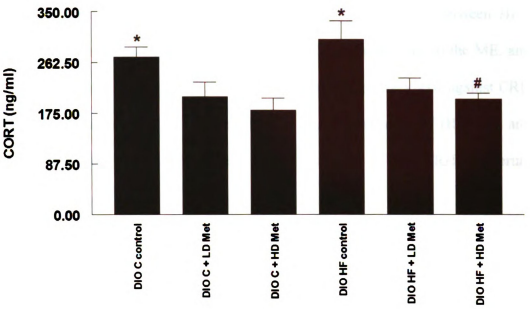


Fig. 5-10C. Serum corticosterone levels (CORT; Mean±SE; ng/ml) in chow and HF-fed groups. Serum was collected from the trunk blood at sacrifice and CORT was measured by RIA. High-dose metformin in both chow and HF-fed groups induced a significant decrease in serum CORT levels compared to the respective control groups. Note that there was no difference between animals with different doses. Also, no difference was found between chow and HF-fed control groups. * $p<0.05$ compared to DIO C+HD Met; # $p<0.05$ compared to DIO HF control.

Relationship between HPA indices in Sprague-Dawley, DR rats and DIO rats

The some of the following data were borrowed from Chapter 4. Type II regression analyses were performed to explore the relationships between HPA axis indices, namely, NE levels in the PVN, CRH concentrations in the ME, and serum CORT (Fig. 5-11A to E). NE values are plotted on the x-axis against CRH and CORT values due to the causal effect of NE on other arms of HPA axis and tested how well PVN NE levels can predict the levels of ME CRH and serum CORT.

Sprague-Dawley male rats were treated with saline, 100μg, or 500μg of rat recombinant leptin i.p., and PVN NE concentrations and serum CORT were measured in the previous study by Dr. Kimberly A. Clark et el. in 2006.

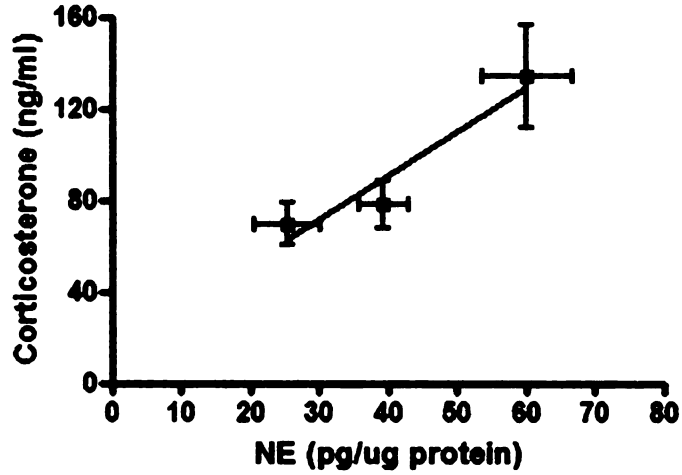
The relationship between PVN NE and CORT (Fig. 5-11A) showed a positive association without much variation. These associations were not statistically significant, however.

Fig. 5-11B and C shows relationships between NE and CRH, or NE and CORT in DR animals (Refer to Chapter 4). These rats also show a positive association with very less variation. In fact, regression coefficient was close to 1 (r^2=0.99, F=272.4, p=0.0037; r^2=0.99, F=178.4, p=0.0056 for NE:CRH and NE:CORT, respectively) where increased PVN NE concentrations were statistically strongly associated with both elevated CRH and CORT.

Fig. 5-11D and E shows relationships between NE and CRH, or NE and CORT in HF-fed DIO animals (from Chapter 4) or HF-fed metformin-treated animals. The metformin-treated DIO animals showed a positive association

between the variables with much less variation (r^2=0.99, F=69.8, p=0.07; r^2=0.89, F=8.425, p=0.21 for NE:CRH and NE:CORT, respectively) compared to HF-fed DIO animals (r^2=0.0017, F=0.0035, p=0.96; r^2=0.18, F=0.44, p=0.58; NE:CRH and NE:CORT, respectively). The slopes of regression lines between the two groups were not significantly different, most likely because of large variations in HF-fed DIO group. The PVN NE values were able to better predict the CRH or CORT values in metformin-treated animals. The regression line slopes also resembled those of Sprague-Dawley or DR rats (Fig. 11-A to C).

A.



B.

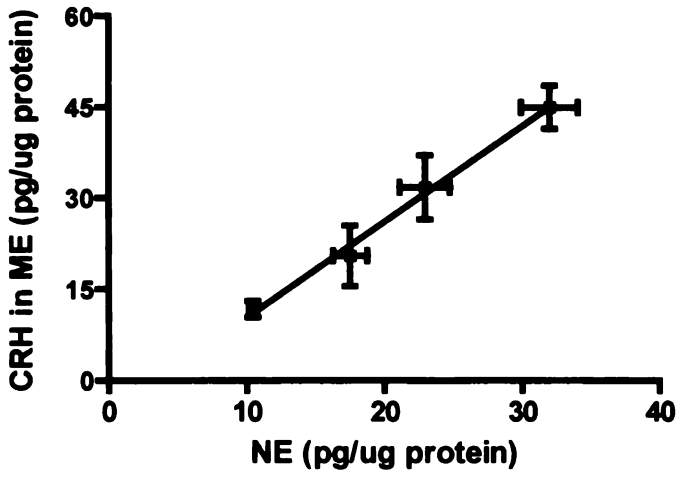
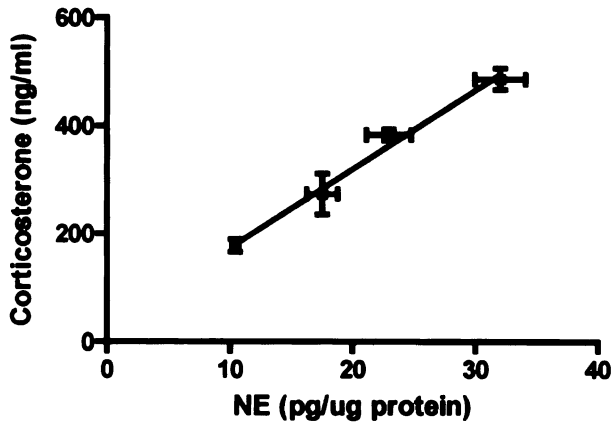


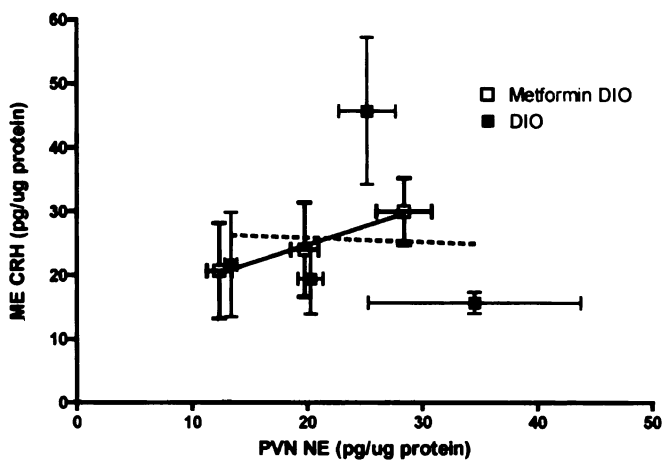
Fig. 5-11. The relationships between PVN NE, ME CRH, and serum CORT in Sprague Dawley & DR (from Chapter 4), or DIO animals are shown. A-C shows how well NE values can predict CRH and CORT values in either Sprague Dawley or DR rats. D-E shows the same relationships in either HF-fed DIO animals (Chapter 4; dashed line) or HF-fed metformin-treated animals (solid line). The relationships observed in metformin-treated DIO group show positive associations. PVN NE in metformin-treated DIO group can predict better the values of CRH or CORT than that of HF-fed DIO group, and the predictions more resemble those of Sprague-Dawley or DR rats.

Fig. 5-11 continued

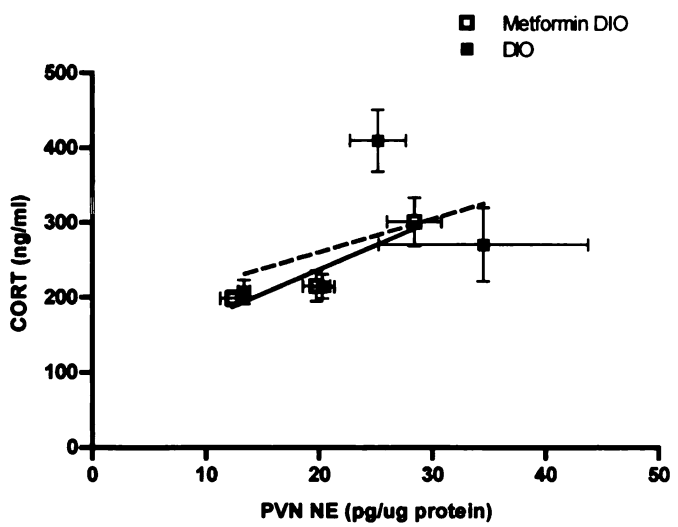
C.



D.



E.



Protein expression analysis of pSTAT-3 in the brainstem

pSTAT-3 protein expression in A1: Protein expression of pSTAT-3 in the A1 (VLM) noradrenergic region of the brainstem is shown in Fig. 5-12A. It is expressed as values of optical density after chow-DIO group value was normalized to 1.0. No difference was found between chow-fed animals. There was a trend of increased expression in HF-fed groups exposed to metformin treatment, but it was not statistically significant due to the large variability. Also, no difference was found between different dietary groups.

pSTAT-3 protein expression in A2: Fig. 5-12B shows pSTAT-3 protein expression in A2 (NTS) region of chow and HF-fed DIO animals analyzed by densitometry. There was no effect of metformin on pSTAT-3 expression.

pSTAT-3 protein expression in A6: The expression in A6 (LC) noradrenergic region of the braintem of chow and HF-fed DIO animals is shown in Fig. 5-12C. No statistical difference was found between any of the groups. There was a tendency for increased expression of pSTAT-3 in DIO HF+HD Met group compared to the HF-fed controls, but the difference was not significant.

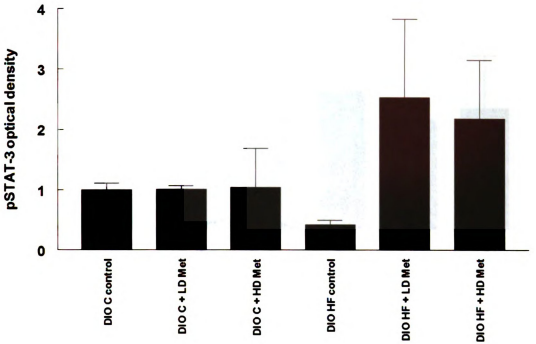


Fig. 5-12A. pSTAT-3 protein expression in the A1 noradrenergic region of the brainstem in chow and HF-fed DIO groups. Protein was detected by western blot and analayzed by densitometry. In spite of a trend of increased pSTAT-3 expression in HF-fed metformin-treated groups compared to HF-fed controls, the difference was not significant.

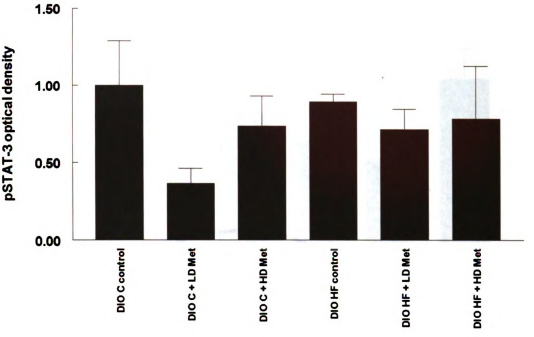


Fig. 5-12B. pSTAT-3 protein expression in A2 noradrenergic region of the brainstem in chow and HF-fed DIO groups. The protein was detected by western blot and analayzed by densitometry. No difference was found between groups.

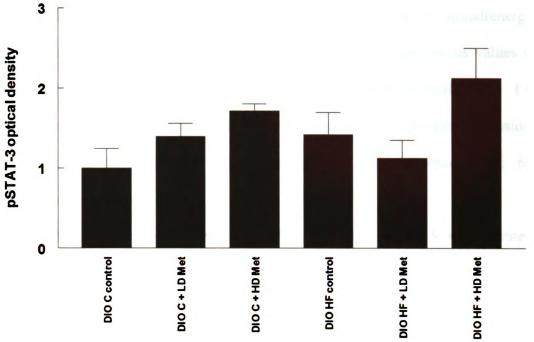


Fig. 5-12C. pSTAT-3 protein expression in the A6 noradrenergic region of the brainstem in chow and HF-fed DIO groups. The protein was detected by western blot and analayzed by densitometry. There was a trend of increased expression after high-dose of metformin treatment in HF-fed animals, but the difference was not significant.

Protein expression analysis of SOCS-3 in the brainstem

SOCS-3 protein expression in A1: Protein expression of the negative feedback inhibitor of leptin signaling, SOCS-3, in the A1 (VLM) noradrenergic region of the brainstem is shown in Fig. 5-13A. It is expressed as values of optical density after the chow-DIO group values were normalized to 1.0. Metformin-treated animals tended to have lower SOCS-3 protein expressions compared to the respective control groups, but the differences were not statistically significant.

SOCS-3 protein expression in A2: Fig. 5-13B shows SOCS-3 protein expression in A2 (NTS) region of chow and HF-fed DIO animals analyzed by densitometry. No difference was found between groups.

SOCS-3 protein expression in A6: The SOCS-3 expression in A6 (LC) noradrenergic region of the braintem in chow and HF-fed DIO animals is shown in Fig. 5-13C. No statistical difference was found between any of the groups.

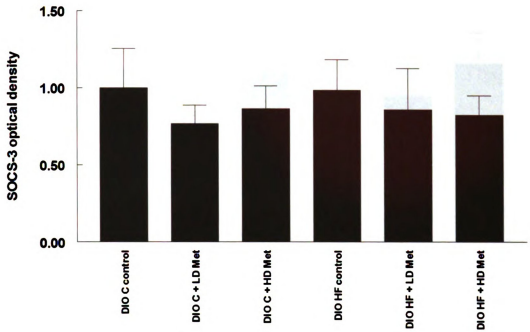


Fig. 5-13A. SOCS-3 expression in the A1 (VLM) noradrenergic region of the brainstem. Metformin-treated animals tended to have lower SOCS-3 protein expressions compared to the respective control groups, but the differences were not statistically significant.

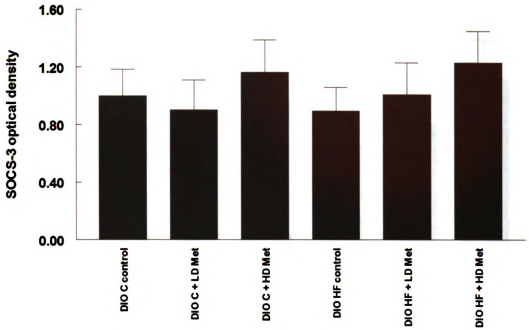


Fig. 5-13B. SOCS-3 protein expression in the A2 (NTS) region of chow and HF-fed DIO animals. SOCS-3 levels were measured by western blotting followed by densitometry. No difference was found between groups.

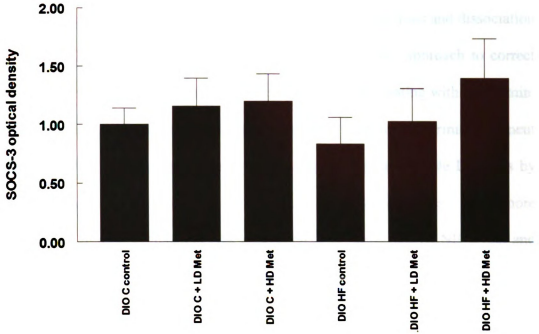


Fig. 5-13C. SOCS-3 protein expression in the A6 (LC) noradrenergic region of the brain stem in chow and HF-fed DIO animals. No statistical difference was found between any of the groups.

D. Discussion

The purpose of the current study was to extend on the previous findings (Chapter 3 and 4) on leptin insensitivity in noradrenergic neurons and dissociation between NE and the HPA axis, and propose a mechanistic approach to correct these abnormalities and the resulting obese phenotype by treating with metformin. The current findings show for the first time that chronic metformin treatment partially reversed the obese phenotype of polygenically susceptible DIO rats by reducing body weight gain, caloric intake, and visceral fat depots. A far more interesting finding is that this was associated with correction of NE levels and HPA axis function independent of pSTAT-3 signaling in brainstem noradrenergic neurons.

Throughout these series of studies, it was assuring that the measurements of parameters such as body weight, visceral fat, caloric intake, central pepdide or serum hormone levels were reproducible between chow-fed and HF-fed DIO animals. The HF-fed DIO control group had markedly high weekly caloric intake compared to the chow-fed control group (Fig. 5-5A). There was a sudden decrease in caloric intake starting on week 2 in HF-fed DIO controls while chow-fed controls maintained the amount of consumption. This could be a compensatory mechanism accommodating the increased energy density in the HF diet. Nevertheless, the caloric intake in HF-fed controls was never close to the amount consumed by chow-fed controls. Accompanying the increased total caloric intake (Fig. 5-5B) was increased body weight gain (Fig. 5-2E) and visceral fat (Fig. 5-7A) at the end of 7 weeks compared to chow-fed controls. Increased

body weight in these animals can be attributed to increased caloric intake, but more importantly, increased feed efficiency (Fig. 5-6), indicating that these animals are metabolically more efficient than chow-fed controls. The bulk of the increased energy retention in HF-fed controls was mainly deposited in adipose stores, as shown by markedly increased weight of visceral fat depots. These are in line with previous findings in Chapter 4 and suggest that the HF diet exposure results in increased food consumption, and anabolic efficacy mainly in adipose depots, resulting in the obese phenotype in DIO rats.

Metformin is widely known as an insulin-sensitizing agent that has shown promise in enhancing insulin sensitivity, improving lipid profile, and reducing body weight in both obese humans and rodent models (125-133). Recent findings by Kim et al. shed light on possible mechanisms by which metformin elicits its beneficial effects in obesity (134). In that study, diet-induced obese Sprague-Dawley male rats had a marked reduction in body weight, food intake, and retroperitoneal fat deposition after leptin injections following treatment with metformin for 4 weeks. These changes were associated with improved leptin signaling as indicated by increased POMC mRNA expression in a chronic experiment as well as increased pSTAT-3 protein expression in an acute metfomin experiment in the mediobasal hypothalamus. Since metformin can improve leptin sensitivity in the hypothalamus, it is also likely that metformin can do the same in brainstem noradrenergic neurons. This could reduce noradrenergic activity and restore the responsiveness of the HPA axis to NE levels in HF-fed polygenic DIO rats. Results from the current study show that metformin indeed

reduced final body weight, weight gain, caloric intake, visceral fat depots, and feed efficiency, both in chow and HF-fed DIO animals. This was associated with reduced PVN noradrenergic levels and improved the responsiveness of CRH neurons to NE levels. The following paragraphs will discuss several possible mechanisms which could have contributed to these improvements after metformin treatment.

Noradrenergic function measured by NE concentrations in the PVN was reduced after metformin treatment (Fig. 5-10A). The decrease was more pronounced at higher doses of metformin in both chow and HF-fed DIO animals. Besides its role as an insulin-sensitizer, metformin also has been shown to play a role in regulation of the autonomic nervous system (181-183). It is able to directly inhibit the sympathetic nerve activity and decrease arterial blood pressure (181). Although it remains to be shown, these depressor effects may be mediated by either peripheral or central actions. Peripheral mechanisms could include modulation of ganglionic transmission or direct vasodilation/inhibition of vasoconstriction. Central effects could include alterations in receptor sensitivity or sympathetic neurotransmitter or other peptide activities. This sympathoinhibitory effect of metformin may explain the reduction in noradrenergic function in the PVN since central NE serves as an important activator of the sympathetic nervous system. NE levels in the PVN, on the other hand, are also associated with feeding behavior (174). NE injections into the PVN and treatment with α-2 adrenergic agonists such as clonidine are known to stimulate feeding (175-177). Moreover, lesioning of noradrenergic fibers that

innervate the PVN have suppressed feeding behavior, suggesting that NE levels in the PVN are critical for feeding (178). The metformin-induced reduction in PVN NE in DIO animals may explain the decrease in caloric intake and subsequent reduction in body weight gain and visceral fat depots. Leptin levels decreased probably because of significant reduction in fat mass, especially in HF-fed DIO animals. With this interpretation, it is possible that the metformin decreased PVN NE independent of the suppressive action of leptin.

On the other hand, there may be an indirect action of metformin that can decrease NE concentrations in the PVN in these animals. Even though the precise mechanisms by which metformin increases insulin sensitivity is not clear, studies indicate that metformin reduces free fatty acid (FFA) concentrations in the circulation, possibly by inhibiting lipolysis in adipose tissues (184-186). Obese subjects have large central fat stores resulting from an overload of abdominal adipocytes with triglycerides. In addition to the high basal lipolysis and increased sensitivity to fat-mobilizing hormones, the enlarged visceral fat cells release abundant FFA into the portal circulation. This exposes the non-adipose tissues like skeletal muscle, liver, and pancreas to the FFA excess, which will accumulate in these tissues and develop insulin resistance and cause pancreatic beta cells to become dysfunctional (187). That could be a possible mechanism by which metformin or other therapeutic drugs could improve insulin sensitivity through suppression of fat mobilization and the release of FFA.

This inhibitory action of metformin is of importance in metabolic diseases including obesity when we consider possible detrimental effects of circulating

lipids, in the form of FFA or triglycerides. Circulating lipids are known to disrupt the peripheral metabolic and central homesostatic processes that can consequently lead to excess caloric consumption and body weight gain. They can interfere with normal physiological processes by affecting neuropeptides or neurotransmitters in the brain (188). Thus, it is feasible that FFA can enhance the noradrenergic function in the PVN to increase feeding behavior. This idea is supported by evidence showing increased sympathetic nervous system activity along with increased HPA axis activation following excess portal fatty acids supply, indicating that the action of neurotransmitters that regulate sympathetic tone such as NE may be upregulated by excess circulating lipids (189). Even though we did not measure it in this study, the chronically HF-fed DIO control animals are very likely to have elevated circulating FFA concentrations, which may be partially responsible for inducing/promoting increased NE concentrations in the PVN and subsequently increase caloric intake and body weight. Hence, the reduction of PVN NE and the HPA axis observed in metformin-treated DIO animals can be logically explained by the inhibition of lipolysis and improvement of the lipid profile by metformin treatment.

One interesting finding was the lack of decrease in serum glucose levels (Fig. 5-8). Even though the possibility that metformin may have differential or more pronounced peripheral effects depending on longer duration or higher doses cannot be ruled out, it is unlikely that glucose metabolism was not influenced by metformin. A more practical explanation is that the glucose measurement was made in serum samples and not in whole blood. Not only do we not know the

validity of this specific kit, but there is also no assurance of how well the serum glucose readings correlate with the readings from glucometer, which measures glucose from whole blood. These concerns need to be resolved before relying on the data with more confidence.

In Chapters 3 and 4, a clear dissociation between leptin and noradrenergic activity was observed in the brainstem as indicated by decreased pSTAT-3 expression and increased TH mRNA expression in A1 noradrenergic neurons. As hypothesized, the current study shows that the pSTAT-3 protein expression was elevated in only A1 region in metformin-treated HF-fed animals compared to the chow-fed controls, but it was not statistically different. The increase in leptin signaling makes sense, given the notion of leptin's suppressive effect, because there is a corresponding reduction in NE levels in the PVN, CRH levels in the ME, and serum CORT in HF-fed high-dose animals compared to chow-fed controls. An increase in sample size could reduce the variation within the groups. On the contrary, it is likely that there is no change in pSTAT-3 expression between groups. Or, the metformin-induced reduction of PVN NE may be mediated through another leptin signaling pathway that involves the induction of phosphatidylinositol-3 kinase (PI3K). Leptin is known to stimulate the PI3K activity in the hypothalamus, and PI3K inhibitors reverse the anorectic action of leptin (190, 191). The importance of this particular leptin signaling pathway is supported by evidence that showed impaired PI3K-induced leptin signal pathway during development of diet-induced obesity in mice (192). This suggests the importance of both pSTAT-3 and PI3K-mediated pathways for the induction of

leptin's anorectic effect. It further indicates a strong possibility for cross-talk and convergence between two distinct pathways. Therefore, it is possible that the reduced NE levels in the PVN in metformin-treated animals, produced as a result of decreased TH mRNA production in brainstem A1 region, is mediated through increased activation of PI3K, but not the pSTAT-3-mediated pathway. This may explain the increased levels of serum leptin and decreased NE, CRH, and CORT levels in DIO HF + HD Met group compared to the DIO Chow control group (Fig. 5-9 and 5-10). While HF-fed DIO controls have increased PVN NE levels due to leptin resistance in the brainstem A1 noradrenergic region (Chapter 4), metformin-treated HF-fed DIO animals had improved leptin sensitivity and lower noradrenergic activity. Lack of the inverse relationship in chow-fed metformin-treated groups like in HF-fed metformin treated groups may be because the leptin levels were not high enough in these animals to suppress noradrenergic neurons.

The effect of metformin on the neuroendocrine axis is much clearly illustrated in type II regression plots (Fig. 5-11). As seen in Chapter 4, normal Sprague-Dawley male rats show a positive relationship between PVN NE and serum CORT (Fig. 5-11A). The same is true with DR rats, showing a significantly positive relationship with very little variation between NE and CRH or CORT (Fig. 5-11B and C). Surprisingly, the relationships observed in metformin-treated DIO group show positive associations. NE levels in the PVN in metformin-treated DIO group can predict better the values of CRH or CORT than that of HF-fed DIO group. The predictions resemble those in Sprague-Dawley or DR rats. These findings indicate that metformin can restore the

association between NE and other arms of the HPA axis in DIO rats that are under chronic HF diet. The underlying mechanisms for the restoration are not clear. Since DIO rats have postsynaptic noradrenergic deficit in the PVN (156), it is possible that metformin corrected the deficit and improved α2-adrenoceptor binding capacity, thus re-establishing a proper connection between NE and CRH neurons in the PVN. It is also possible that the reduction in HPA axis activity may be independent of the decrease in NE. As mentioned before, lipids in the circulation are able to increase sympathetic tone as well as the HPA axis (189). Hence, metformin could have acted on different levels of the HPA axis – either on brainstem noradrenergic neurons, CRH neurons in the PVN, or directly on adrenal glands to reduce glucocorticoid production.

In summary, metformin treatment significantly reduced body weight, weight gain, caloric intake, amount of visceral fat, and feed efficiency in both chow and HF-fed DIO animals. Pronounced effects were seen with the higher dose of metformin. This was accompanied by a marked reduction in NE concentrations in the PVN as well as CRH levels in the ME and serum CORT. Most importantly, metformin treatment partially restored the relationship between NE and the HPA axis. However, this was independent of pSTAT-3 expression. Reversal of NE, CRH, and CORT levels and the restoration of the NE-HPA axis circuitry with metformin treatment may contribute to the partial reversal of the obese phenotype in HF-fed animals. This leaves a number of possibilities concerning the underlying mechanisms and sites of action of metformin at the central nervous system and the periphery. This will be the focus of future studies.

E. Summary

This chapter was designed to propose a potential therapeutic approach with minimal health risks by introducing a widely known insulin-sensitizing agent, metformin. In the last chapter, we found that HF diet exposure produces leptin resistance with concurrent increase in TH mRNA levels in brainstem A1 noradrenergic neurons. This was associated with leptin insensitivity in noradrenergic neurons, increased noradrenergic activity in the PVN, and the HPA axis, which may be responsible for development and promotion of obesity. Metformin has been recently discovered to have leptin-sensitizing properties. Thus, I hypothesized that metformin treatment in HF-fed DIO rats would restore the leptin sensitivity in the brainstem noradrenergic neurons and reduce PVN NE levels, as well as restore the HPA axis regulation. This may help correct the obese phenotype.

As in previous chapters, we were able to observe increased fat depots, body weight, caloric intake, and feed efficiency in the HF-fed control group. Moreover, serum leptin and CORT levels, NE levels in the PVN and CRH levels in the ME were consistently higher in chow and HF-fed control groups. Exposure to metformin for 4 weeks, however, decreased all of these parameters, with the effect being more pronounced after high-dose metformin treatment. Regardless of dietary treatment, metformin decreased body weight, visceral fat depots, caloric intake, and feed efficiency. This was associated with dramatically reduced serum leptin levels. Moreover, all arms of the HPA axis, including NE concentrations in the PVN, CRH levels in the ME, and serums CORT were

normalized with metformin treatment. Metformin brought about these effects without altering the pSTAT-3-mediated leptin signaling in brainstem noradrenergic neurons. There was also no change in SOCS-3 protein expression. The possible underlying mechanisms for metformin's action are not clear and may be at multiple levels in both periphery and the brain. Metformin may act directly at the level of the PVN in the hypothalamus to alter noradrenergic activity and decrease NE concentrations to reduce feeding. It may also restore the noradrenergic deficit present in the PVN in order to re-establish the association between NE and the HPA axis. At the level of brainstem noradrenergic regions, metformin may be selective and activate the PI3K-mediated pathway rather than the pSTAT-3 pathway to restore leptin sensitivity and reduce NE levels in the PVN and HPA axis activity. On the other hand, metformin may also act on visceral adipose stores to inhibit lipolysis and reduce circulating FFA concentrations that will lead to suppression of the sympathetic nervous system and the HPA axis. More questions need to be answered and the drug tested before used in clinical settings, but the current findings clearly shed light on the pathogenesis of obesity and establishes the beneficial effects of metformin in partial reversal of the neuroendocrine changes and the associated obese phenotype.

Chapter 6. Summary and Conclusions

The awareness of and participation in diverse weight loss programs and changes in lifestyles have increased recently since the prevelance of obesity has increased from 15.0% in 1980 to 32.9% in 2004. However, the obese population seems to increase every year (23). Developing robust research for understanding the mechanisms by which obesity develops is of the utmost importance.

The goal of the research described in this dissertation was to investigate the role of leptin, and brainstem noradrenergic neurons in the dysregulation of the HPA axis, a prominent pathophysiological hallmark, in diet-induced obesity. For this purpose, polygenically susceptible diet-induced obese (DIO) and diet-resistant (DR) rats were used and *in vivo* experiments were conducted.

The purpose of the first study was to characterize the changes in stress axis activity in DIO and DR rats after chronic exposure to a HF diet, and investigate the possible mechanisms that are involved in producing changes in the brain. Chapter 3 describes an experiment in which I showed that chronic HF diet results in increased weight gain, increased serum leptin levels in DIO rats. This was accompanied by elevated NE concentrations in the PVN, but there was no change in CRH concentrations in the ME or serum corticosterone levels compared to chow-fed DIO controls. On the other hand, HF-fed DR animals had low levels of serum leptin, but higher NE concentrations in the PVN, CRH levels in the ME and serum CORT compared to the chow-fed DR controls. This was the first study to characterize the changes in HPA axis activity in these selectively bred animals, and provide evidence that the HPA axis is dysregulated in DIO rats. Also, results

from this study demonstrated leptin resistance in the brainstem noradrenergic neurons in HF-fed DIO rats. This was supported by increased TH mRNA expression in brainstem A1 noradrenergic neurons in DIO rats after HF diet exposure. However, ObRb mRNA expression was not different between groups in all three brainstem noradrenregic regions, suggesting that the presumed leptin resistance is not due to downregulation of functional leptin receptors, but rather, it may be due to defective leptin signaling.

To further characterize the dysregulation of the HPA axis and to understand its onset, DIO rats were subjected to acute HF diet exposure as well as exogenous leptin treatment. DIO animals consumed more calories than DR animals, and HF diet exposure resulted in hyperphagia in both phenotypes. This was followed by increased weight gain and visceral fat depots. As expected, while DR rats showed an intact positive association between noradrenergic function in the PVN and the HPA axis in all treatments, DIO rats showed dissociation between these variables as indicated by continuous increase of NE in the PVN, but different levels of CRH and CORT. The dysregulation of the HPA axis became worsened following longer duration of HF diet exposure. However, these animals responded normally to exogenous leptin by reducing the PVN NE concentrations, indicating that it is only after HF treaetment that they develop leptin signal impairment in the brainstem noradrenergic neurons. This notion was supported by a significant decrease in pSTAT-3 protein expression in A1 noradrenergic neurons in chronically HF-fed DIO rats. This finding along with increased TH mRNA expression in the A1 region from the previous chapter

strongly supports the notion that normally leptin indeed suppresses NE functions, and confirms the relevance of leptin resistance in the selective brainstem noradrenergic neurons. Furthermore, the continuous increase in PVN NE levels and other HPA axis indices in DR animals with HF exposure suggest that HF diet may play a role as a stressor. It is very likely that on top of leptin resistance in the brainstem, DIO rats may perceive the HF diet as a stressor also, as indicated by the consistent elevation in NE levels in the PVN.

Now that neuroendocrine perturbation of the HPA axis and leptin resistance in the brainstem in HF-fed DIO rats are confirmed in detail, the obvious next step was to see if the association between leptin and noradrenergic function in the PVN can be restored by improving leptin sensitivity. Because the insulin-sensitizing agent metformin has been successful in promoting weight loss (129, 130) and has recently shown to increase leptin sensitivity in the hypothalamus of diet-induced obese rats (134), I hypothesized that metformin treatment will improve the leptin signaling in the brainstem noradrenergic neurons, which would correct the dissociation between leptin and PVN NE we observed in previous chapters. I also hypothesized that due to a number of beneficial effects in many physiological processes, metformin also may correct the HPA axis dysregulation and restore the association between NE and the HPA axis. DIO animals were divided into 6 groups, half of the groups being on chow and the other half on HF diet for 7 weeks. Low or high-dose metfomin treatment was initiated at the end of week 3 and terminated at the end of week 7, exposing the animals for 4 weeks of metformin. As shown previously, chow and HF-fed animals showed reproducible

distinctive patterns of weight gain, caloric intake, and visceral fat depots, as well as leptin and HPA axis indices. In support of current literatures, this study showed for the first time that metformin reduced the weight gain, caloric intake, visceral fat depots, and feed efficiency in these polygenetically susceptible DIO animals regardless of dietary regimen. High-dose metformin treatment produced more pronounced effects in all parameters measured. Decreased serum leptin levels following metformin treatment can be explained by reduced fat depots in the carcass. Surprisingly, serum glucose levels did not change. Also, PVN NE was reduced in HF-fed DIO rats following metformin treatment that was independent of leptin signaling in the brainstem noradrenergic neurons, as indicated by no change in pSTAT-3 or SOCS-3 protein expression between any of the groups. Likewise, there was a corresponding reduction in CRH levels in the ME and serum CORT in these animals compared to the HF-fed controls. Similar results were observed in chow-fed DIO animals. This and the lack of improvement in pSTAT-3-mediated leptin sensitivity in brainstem noradrenergic neurons after metformin treatment add a dimension of complexity in interpreting the current findings. Metformin is known to increase insulin sensitivity, but the mechanism is not clear. Current literatures suggest that the insulin-sensitizing action of metformin is mediated through inhibiton of lipolysis from adipose tissues (184-186). Since circulating lipids in the form of fatty acids or triglycerides are known to disturb the central physiological processes that would lead to increased consummatory behavior, such as stimulating activity of neuropeptides or neurotransmitters that activate feeding response, reducing the

levels of circulating lipids via metformin is a feasible mechanism by which PVN NE is reduced. On the other hand, metformin may directly suppress noradrenergic activity in the PVN and reduce feeding. The reduction of the HPA axis can be explained by the HPA axis and sympatho-activational role of lipids (189). If metformin improved the circulating lipid profile, the activation of the HPA axis may have been absent or even suppressed by the lack of activators such as lipids. It is also possible that metformin could have corrected or improved the postsynaptic noradrenergic deficit in the PVN of DIO rats, thus restoring the association between NE and the HPA axis and decrease CRH and CORT. Hence, it seems that metformin could have acted directly on different levels of the HPA axis – either on brainstem noradrenergic neurons, CRH neurons in the PVN, or even directly on adrenal glands to reduce glucocorticoid production. Circulating glucocorticoids act at the level of PVN, anterior pituitary, hippocampus, and also brainstem noradrenergic neurons to suppress further activation of the HPA axis and the noradrenergic system. Considering the synergistic positive effects between HPA axis and the noradrenergic system, the abolishment of negative feedback inhibition of brainstem noradrenergic system and hippocampus by circulating glucocorticoid can explain the consistant increase in NE levels in the PVN, which may be corrected by metformin treatment. It is also possible that the restoration of the leptin-NE-HPA axis circuitry following metformin treatment is indeed due to the enhanced leptin signaling not by pSTAT-3, but by PI3K-mediated pathway. This is supported by the current findings that the increase in

leptin levels in HF-fed metformin-treated animals was accompanied by reduced PVN NE and other HPA axis indices compared to chow-fed controls.

In summary, the findings from these studies demonstrated that the chronic HF diet exposure in DIO rats result in leptin signal impairment and increased TH gene expression in the brainstem noradrenergic neurons. This was associated with increased NE in the PVN and reduction in CRH levels in ME and serum CORT, indicative of leptin insensitivity in the brainstem and dysregulation of the HPA axis. Indeed, the DIO animals displayed a full-blown obese phenotype. Also, the findings showed that the genetic predisposition of DIO animals plays a role in neuroendocrine changes of the HPA axis. There can be a number of HF-induced factors that can bring about these changes in these animals (Fig. 6). HF-induced hyperleptinemia may be responsible for the defective leptin signaling at the level of the brainstem. On the other hand, HF diet may also prompt the release of proinflammatory mediators such as IL-1β, IL-6, TNF-α, and CRP that can participate in the activation of the brainstem noradrenergic neurons and the HPA axis. And the source of these mediators need not necessarily be white adipose tissues. Prolonged HF diet regimen also can affect the immune system and trigger release of these cytokines along with other reactive oxygen species from leukocytes and macrophages. These in turn may activate the HPA axis and also directly affect other peripheral organs and vasculature to induce sympathetically activated state. Also, the interaction between macrophages and the white adipose tissues in generating excess proinflammatory cytokines ought to be considered. Moreover, chronic HF diet can impair the glucosensing neurons in

the hypothalamus including the PVN and the brainstem noradrenergic neurons to induce activation of NE system as well as the HPA axis. As forementioned, HF-induced elevation in circulating lipids in forms of FFA, TG, or long-chain fatty acids all can be actively involved in the neuroendcorine changes that are seen during development of obesity. The genetic predisposition of the DIO animals may have these peripheral signs from the beginning, and upon HF diet these may worsen to induce changes in the neuroendocrine system. In conclusion, it seems likely that the peripheral changes induced by chronic HF diet exposure can interact with different levels of the neuroendocrine system in a reciprocal positive feedback manner towards the development of obesity. Metformin probably affects the peripheral factors to correct the leptin insensitivity in the brainstem, reduce NE levels in the PVN, and restore the HPA axis regulation, thereby partially correcting the obese phenotype in these DIO animals.

However, this is not to say that the brainstem neurons and the neurons in the arcuate nucleus of the hypothalamus act by completely separate mechanisms. They are indeed associated with somewhat distinct mechanisms for regulation of energy homeostasis: primary use of brainstem for short-term regulation of food intake vs. primary use of brain acruate neurons for long-term regulation of food intake and hence body weight. While brainstem mainly receives signals about energy availability such as blood glucose and cholecystokinin (CCK) from the GI tract, as well as mechanical visceral inputs from the stomach, arcuate nucleus along with other hypothalamic regions receive peripheral signals such as ghrelin, leptin, and insulin to induce the long-term anorexigenic effects. However,

considering the extensive innervation between brainstem neurons and the various regions of the hypothalamus including the PVN and the arcuate nucleus, it is hard to imagine that the perturbation in the brainstem functions does not influence the hypothalamic regions that control the long-term regulation of the food intake and body weight, which is supported by the current findings. Substatntial literature also points out the activation of non-shivering thermogenesis via brainstem that is regulated by hypothalamic areas, further supporting the importance of the interaction between the brainstem and hypothalamic regions for proper regulation of food intake and body weight. Even though not investigated, it is very likely that there is a degree of dysfunction in the arcuate and other associated regions in the hypothalamus such as reduction in insulin/leptin signal capacity or upregulation of orexigenic activities that can lead to obesity development in these DIO animals. Conversely, the disruption in the reward pathway that involves non-homeostatic mechanisms by abnormal brainstem functions is a viable explanation for the observed constant increase in food intake and body weight gain. Clearly how these changes are brought about and influenced by the dysfunctional brainstem neurons are yet to be elucidated.

With millions of people suffering from obesity and the significant economical, emotional, and physical burden it brings to the patients and their families, we hope that the current results presented in this dissertation will help provide a greater understanding of the pathogenesis of obesity and the mechanisms of obesity development. The findings in the dissertation propose

metformin as a possible low-risk therapeutic approach for correcting dysregulation of the HPA axis in obese patients.

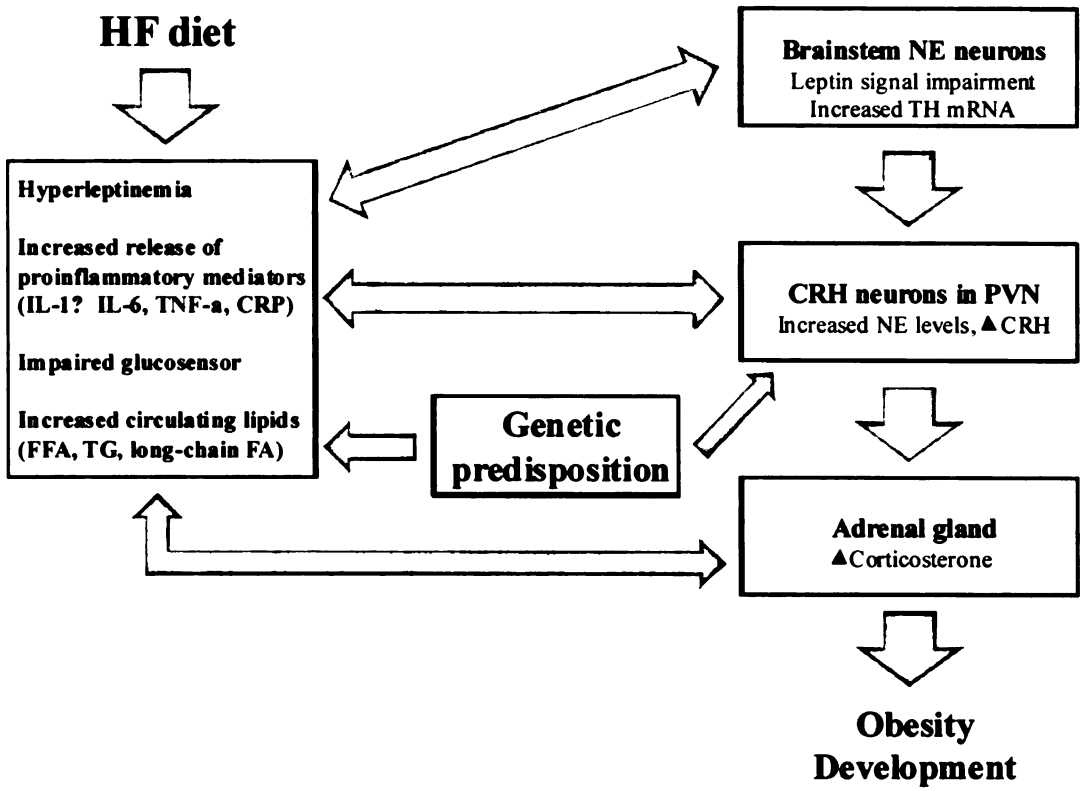


Fig. 6. Possible explanation for the underlying mechanisms of obesity development in DIO animals. The interaction between peripheral signals and the neuroendocrine system such as the HPA axis in the brain may trigger and/or promote obesity development. DIO animals also have genetic predisposition for obesity that may affect a myriad of energy homeostasis-related factors in both periphery and the brain to result in obese phenotype.

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