2000

Glucocorticoid Regulation of the Leptin Receptor Signaling System in the Rat.

Abram Madimabe Madiehe
Louisiana State University and Agricultural & Mechanical College

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DEDICATION

To my beloved brother and my friend: William Mogapi Madiehe

Remembering the good times
And the sad ones too,
Because if it weren't for all of them
I would have no memory of you.

To my grandfather and my first teacher: Samuel Madiehe

Memories are treasures
time cannot take away.
You made this life seem hopeful
And promising in many ways
For you taught me to...

"...Prove all things; hold fast that which is good." (1 Thes. 5:21)

To Dineo Phasha

Though most of the world, let alone South Africa,
ever had a chance to know you
You could have never imagined how you affected some of us
when you were at the United States Information Services
Though you never set up to become a hero,
You were content to help others become heroes!
It is this which our minds and history will record.
ACKNOWLEDGEMENTS

“Enjoyment is always bound up with gratitude; if this gratitude is deeply felt it includes the wish to return goodness received and is thus the basis of generosity” (Melanie Klein).

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<tr>
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<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADX</td>
<td>adrenalectomy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
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<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CAAT/enhancer binding protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3',5'-monophosphate</td>
</tr>
<tr>
<td>CART</td>
<td>cocaine- and amphetamine-regulated transcript</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CIS</td>
<td>cytokine-inducible SH2 protein</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin releasing hormone</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimal essential medium</td>
</tr>
<tr>
<td>DMN</td>
<td>dorsomedial hypothalamic nucleus</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetic acid</td>
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<td>Abbreviation</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetra-acetic acid</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>Gin</td>
<td>glutamine</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
</tr>
<tr>
<td>GTG</td>
<td>gold thioglucone</td>
</tr>
<tr>
<td>HF</td>
<td>high fat</td>
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<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
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<td>HPG</td>
<td>hypothalamic-pituitary-gonadal</td>
</tr>
<tr>
<td>HRP</td>
<td>horse-radish peroxidase</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IgG</td>
<td>gamma-immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneal</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>rLeptin</td>
<td>recombinant leptin</td>
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<tr>
<td>LF</td>
<td>low fat</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCH</td>
<td>melanin-concentrating hormone</td>
</tr>
<tr>
<td>ME</td>
<td>median eminence</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSG</td>
<td>monosodium glutamate</td>
</tr>
<tr>
<td>α-MSH</td>
<td>α-melanocortin stimulating hormone</td>
</tr>
<tr>
<td>NIDDM</td>
<td>non-insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
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<tr>
<td>ob</td>
<td>leptin gene</td>
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<tr>
<td>OBR</td>
<td>leptin receptor</td>
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<tr>
<td>OM</td>
<td>Osborne-Mendel</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
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<td>phosphate-buffered saline</td>
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<tr>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PIAS</td>
<td>protein inhibitor of activated STAT</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferation activated receptor</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular hypothalamic nucleus</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPA</td>
<td>ribonuclease protection assay</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>sc</td>
<td>subcutaneous</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>SH-PTP</td>
<td>SH2-containing protein tyrosine phosphatase</td>
</tr>
<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>VMN</td>
<td>ventromedial hypothalamic nucleus</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
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ABSTRACT

Obesity, a recognized health problem, is associated with cardiovascular disease, diabetes and hypertension. Removal of adrenal steroids by adrenalectomy (ADX) slows the development of obesity. Leptin decreases food intake through effects initiated by activation of the JAK-STAT pathway. Defects in leptin signaling lead to obesity, which is associated with hyperleptinemia and leptin resistance. In chapter 4, leptin induces phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression and increases glucose production in hepatocytes from lean but not obese Zucker rats. This suggests that hyperleptinemia in obesity causes hyperglycemia. In chapter 5, ADX decreased the levels of PEPCK mRNA, and increased leptin receptor mRNA expression of obese Zucker rats. The decrease in PEPCK mRNA is presumed to correct the hyperglycemia in these rats. ADX decreases leptin levels and increases leptin receptor (OBR) mRNA in obese Zucker rats, suggesting that leptin downregulates OBR. ADX was reported to increase sensitivity to leptin treatment. I hypothesized that high glucocorticoids in obesity impaired OBR signaling, and removal thereof will activate this pathway. In chapter 6, ADX increased the expression of leptin receptor mRNA in Sprague Dawley rats. ADX increased STAT-3 mRNA and protein levels, and induced constitutive STAT-3 phosphorylation and DNA binding activity. ADX also reduced SOCS-3 mRNA and protein levels. ADX and leptin treatment increased STAT-3 phosphorylation, but with no concomitant increase in DNA binding activity. We speculate that PIAS-3, a protein inhibitor of activated STAT-3, inhibited DNA binding of the activated STAT-3 as a protective measure of cytokine hypersensitivity in the absence of SOCS-3. Leptin and ADX decreased NPY mRNA expression, but their combination did not further decrease NPY mRNA. In conclusion,
ADX mimicked leptin in activating STAT-3, and ADX decreased SOCS-3 inhibitory system. Leptin sensitivity could also result from the release of anorexigenic and orexigenic neuropeptides, but more research is warranted. In chapter 7, high-fat diet induced leptin resistance could be due to downregulation of the leptin receptor proteins in the hypothalamus. Further research is required to define the leptin resistance in OM rats induced by high fat diet, such as changes in leptin receptor signaling and SOCS-3 expression.
CHAPTER ONE

INTRODUCTION

Obesity, the prevalence of which has been progressively increasing, has reached epidemic proportions in the developed countries. This phenomenon is usually ascribed, through epidemiological studies, to the combination of excess consumption of processed foods and decreased physical activity (Ravussin & Tataranni, 1997). The pathogenesis of obesity remains largely unknown, but obesity is closely associated with increased morbidity and mortality caused by several of the most common diseases in the western world, including type 2 diabetes, hypertension, dyslipidemia, atherosclerosis, cardiovascular disease and cancer (Björntorp, 1998). The increasing rates of obesity cannot be explained exclusively by the high availability of energy- and fat-dense foods and increased food intake, or decreased energy expenditure due to the increasingly sedentary lifestyle. The study of factors such as genetics and lifestyle implicated in obesity is crucial for predictions of the impact of the obesity epidemic, and provides an opportunity for the implementation of preventive measures (Grundy, 1998).

Variations in obesity-related phenotypes has been shown to be heritable (Commuzie and Allison, 1998), and environmental factors are becoming important determinants in the development of obesity (Bray, 1998). A gene x environment effect arises when the response of a phenotype to environmental changes depends on the genotype of the individual. It is well known that there are individual differences in the response to changes in energy balance. Therefore, gene x environment interactions are shown to be ubiquitous, as monozygotic twins responded similarly to variations in energy balance (Bouchard & Tremblay, 1997). The epidemic of obesity is therefore attributed
largely to environmental factors, namely, the abundance of high energy foods and a sedentary lifestyle. It is possible that, in the nearest future, candidate genes predisposing individuals with susceptibility or resistance to the development of obesity in response to changes in environmental factors will be identified. Since obesity rates are high, the continuous efforts by scientists in genetic and molecular research are warranted in order to identify the genes responsible for the susceptibility to obesity and to delineate further the cause of obesity, which involves neuroendocrine, genetic and dietary factors, in addition to lifestyle (York, 1992b; York, 1996; Weinsier, et al., 1998; Williamson, 1996; Bray and Bouchard, 1997). The role of a genetic predisposition to obesity has long been recognized (Bray and Bouchard, 1997), with evidence from human single gene mutations (e.g. leptin, proopiomelanocortin, peroxisome proliferator-activated receptor-γ, melanocortin-4 receptor), mendelian syndromes (e.g. Cushing's, Prader-Willi), animal models (genetically obese rodents, transgenic animals) and linkage and association studies (twins and adoption, nuclear families).

In animals, obesity phenotypes have been categorized into dietary, neuroendocrine and genetic, depending on the origin of the obesity (York, 1992; York, 1996). Dietary obesity is produced by a variety of dietary manipulations, which include increasing the dietary fat or sugar and increasing palatability of foods (Sclafani, 1992). Little is known of the factors that predispose individual humans to become obese, because obesity is a complex syndrome which appears to be polygenic rather than monogenic, and may be affected by environmental influences. The myriad of central neurotransmitters and peptide systems involved in the regulation of body weight in humans remain to be assessed, because these may regulate or be regulated by susceptible
genes which in turn may alter appetite or food preference and fuel metabolism. Animal models have become useful alternates for such studies. Single gene mutations producing obesity in rodents provide insights into the genetically modulated development of obesity. On the other hand, models of dietary-induced obesity provide important insights into defects that might predispose rats to become obese, and these models can be identified prospectively before they express their respective phenotypes. These models also allow manipulations of the environment so as to alter metabolic and neural functions of rodents of a known phenotype or genotype. These studies will therefore help decipher whether diet-induced obesity in humans is an inherited polygenic trait or not.

Studies of the interactions between heredity and physical activity have shown that the genetic background may modify the effect of physical activity on weight change (Heitman, et al., 1997; Samaras, et al., 1999), and that lifestyle may have an obesity-promoting effect, depending on a genetic predisposition (Hill, 1998; Hill and Peters, 1998). Genotype-environment interactions, which arise when a phenotype responds to environmental changes based on the genotype of an individual, may affect body weight and energy expenditure induced by overeating or by high fat diet consumption. Therefore, the examination of factors such as genetics and lifestyle, implicated in weight gain and obesity, is crucial in our understanding of obesity development. This will impact on the integrated approach for the effectiveness of prevention and management strategies for obesity. The main challenge for the future is, therefore, to precisely define the genotype-environment interactions that are fundamental to the development of obesity, and to show that this leads to the obesity phenotype observed due to changes in susceptibility genes that remained silent until the conducive environment was attained.
This dissertation describes studies on 1) the effects of leptin and glucocorticoids on the liver phosphoenolpyruvate carboxykinase, 2) the effects of glucocorticoids on the leptin receptor gene expression in lean and obese Zucker rats, and 3) the increased sensitivity to leptin treatment in adrenalectomized rats. These experiments tested the hypotheses that PEPCK gene expression contributes to hyperglycemia observed in obese Zucker rats, and that glucocorticoids also regulate the leptin receptor signaling system by playing a part in the leptin resistance observed in obesity.

In the following chapters, each of the various effects of leptin and glucocorticoids are described in detail. There are a total of eight chapters. Chapter two gives a detailed review of the literature. Chapter three presents the general methods and techniques used in studies in this dissertation. Chapter four discusses the effects of leptin on liver gene expression and metabolism. Chapter five investigates the effects of glucocorticoids on PEPCK and leptin receptor gene expression in lean and obese Zucker rats. Chapter six describes the effects of adrenalectomy on the leptin receptor signaling system. Chapter seven describes the modulation of leptin receptor signaling system by high fat and low fat diets in Osborne-Mendel and S5B/P1 rats. Chapter eight offers the author’s summary and future research directions.

Final conclusions that can be drawn from the presented dissertation research are 1) that glucocorticoids cause significant changes in the leptin receptor signaling system in the hypothalamus during obesity, and that these changes may account to some extent for the establishment of leptin resistance in obesity, and 2) that high fat diet induces leptin resistance by downregulating the leptin receptors in the hypothalamus of obesity-susceptible Osborne-Mendel rats.
CHAPTER TWO

REVIEW OF THE LITERATURE

2.1 OBESITY

Obesity is the major nutritional problem affecting industrialized societies. The specific syndromes of obesity both in animal models and in humans are associated with identified dietary, endocrine and/or genetic causes (York, 1996). However, the pathogenesis of obesity in humans is still unknown, apart from unexplained chronic excess in caloric intake relative to energy needs. Therefore, an understanding of obesity and its consequences requires the investigation of the many factors that control energy intake and energy expenditure. Such factors may be genetic, environmental, physiological, psychological, social or cultural, all of which are known to influence the presence of obesity in modern societies.

2.2 ANIMAL MODELS OF OBESITY

Using animal models, obesities have been categorized into dietary, neuroendocrine and genetic, depending on the origin of obesity (York, 1992b; York, 1996). Dietary obesity can be produced in laboratory animals by a variety of dietary manipulations, which include increasing the dietary fat or sugar and increasing palatability of foods (Sclafani, 1992). Although the role of dietary factors in human obesity has not been precisely identified, this research with laboratory animals clearly shows that certain diets promote hyperphagia and obesity in animals. Epidemiological studies show that obesity is more prevalent in humans consuming high fat diets, a characteristic of westernized diets, accompanied by decreased physical activity (Ravussin & Tataranni, 1997).
The ventromedial hypothalamic (VMH) region is responsible for integrating information about energy stores, and hypothalamic bilateral lesions of the ventromedial nuclei (VMN) produce obesity in laboratory animals (Bray, et al., 1990). The VMH obesity was produced by lesions of the VMN in rats by passing an electric current through an electrode, whereas in mice the lesions were produced by intraperitoneal (ip) injection of gold thioglucone (GTG) (Brecher & Waxler, 1949). Obesity can also be produced by destroying the paraventricular hypothalamic nuclei (PVN) (Leibowitz, et al., 1981) or by damaging the arcuate nucleus (ARC) bilaterally using monosodium glutamate (MSG) (Olney, 1969).

Apart from induction of obesity by diet and hypothalamic lesions, a large number of genetically inherited forms of obesity have been described (Trayhurn, 1984; York, 1996). Genetically obese animals have been divided according to their mode of inheritance into the single-gene dominant or single-gene recessive strains, and the polygenic inbred or hybrid strains due to a number of mutations in various genes (see Table 2.1). While great interest has justifiably been focused on the single gene mutations which result in obesity in rodents, and recently humans (Licinio, et al. 1998), it is likely that most instances of human or animal obesity reflect the interaction of a number of genes with a permissive environment. Recently, gene linkage analysis of animal models of obesity has identified over 80 linkages of chromosomal locations with obesity and/or body fat distribution in polygenic forms of obesity (Chagnon, et al., 1999).

Classical parabiosis studies by Coleman (1978), in which blood circulation of obese (ob/ob) mice was joined to that of obese (db/db) mice, showed that the ob/ob mice reduced food intake and lost weight, whereas db/db mice became more obese.
Table 2.1 Animal models of genetically inherited obesity

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Chromosome</th>
<th>Gene defect</th>
<th>Gene product</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recessive inheritance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ob/ob</td>
<td>6</td>
<td>stop codon 105 produces truncated leptin</td>
<td>Leptin (167 amino acids)</td>
<td>Leptin signal from fat to brain and peripheral organs</td>
</tr>
<tr>
<td>db/db</td>
<td>4</td>
<td>splicing defect</td>
<td>Leptin receptor (505 amino acids)</td>
<td>Impaired leptin receptor</td>
</tr>
<tr>
<td>fa/fa</td>
<td>5</td>
<td>Gln to Pro mutation</td>
<td>Leptin receptor</td>
<td>Impaired leptin receptor</td>
</tr>
<tr>
<td>Fat</td>
<td>8</td>
<td>Insert</td>
<td>Carboxypeptidase E</td>
<td>Pro-peptides not cleaved</td>
</tr>
<tr>
<td>tub</td>
<td>7</td>
<td>Insert</td>
<td>Phosphatase</td>
<td>Phosphate not cleaved</td>
</tr>
<tr>
<td><strong>Dominant inheritance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow mouse (Aγ)</td>
<td>2</td>
<td>Agouti signaling protein ectopic expression</td>
<td>Asp (133 amino acids)</td>
<td>Competes with MSH for receptors</td>
</tr>
<tr>
<td><strong>Polygenic inheritance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZO mouse</td>
<td>?</td>
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<td>?</td>
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<tr>
<td>KK mouse</td>
<td>?</td>
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</tr>
</tbody>
</table>
When *ob/ob* mice were joined to lean mice, the *ob/ob* mice lost weight and the lean mice remained normal. Upon joining the *db/db* mice to normal lean mice, the *db/db* mice became more obese and the lean rats had decreased glucose and insulinemia. This suggested either an absence of a circulating factor or an inability to respond to a circulating factor was fundamental to the development of obesity in these animal models (*ob/ob*, *db/db* and *fa/fa*). This factor was recently discovered to be leptin in *ob/ob* mice, while mutations in the leptin receptor in *db/db* mice and *fa/fa* rats (Zhang, et al., 1994; Tartaglia, et al., 1995; Chua, et al., 1996; Lee, et al., 1996; Iida, et al., 1996) explained their inability to respond to leptin.

![Figure 2.1. Model of the parabiosis experiment of Coleman (1978). When obese (ob/ob) mice were joined to obese (db/db) mice, the ob/ob mice reduced food intake and lost weight. These experiments led Coleman to hypothesize that ob/ob mice fail to produce a circulating factor (OB protein from their fat cells), but their brain remains sensitive to it, while db/db mice produced the circulating factor (functional OB proteins), but their brain was unresponsive. Adapted from Coleman (1978).](image)
2.3 EFFECTS OF GLUCOCORTICOIDS ON OBESITY

Adrenal hormone secretion plays a major role in the control of substrate supply to tissues and in the partitioning of dietary energy between storage compartments. In general, glucocorticoids are catabolic toward protein, increasing muscle protein breakdown and enhancing gluconeogenesis in stress and starvation to maintain the glucose supply for the brain. They are also catabolic toward fat at high doses (Woodward & Emery, 1989), but anabolic in fat metabolism at low doses (Diamant & Shafrir, 1975). Glucocorticoids enhance the induction of lipogenic enzymes by insulin, an effect illustrated by the submaximal induction of lipogenic enzymes on refeeding of adrenalectomized rats (Wurdeman, et al., 1978).


Glucocorticoids regulate the autonomic nervous system by suppressing the sympathetic drive to brown adipose tissue (BAT) and enhancing the parasympathetic drive to the pancreatic islets and thus promoting insulin secretion (Holt & York, 1989; Stubbs & York, 1991). The effects are exaggerated in obese rats and mice compared to
lean animals (Bray, et al., 1990a; Bray, et al., 1990b). The low thermogenic response of BAT to diet contributes to the increased energy efficiency associated with obesity (Marchington, et al., 1983; York, 1989). Adrenalectomy enhances BAT thermogenesis to diet stimuli elevating the sympathetic drive to the tissue (Marchington, et al., 1983; York, 1989; York, et al., 1985). Conversely, corticosterone suppresses BAT activity in lean rats, which is associated with the impairment in sympathetic drive to the tissue (York, 1989; York, et al., 1985). This impairment is specific to diet-related BAT thermogenesis in fa/fa rats and does not affect cold-responsive nonshivering thermogenesis (Bray, et al., 1990a; Bray, et al., 1990b; Holt, et al., 1983), showing that the glucocorticoid site of action is prior to the efferent sympathetic pathway. Glucocorticoids also interfere with the central responses to glucose metabolism through suppression of both the feeding and sympathetic responses to 2-deoxyglucose (York, et al., 1985; Allars & York, 1986).

ADX reduces body weight gain in Zucker fa/fa rats concomitant with reduction in food intake and marked attenuation of fat accumulation (Yukimura, et al., 1978; Bray, et al., 1992). In the obese (ob/ob) mouse, ADX increases energy expenditure (Vander Tuig, et al., 1984), restores norepinephrine turnover to normal in BAT and heart (Vander Tuig, et al., 1984), abolishes insulin resistance in muscle (Oshima, et al., 1984), lowers circulating levels of insulin and glucose (Yukimura & Bray, 1978a) and normalizes glucose tolerance (Solomon, et al., 1977; Bailey, et al., 1986). Similar changes have been reported for the diabetes (db/db) mouse (Bray, 1982) and the fatty rat (Marchington, et al., 1983). It is, therefore, likely that changes in the hormonal milieu, which include the effects of glucocorticoids and insulin, contribute to the altered food intake, energy expenditure and obesity.
2.4 GLUCOCORTICOID RECEPTOR

Glucocorticoids are steroid hormones synthesized and released by the adrenal cortex that influence a variety of physiological and developmental processes including intermediary metabolism, differentiation, response to stress and immunity. Glucocorticoids, like all steroid hormones, exert their effects on responsive cells by interacting with a specific cytosolic glucocorticoid receptors (GR), which in turn regulate glucocorticoid-responsive genes through interactions with hormone-response elements on the gene (Yamamoto, 1985). Moreover, studies using the GR antagonist, RU486, which inhibits obesity, showed that the development of obesity is due to the overactivity of the GR receptor (Langley & York, 1990; Okada, et al., 1990).

2.4.1 GLUCOCORTICOID RECEPTOR STRUCTURE

GR shares a similar structure to nuclear receptors, for retinoic acid, vitamin D, estrogen, androgen, progesterone and thyroid hormone. This group collectively forms a superfamily referred to as ligand-activated transcription factors (Evans, 1988). Members of this superfamily share a common domain structure composed of three distinct regions, each required for a specific function (Figure 2.2). The amino terminal end of the receptor, the transactivation domain, is responsible for its ability to activate transcription. The precise function of this domain is not known, but it appears to be involved in interactions with other transcription factors. The deoxyribonucleic acid (DNA) binding domain is located in the center of the receptor protein and comprises 70 amino acids. This sequence is required for interaction with the glucocorticoid response element (GRE), a short 15 base-pair regulatory DNA sequence located near the promoter, to which the receptor binds to modulate transcription. This domain also contains sequences involved
in nuclear translocation of GR and for dimerization of the receptor. The carboxy terminal region of the GR comprises the hormone-binding domain. This region also contains residues important for transcription activation, nuclear translocation and dimerization (Gehring, 1993).

2.4.2 GLUCOCORTICOID RECEPTOR FUNCTION

The effect of steroid hormone results from the hormone passing through the cell membrane by passive diffusion and binding to the receptor. Unbound GR is found predominantly in the cytosol. Unlike other hormones, eg. insulin, which transduce the hormonal signal as second messengers into the cell, glucocorticoids activate GR directly and influence transcription of glucocorticoid-responsive cells. The mechanism of action of GR involves ligand binding to the receptor, which then results in dimerization and activation of GR (Figure 2.2). In the absence of ligand, the GR is composed of multiple proteins in an aggregate with various components. The aggregates are formed by noncovalent interactions of a monomeric GR with a heat shock protein 90K (Hsp90) dimer (Pratt, et al., 1992). In this state, the GR is unable to bind to DNA. Following ligand binding to GR, the Hsp90 dissociates from the ligand-GR complex, the ligand-GR complex dimerizes and then translocates to the nucleus, where the GR binds to the GRE. The consensus sequence for the GRE is 5'-GGTACAnnnTGTTCT-3', where n is any nucleotide (Beto, 1989).

2.4.3 GLUCOCORTICOID RECEPTOR REGULATION

Cellular responsiveness to glucocorticoids depends upon GR levels. Therefore, mechanisms that modulate the concentration of functional GR can be pivotal controllers
Figure 2.2. A model for the mechanism of glucocorticoid receptor action. GR is folded into three domains: hormone-binding domain at the C-terminus, DNA binding domain in the middle, and the gene transcription activation domain at the N-terminus. See text for description of the mechanism. Adapted from Boumpas, et al. (1991).

of glucocorticoid action. All steroid hormone receptors are regulated by their specific hormone, leading to autoregulation (Haddock and Malbon, 1991). This process involves hormone-mediated transcriptional (mRNA synthesis), post-transcriptional (mRNA stability) and post-translational (protein turnover) mechanisms. Sequence analysis studies of human GR, by Burnstein and colleagues (1993), showed that the gene contains sufficient sequence information to confer glucocorticoid-inducible GR down regulation, which could involve either the rate of transcription or the stability of the GR mRNA.

Most steroid hormone receptors are phosphoproteins (Orti, et al., 1992). The GR is phosphorylated predominantly in the amino terminal transactivation domain. All the
phosphorylated amino acids are serines except for one threonine (Bodwell, et al., 1991). So far, the function of this phosphorylation of GR is unknown. It is hypothesized that phosphorylation-dephosphorylation mechanisms could be involved in the regulation of transcription.

2.4.4 GLUCOCORTICOIDS AND THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Cortisol, the major glucocorticoid secreted by the human adrenal cortex, (corticosterone in the rat) mediates a number of metabolic functions essential for life. The secretion of cortisol is narrowly regulated because both deficient and excess plasma concentration of cortisol are associated with disease and mortality. Hypothalamic corticotropic releasing hormone (CRH) stimulates the pituitary to secrete adrenocorticotrophic hormone (ACTH) which in turn stimulates the secretion of cortisol from the adrenal cortex into the systemic circulation. Circulating cortisol exerts negative feedback on the hypothalamus and pituitary by inhibiting the secretion of CRH and ACTH. These functionally linked units, composed of the hypothalamus, pituitary and adrenal cortex, form a regulatory system for plasma cortisol, termed the hypothalamic-pituitary-adrenal (HPA) axis (Björntorp, 1995; Chalew, et al., 1995).

Glucocorticoids impair insulin action and increase circulating glucose levels. They inhibit glucose uptake by peripheral tissues (Bowes, et al., 1991; Nosadini, et al., 1993) and augment the effect of glucagon and epinephrine to enhance gluconeogenesis in the liver (DeFeo, et al., 1990). Therefore, the effects of cortisol work to render insulin action ineffective. Furthermore, glucocorticoids promote the differentiation of human adipocyte precursor cells into fat cells (Hauner, et al., 1987) and is associated with increased adipocyte lipoprotein lipase activity and hyperlipidemia (Taskinen, et al.,

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1983). The pathological role of cortisol in the development of glucose intolerance and other complications of obesity makes it imperative to study the HPA axis and its role in obesity.

2.5 THE \textit{ob} GENE AND LEPTIN

The \textit{ob} (obese) gene was recently identified (Zhang, et al., 1994) and initially found to be expressed and the protein secreted primarily in the adipose tissue, where it encodes a 4.0-4.5 kb mRNA with a 167-amino acid open-reading frame without any similarities to other proteins. The search of a 3-dimensional structure database, to determine whether the \textit{ob} protein might adopt a fold similar to any known structure, revealed that the \textit{ob} sequence is comparable with structures from the family of helical cytokines that includes interleukin-2 and growth hormone (Madej, et al., 1995; Zhang, et al., 1994; Murakami & Shima, 1995). Whether the gene is also expressed in the brown adipose tissue is controversial (Frederich, et al., 1995; Moinat, et al., 1995; Deng, et al., 1997). The \textit{ob} mRNA encodes an 18 kDa protein which is secreted, after cleavage of the signal peptide, from the adipocytes as a 16 kDa glycoprotein named leptin, from the Greek \textit{leptos}, meaning thin or small (Halaas, et al., 1995). Leptin is single-stranded with a disulfide bridge at the C-terminus, which is pivotal for stability and bioactivity (Rock, et al., 1996).

Leptin mRNA and protein levels increase with adipose stores (Maffei, et al., 1995a; Frederich, et al., 1995) and therefore, leptin is considered to signal the size of the white adipose tissue (WAT) depots. This provides a molecular basis for the lipostat theory of energy regulation (Kennedy, 1953), proposing that there is a feedback loop. The existence of such a chemical feedback signal was supported by the classical parabiosis experiments of Coleman (Coleman, 1973; Coleman, 1978). Leptin may work as a satiety
factor because administration of the recombinant leptin to \textit{ob/ob} mice decreased food intake and body weight (Halaas, et al., 1995; Pellemounter, et al., 1995; Campfield, et al., 1995; Stephens, et al., 1995).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2.3}
\caption{Model of the feedback loop proposed by Kennedy (1953). The hypothalamus is depicted as the central controller of food intake, and leptin, which increases due to increased white adipose tissue mass, sends a satiety signal to the hypothalamus, thus causing the decrease in food intake.}
\end{figure}

There are site-specific variations in the expression of the \textit{ob} gene (Trayhurn, et al., 1995; Maffei, et al., 1995a; Masuzaki, et al., 1995; Frederich, et al., 1995), and the highest level of expression is evident in the epididymal and perirenal adipose tissue (Trayhurn, et al., 1995; Maffei, et al., 1995a; Masuzaki, et al., 1995). Leptin has also been shown to be expressed in the stomach (Bado, et al., 1998), and placenta (Masuzaki, et al., 1997; Hoggard, et al., 1997b). Increased levels of \textit{ob} mRNA have been reported for \textit{ob/ob} mouse, \textit{db/db} mouse and the Zucker \textit{fa/fa} rat (Zhang, et al., 1994; Trayhurn, et al., 1995; Maffei, et al., 1995; Frederich, et al., 1995; Funahashi, et al., 1995). Therefore,
obesity seems to be associated with increased expression of the ob gene. Classic parabiotic experiments between ob/ob and wild-type mice indicated that a factor circulating in the bloodstream of lean (ob/ob littermates) or db/db mice reverses the effects of the ob mutation (Coleman, 1973; Coleman, 1978). Thus, the elevated levels of ob mRNA may reflect the strength of the feedback signals to adipose tissue resulting from the fact that a truncated, non-functional leptin is synthesized in this mutant (Zhang, et al., 1994). This is true for the db/db mouse and the fa/fa rat because the mutants produce defective receptor systems for leptin. Treatment of ob/ob mice with leptin reversed the obesity phenotype (Weigle, et al., 1995; Halaas, et al., 1995; Pelleymounter, et al., 1995).

2.6 REGULATION OF LEPTIN PRODUCTION

Leptin biosynthesis and release is governed by a complex array of neuroendocrine, endocrine and paracrine signals that impinge on the adipocyte. Fasting decreases the level of ob mRNA expression, which is rapidly reversed on refeeding (Trayhurn, et al., 1995; Frederich, et al., 1995; Hardie, et al., 1996; MacDougald, et al., 1995; Saladin, et al., 1995). In contrast with normal rats and mice, fasting does not induce the decrease in the ob mRNA in ob/ob mice (Trayhurn, et al., 1995; Frederich, et al., 1995). The decrease in leptin appears to be central to the neuroendocrine adaptation to starvation (Ahima, et al., 1996). Insulin increases the ob mRNA expression (Wabitsch, et al., 1996) and circulating protein levels (Malmström, et al., 1996), and the idea that insulin may be a controlling factor over leptin expression has been suggested. During starvation, the decrease in insulin may be a key regulatory signal for the suppression of leptin expression (Boden, et al., 1996).
Leptin is also regulated positively by glucocorticoids at high doses (De Vos, et al., 1995; Slieker, et al., 1996; Wabitsch, et al., 1996; Considine et al., 1997) and cytokines (Grunfeld, et al., 1996; Sarraf, et al., 1997), and negatively by β2- and β3-adrenergic receptor agonists and cAMP (Slieker, et al., 1996; Mantzoros, et al., 1996; Trayhurn, 1996b). Thyroid hormones also suppress leptin production from the adipose tissue (Fain, et al., 1997; Escobar-Morreale, et al., 1997; Valvaci, et al., 1997; Yoshida, et al., 1997). The ob gene promoter is positively regulated through a functional binding site for CAAT/enhancer binding protein (C/EBP-α) (He, et al., 1995; Miller, et al. 1996; Hwang, et al., 1996), whereas thiazolidinedione agonists for peroxisome proliferation activated receptor (PPAR-γ) transcription factor suppress leptin expression in rodents (Forman, et al., 1995; De Vos, et al., 1996; Nolan, et al., 1996; Zhang, et al., 1996; Kallen & Lazar, 1996). This may involve a functional antagonism between C/EBPα and PPARγ on the leptin promoter (Hollenberg, et al., 1997). In summary, nutritional status, stress and immune activation influence leptin expression and production.

2.7 LEPTIN RECEPTOR

The receptor for the leptin protein was recently identified by an expression cloning strategy (Tartaglia, et al., 1995; Devos, et al., 1996). A cDNA library was constructed from mouse choroid plexus to clone this leptin binding activity. From this screen, cDNAs that encoded a cell surface leptin receptor (OB-R) were identified (Tartaglia, et al., 1995). The majority of the transcripts are those encoding the short forms of the receptor (Ghilardi, et al., 1996). The transcript that encodes the long form is less abundant even though it was detected in nearly all tissues in mice and humans by ribonuclease protection analysis (RPA) and polymerase chain reaction (PCR) (Ghilardi,
et al., 1996). Nevertheless, the long form is expressed mainly in the hypothalamus. This long form transcript is found in regions that are thought to control body weight, namely, the ARC, VMN and PVN (Mercer, et al., 1996a).

The murine cDNA clones identified above revealed a single membrane-spanning receptor that is similar to the class I cytokine receptor family (Tartaglia, et al., 1995). The closest relatives of OB-R were shown to be gp130 (Taga, et al., 1989; Hirano, et al., 1994), the granulocyte colony stimulating factor (G-CSF) receptor (Larsen, et al., 1990), and leukemia inhibitory factor receptor (LIF-R) (Gearing, et al., 1991).

Figure 2.4. Short form (OB-R$_S$) and long form (OB-R$_L$) leptin receptors. The similarities and differences between the extracellular, transmembrane, and intracellular domains of OB-R$_S$ and OB-R$_L$ are shown schematically. aa: amino acids, 100% is similarity between extracellular domains. 23 aa represents the transmembrane domain of the OB-R$_S$ and OB-R$_L$. OB-R$_S$ has 34 aa and OB-R$_L$ has 303 aa in the intracellular domain. Adapted from Tartaglia (1997).
The extracellular domain of OB-R is about 816 amino acids and the intracellular domain of the short form is composed of 34 amino acids. The long form has an intracellular domain made of approximately 303 amino acids (Tartaglia, et al., 1995; Chen, et al., 1996; Lee, et al., 1996). The extracellular domains of the short and long forms are identical throughout their entire length, except in the fa/fa rat where a mutation has been described (Chua, et al., 1996; Iida, et al., 1996a; Iida, et al., 1996b). Differences only arise from the alternative RNA splicing at the carboxy-terminal coding exon, which results in the leptin receptor intracellular domains with differing length and sequence composition (Chen, et al., 1996; Lee, et al., 1996; Tartaglia, 1997). Additional short forms have been identified, all of which terminate shortly after the point of divergence at Lys889, i.e., after amino acid 29 of the intracellular domain (Chen, et al., 1996; Chua, et al., 1996). A transcript that potentially encodes a soluble form of the receptor was also described (Lee, et al., 1996). This protein lacks the transmembrane domain found in the other OB-R proteins. Genetic mapping of the gene encoding OB-R localized its position to mouse chromosome 4 (Tartaglia, et al., 1995). The position is within the genetic interval to which the db locus has been mapped (Chen, et al., 1996).

2.8 LEPTIN RECEPTOR MUTATIONS

The gene encoding the leptin receptor was sequenced from the wild type and db/db mice and revealed a mutation in db/db mice (Chen, et al., 1996; Lee, et al., 1996). The mutation is a single nucleotide substitution, G→T, within the exon containing the carboxy terminus and 3'-UTR of the short form of OB-R. The mutation consequently resulted in the generation of a new splice donor site, creating an exon that is inappropriately spliced into the transcript encoding the long form of OB-R. This new
exon is made up of the last six codons and the first 88-bp of the 3'-UTR of the primary short form and is inserted where the long form and short form domains diverge. As a result of this insertion, the long form transcript in \( db/db \) mice encodes a protein in which the majority of the intracellular domain is truncated and thus identical to the major short form. The defect in \( db/db \) mice is in the OB-R gene providing evidence for the importance of this receptor in body weight control (Chen, et al., 1996).

In the \( fa/fa \) Zucker rat, the genetic mutation is a single nucleotide substitution, \( A^{880} \rightarrow C \) that leads to a single amino acid substitution, from Gln\(^{269} \) to Pro\(^{269} \), in the extracellular domain. This mutation causes the obesity phenotype as it affects cell surface expression and binding affinity of leptin at the cell surface (Chua, et al., 1996a; Chua, et al., 1996b; Philips, et al., 1996, Iida, et al., 1996a; Iida, et al., 1996b). The expression levels of the OB-R mRNA in the brain of Zucker \( fa/fa \) rat was higher than for lean littermates (Iida, et al., 1996). The \( fa \) mutation was mapped to rat chromosome 5, a region syntetic with the \( db \) region on mouse chromosome 4 (Truett, et al., 1991).

Another mutation of the leptin receptor was found in the inbred genetic model of non-insulin-dependent diabetes mellitus (NIDDM), an NIH-corpulent rat \( (cp/cp) \), (Koletsky, 1973; Lee, et al., 1997). This mutation is a \( fa \) allele \( (fa^{cp}/fa^{cp}) \) since crosses of rats carrying the \( fa \) mutation with rats carrying the \( cp \) mutation yielded obese progeny (Yen, et al., 1977). The mutation is a single base change of T\(^{2289} \) to A\(^{2289} \) in the obese corpulent rats resulting in the conversion of Tyr\(^{763} \) to a stop codon (Lee, et al., 1997). This nonsense mutation results in the termination of translation at the amino terminal end of the transmembrane domain. As a result, none of the leptin receptor isoforms in \( cp/cp \) rats are predicted to encode a transmembrane domain, nor any of the motifs necessary for
signal transduction. The mutation in the corpulent rat and the frameshift mutation in 129 db^{3f/3f} mice confirm that defects in the leptin receptor lead to abnormalities in the leptin-leptin receptor pathway and an obese phenotype.

2.9 LEPTIN RECEPTOR FUNCTIONS

The functions of the long and short intracellular domains of the leptin receptor are currently being defined. The high expression levels of the short form have been speculated to play a role in transporting leptin from the blood to the cerebrospinal fluid (CSF), where leptin can then move by diffusion to the ARC, PVN or VMN to regulate body weight. This is supported by the observation that leptin enters the brain by a specific and saturable transport mechanism (Banks, et al., 1996). However, the role of the leptin receptors in this transport system has not been described. The short form of OB-R may also play a role in clearance due to their distinct tissue distribution (Cumin, et al., 1996).

The long form of the receptor shows sequence homology to members of the class I cytokine receptor superfamily (Tartaglia, et al., 1995; Bazan, 1990). This provides important information as to possible intracellular mediators of leptin receptor activation (Figure 2.5). The cytoplasmic domains of the class I cytokine receptors lack enzymatic activity and instead they associate with the cytoplasmic tyrosine kinase of the Janus kinase (JAK) family (Ihle, 1995). Ligand binding leads to activation of receptor-bound JAK kinases, which phosphorylate tyrosines in the cytoplasmic domain of the receptor as well as other cytoplasmic target proteins, including the signal transducers and activators of transcription (STAT), ras/mitogen activated protein (MAP) kinase, and phosphoinositide-3-kinase pathways (Ihle, 1996; Darnell, et al., 1994). The activated STAT proteins then translocate to the nucleus and stimulate gene transcription.
Figure 2.5. Schematic diagram of the leptin receptor and its molecular signaling pathways. Homodimerization of the OBR complex by leptin binding to leptin binding domain (LBD) activates JAK kinase activity, which phosphorylates OBR on tyrosine residues in the cytoplasmic domain. Phosphorylated tyrosine residues provide specific docking sites for the SH2 domains of STAT-3 proteins, causing receptor association and subsequent phosphorylation of STAT-3. After activation, STAT3 translocates to the nucleus to modulate transcription. Negative regulators of JAK/STAT signaling, e.g., SOCS and PIAS, interfere at specific sites in the signaling cascade, with SOCS-3 inhibiting JAK association with the OBR and inhibit OBR activation and STAT-3 phosphorylation, and PIAS-3 inhibiting the DNA binding of activated STAT-3. In addition to activating the JAK/STAT cascade, leptin also stimulates the MAP kinase pathway. This pattern of JAK/STAT protein activation by leptin is predominantly in the hypothalamus. Adapted from Auernhammer and Melmed (2000).
Thus, the long form OB-R is likely to be the active signaling receptor because it has features similar to that of the class I cytokine receptors that are essential for signaling. Furthermore, it is preferentially expressed in the hypothalamus, the primary site of leptin action.

Co-transfection studies have shown that the long form of OB-R is capable of activating the STAT proteins in response to ligand binding (Baumann, et al., 1996; Ghilardi, et al., 1996). This effect is not observed in the presence of the short form receptor. The short form of the receptor was unable to bind to or activate JAK kinase (Ghilardi & Skoda, 1997) which is consistent with the inability to activate STAT proteins. In contrast to the inability of the short form leptin receptor to bind and activate JAK/STAT proteins, the short form does induce gene transcription of *c-fos* and *c-jun* (Murakami, et al., 1997; Yamashita, et al., 1997). This is similar to transcription induced by the truncated G-CSF receptor, which contains a short cytoplasmic domain (Baumann, et al., 1996). This suggests, therefore, that the leptin-induced mRNA expression in CHO cells transformed with the short form OB-R was signaled through the short cytoplasmic domain (Murakami, et al., 1997). The Zucker rat leptin receptor not only exhibits a slightly reduced leptin binding affinity, it was also shown to perform reduced signal transduction by stimulating the transcription of *c-fos*, *c-jun* and *jun-B* (Yamashita, et al., 1997).

2.10 PERIPHERAL METABOLIC ACTIONS OF LEPTIN

The presence of receptors for leptin not only in the hypothalamus, but also in the peripheral tissues, including adipose tissue, liver, skeletal muscle and islet cells, suggests that leptin has peripheral as well as central actions (Tartaglia, et al., 1995; Tartaglia,
Such peripheral actions were confirmed by studies which showed that leptin can impair insulin signaling, both in skeletal muscle and adipocytes. For example, leptin impairs insulin-mediated glucose uptake in adipocytes (Muller, et al., 1997). Furthermore, in both HepG2 cells and skeletal muscle myotubules, leptin was found to inhibit phosphorylation of insulin receptor substrate-1 (IRS-1) (Berti, et al., 1997; Muller, et al., 1997).

In addition to its effect on insulin-mediated glucose uptake, leptin inhibits insulin-mediated lipogenesis, and stimulates lipolysis and protein kinase A (PKA) (Wang, et al., 1997a). These studies suggest a role for leptin in peripheral metabolic regulation, and have raised the possibility that the impaired insulin signaling in obese subjects might result, in part, from increased circulating leptin levels.

2.11 LEPTIN AND THE HPA AXIS

Conflicting results have been obtained in studies looking at the relationship between plasma leptin and cortisol levels (Licinio, et al., 1997; Haffner, et al., 1997; Vettor, et al., 1997). The diurnal variation of leptin and cortisol is reciprocal, leptin levels peak during the nadir of cortisol secretion. In cultured adrenocortical cells, leptin was found to dose-dependently inhibit ACTH-stimulated cortisol production and P450 17α-hydroxylase mRNA expression (Bornstein, et al., 1997). In contrast, leptin increases CRH expression in the PVN (Schwartz, et al., 1996). Despite the observation that dexamethasone can stimulate leptin production in culture adipocytes, peripheral CRH administration in humans does not influence leptin levels (Cizza, et al., 1997). It is therefore apparent that leptin and HPA axis may be reciprocally related, but with interactions at several levels.
An intact HPA axis has been suggested to be necessary for the normal actions of leptin on energy balance. Leptin feeds back to the HPA axis to inhibit release of CRH. Stress is the classic stimulus to the HPA axis (Dallman, M.F. 1993). Starvation and immobilization stress markedly stimulate this endocrine axis (Chowers, et al., 1969; Akana, et al., 1992). Administration of leptin to fasting mice or to restraint-stressed rats attenuates stimulation of the HPA axis and both ACTH and corticosterone levels are normalized to that of the fed state (Ahima et al., 1996). Heiman and colleagues showed that such inhibition may be exerted at the hypothalamic level, because leptin directly inhibited CRH release in response to hypoglycemia, and because it did not directly change the secretion of ACTH, which is a counterregulatory response to prevent hypoglycemia (Aizawa, et al., 1981; Widmaier, et al., 1988; Heiman, et al., 1997).

Injection of leptin centrally to ADX rats showed that the hypophagic and weight-reducing effects of leptin were significantly amplified, and this effect was abolished by treatment with dexamethasone (Zakrzewska, et al., 1997). These observations suggested that glucocorticoids exert a counter- regulatory effect on leptin action, and that the activity of the HPA axis may set the level of sensitivity to leptin. In humans, however, little is known about the possible effects of leptin on the HPA axis in human obesity. Vettor et al. (1997) showed that no relationship exists between the activity of the HPA axis and serum leptin concentrations in women with different obesity phenotypes.

2.12 LEPTIN AND REPRODUCTION

Studies in rodents have shown that male and female homozygous ob/ob mice are obese and infertile (Hoggard, et al., 1998) and these mice do not produce functional leptin (Chehab, et al., 1996). Whereas male ob/ob mice can reproduce if maintained on a
restricted diet, female ob/ob mice are always sterile (Lane and Dickle, 1964). Early sexual development is normal in ob/ob females; however, ovulation never occurs and the mice remain prepubertal indefinitely, with no occurrence of estrus cycle. Reproductive hormones are reduced in ob/ob female mice, showing a functional defect from the hypothalamic-pituitary axis (Chehab, et al., 1996). When injected with leptin, female ob/ob mice show an increase in serum luteinizing hormone (LH) levels and ovarian weight, and they become fertile (Chehab, et al., 1996; Barash, et al., 1996). The leptin-induced elevation in LH in ob/ob mice suggested that the primary regulatory effect of leptin was on the reproductive neuroendocrine system, with the ovarian effects being secondary to stimulation by LH. The detection of leptin receptor mRNA in human ovary has suggested a direct action of leptin in the ovary (Cioffi, et al., 1996). Zachow and Magoffin (1997) showed that leptin can act directly on the ovarian granulosa cells to selectively decrease estradiol (E$_2$) but not progesterone (P$_4$) production. Interestingly, follicle-stimulating hormone (FSH)-dependent E$_2$ production was not reduced by leptin, whereas leptin impaired the sensitizing effect of insulin-like growth factor-1 (IGF-1) on FSH-dependent E$_2$ synthesis by granulosa cells.
CHAPTER THREE
GENERAL METHODS AND TECHNIQUES

3.1 PREPARATION OF HEPATOCYTES

Perfusion and preparation of hepatocytes was carried out as illustrated in Figure 3.1 using the *in situ* collagenase perfusion method as described previously (Berry and Friend, 1969; Seglen, 1976) with modifications of Pertoft & Smedsrod (1987). Rats were anesthetized by intraperitoneal injection of ketamine (0.125 ml/100 g body weight) and decontaminated with a 70% ethanol solution. The first cannula was placed in the hepatic portal vein, and a second was placed in the inferior vena cava. The liver was perfused, at a rate of 30 ml/min, with calcium-free perfusion buffer [Hank's Balanced Salt Solution (HBSS), pH 7.5; 10 mM glucose] that had been equilibrated at 37°C with 95% oxygen and 5% CO₂. This initial perfusate was allowed to drain to waste. The liver was then

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*Figure 3.1. Diagrammatic representation of liver perfusion.*

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enzymatically digested for 10 min by recirculating 50 ml of collagenase buffer [HBSS; 2 mM CaCl₂; 0.05% (w/v) type 1 collagenase (Worthington Biochemical Corp., Lakewood, NJ)]. The liver was carefully removed, placed in a beaker containing collagenase buffer and minced. The liver lysate was further incubated at 37°C for 15 min with shaking to release hepatocytes, and then placed on ice for 5 min. An equal volume of ice-cold wash buffer [HBSS; 2 mM CaCl₂] was added to the cell suspension, which was then filtered through a 250 μm nylon filter into a sterile centrifuge tube. The cell suspension was centrifuged at 300 rpm for 2 min at 4°C. Cells were washed three times in the wash buffer. The final pellet was resuspended in 20 ml of wash buffer. For cell counting and viability determination, cells were diluted 1:10 with William’s E (WE) medium (Sigma Chemical Co., St Louis, MO) and counted on a hemacytometer using the inverted microscope. Cell viability was determined by using the trypan blue dye exclusion method. Hepatocytes (4 x 10⁶/5ml) were plated onto collagen-coated 60 mm² cell culture dishes (Corning Glass Works, Corning, NY) in WE medium containing 1 nM insulin, 1 nM dexamethasone (DEX) and 5% (v/v) fetal bovine serum (FBS). The cultures were incubated at 37°C in a humidified 5% CO₂/95% air incubator for 4 hr to allow the cells to attach to the substratum. After 4 hr, unattached cells were removed by aspiration of medium, and cells were incubated overnight in serum-free medium containing 1 nM insulin and 1 nM DEX. The spent medium was replaced with either fresh WE medium or fresh glucose-, L-glutamine and sodium pyruvate-free Dulbecco’s minimal essential medium (DMEM) (Life Technologies, GIBCO BRL, Grand Island, NY) containing different hormones as described in the respective experimental setups.
3.2  TOTAL RNA EXTRACTION FROM HEPATOCYTES

Isolation of total RNA was carried out by the standard procedure of Chomczynski and Sacchi (1987, 1992) with modifications. After treatment with various hormones, the culture medium was aspirated and cells were quickly washed with 2 ml of ice-cold phosphate-buffered saline (PBS, pH 7.2) and lysed in 1 ml of ice-cold solution D (4 M guanidinium isothiocyanate; 25 mM sodium citrate; 0.5 % Sarcosyl; 0.72% β-mercaptoethanol). The cell lysate was scraped to one end of the plate using a sterile disposable cell scraper (Fisher Scientific, Pittsburg, PA), passed up-and-down four times through a 1-ml micropipette and transferred into heavy gel phase lock tubes (5 Prime → 3 Prime, Inc., Boulder, CO, USA). The following solutions were sequentially added to the lysate with mixing: 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml water-saturated phenol and 0.5 ml chloroform. The suspension was incubated on ice for 15 min, and then centrifuged at 3,300 rpm for 15 min at 4°C. The aqueous phase was transferred into a fresh polypropylene tube and total RNA was precipitated with an equal volume of isopropanol at -20°C for 1 hr. Total RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The pellet was resuspended in 0.3 ml solution D and transferred into an 1.5 ml Eppendorf tube. The RNA was re-precipitated overnight at -20°C with an equal volume of isopropanol. The precipitate was centrifuged at 14,000 rpm at 4°C in a Eppendorf microcentrifuge. The pellet was washed with 75% ethanol, air-dried and completely dissolved in 0.1 ml RNAsae-free water at 60°C for 10 min. Total RNA was quantified spectrophotometrically at 260 and 280 nm (1 A_{260} unit = 40 μg/mL RNA) and the purity was determined by the 260/280 ratio. The quality of the total RNA was further analyzed by 1% agarose-2.2 M formaldehyde gel electrophoresis at 100 V for 2-3 hr.
3.3 ISOLATION OF TOTAL RNA FROM TISSUES USING TRIZOL REAGENT

Isolation of total RNA was carried out using the TRIzol reagent protocol as described by the manufacturer (GIBCO BRL, Grand Island, NY, USA) and modified recently (Nakajima, et al., 1998). This protocol follows the original method of RNA isolation described by Chomczynski and Sacchi (1987, 1992). Frozen liver tissue (0.1 g) was homogenized in 1 ml TRIzol reagent using the Polytron (model MR3000, Kinematica AG, Switzerland) at maximum speed for 15 sec. The homogenate was transferred into a heavy gel phase lock tube (5 Prime → 3 Prime, Inc., Boulder, CO, USA) and incubated at room temperature for 5 min. After 5 min, 0.2 ml chloroform was added per 1 ml of TRIzol. The suspension was mixed vigorously for 15 sec, incubated at room temperature for 3 min, and then centrifuged at 3,300 rpm for 5 min at 4°C. After centrifugation, the aqueous phase containing total RNA was transferred into a fresh polypropylene tube. Total RNA was precipitated with 0.5 ml isopropanol per 1 ml of TRIzol at -20°C for 1 hr, and centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was resuspended in 0.3 ml Solution D [4 M guanidinium isothiocyanate; 25 mM sodium citrate; 0.5 % sarcosyl; 0.72% β-mercaptoethanol], and transferred into an Eppendorf tube. The RNA was precipitated with an equal volume of isopropanol overnight at -20°C. Total RNA was recovered by centrifugation at 14,000 rpm at 4°C in a microcentrifuge (Eppendorf centrifuge model 5402, Hamburg, Germany). The pellet was washed with 75% ethanol, air-dried and completely dissolved in 1 ml of RNase-free water at 60°C for 10 min. RNA was quantified and quality checked as described above.
3.4 NORTHERN BLOT ANALYSIS

Agarose gel electrophoresis and Northern blotting were performed according to standard procedure (Sambrook, et al., 1989). Total RNA (20 or 40 µg/lane) obtained from tissues or hepatocytes was mixed (1:2 v/v) with RNA sample buffer [62.5% formamide; 9.25% formaldehyde; 1.25X formaldehyde gel running buffer; 50 µg/ml bromophenol blue; 50 µg/ml xylene cyanole FF; 50 µg/ml ethidium bromide (5 Prime → 3 Prime, Inc., Boulder, CO, USA)], denatured at 65°C for 10 min, and immediately chilled on ice for 5 min. Samples were loaded and size-fractionated in a 1% agarose-2.2 M formaldehyde gel at 100 V for 2-3 hr. The quality of the RNA was determined by visualization under UV light using either a UV transilluminator (Spectronics Corp., Westbury, NY) or ChemiImager (Alpha Innotech Corp., San Leandro, CA). The gel was photographed using Polaroid DS34 instant camera (Polaroid Corp., Cambridge, MA), placed in 10 mM NaOH solution for 10 min and the RNA was transferred overnight onto a Hybond N+ nylon membrane (Amersham, Buckinghamshire, UK) by using the standard upward transfer method with 10X SSC as transfer buffer (20X SSC = 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). The efficiency of transfer was determined by checking the gel under UV light after transfer. The 18S and 28S ribosomal RNA positions were marked by nicking the membrane. The membrane was briefly rinsed with 2X SSC, dab-dried and baked in a vacuum-oven at 80°C for 30 min. The membrane was prehybridized for 3 hr or overnight at 42°C (for cDNA probes) or 50°C (for oligonucleotide probes) in a prehybridization solution [50% formamide; 0.12 M Na₂HPO₄; 0.25 M NaCl, 7% (w/v) SDS]. After prehybridization, the membrane was hybridized for 18 hr with radiolabeled probe in prehybridization buffer. The membrane was then stringently washed three times.
as follows: 2X SSC/0.1% SDS for 15 min at room temperature, 0.5X SSC/0.1% SDS for 20 min at room temperature, 0.1X SSC/0.1% SDS for 15 min at 60°C, placed in a sealed plastic bag and exposed to phosphoscreen overnight. The signal was quantified using the Phosphoimager and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Labeling of cDNA probes by random priming

cDNA inserts, obtained from various plasmids used in this study, were labeled by random primer labeling method using the DECAprime II kit (Ambion Inc., Austin, TX). Briefly, 25ng template DNA was mixed with 1 μL of 10X decamers to a final volume of 11 μL and heated for 5 min at 95°C. The solution was snap-frozen in liquid nitrogen before placing on ice. Then, 14 μL of labeling master mix [1X dCTP-minus buffer, [α-32P]dCTP, Klenow large fragment *E. coli* polymerase] was added and incubated at 37°C for 30 min. The reaction was stopped by adding 1 μl of 0.5 M EDTA and the unincorporated label was removed by centrifugation through a Micro Bio-Spin™-30 chromatography column (Bio-Rad Laboratories, Hercules, CA). The specific activity of the probe was determined by counting 1 μl in 10 ml of EcoLite scintillation liquid in the Beckman LS6000 scintillation counter (Beckman Instruments Inc., Fullerton, CA). Before use, the radiolabeled probe (1-5 x 10^6 cpm/ml hybridization buffer) was linearized by heating at 95°C for 5 min, chilled on ice, and added to prehybridization buffer.

3.5 CLONING THE LEPTIN RECEPTOR FRAGMENT

**cDNA synthesis**

Total RNA was isolated from the hypothalamus of a lean Zucker rat using the TRIzol reagent method as described in section 3.3. Reverse transcription polymerase-chain reaction (RT-PCR) was carried out according to the manufacturer’s instructions.
(Promega Corp., Madison, WI) and as described by Ghilardi and colleagues (1996). Five µg of hypothalamic RNA or yeast control RNA was mixed with oligo(dT)$_{12-18}$ primers (Life Technologies, GIBCO BRL, Grand Island, NY, USA) in a sterile 0.5 ml PCR tube and incubated at 70°C for 10 min to denature RNA secondary structure. A 12 µl reverse transcriptase master mix [1x reverse transcriptase buffer, 2.5 mM MgCl$_2$, 0.5 mM deoxyribonucleotides (dNTPs), 10 mM dithiothreitol (DTT), 1 µL Moloney-Murine leukemia virus reverse transcriptase (M-MLV)] was prepared. After denaturation, the tubes were chilled on ice for 1 min and the master mix was added. To ensure that the primers did not prime off of the contaminating genomic DNA, a negative control omitted the reverse transcriptase. The tubes were incubated at 42°C for 55 min for synthesis, and the enzyme was inactivated by heating at 70°C for 15 min. The tubes were chilled at 0°C for 1 min. One µl of RNase H was added to each tube and further incubated at 37°C for 30 min. The reaction was thereafter chilled on ice or stored at −20°C until required for PCR.

Polymerase-chain reaction (PCR) amplification

The leptin receptor cDNA was amplified using the PCR amplification reagents obtained from Promega (Madison, WI, USA). The following primers were used to amplify the leptin receptor cDNA: forward, 5′-TTCCTATCGAGAAATATCAG-3′, reverse, 5′-GGTACCATCTCATCTTTATT-3′, (named leptin I and II, respectively), selected to amplify sequences corresponding to nucleotides from +2473 to +2875 of the rat leptin receptor cDNA (GenBank U52966) as described by Ghilardi, et al. (1996). Two µL of the cDNA synthesis reactions were mixed with 48 µl of the PCR master mix [1x PCR buffer, 1.5 mM MgCl, 0.4 mM dNTP mix, 0.5 µl Taq polymerase, 200 nM each of
leptin I and II primers or control primers for yeast RNA] and amplified for 40 cycles [PCR conditions: initial denaturation at 94°C for 90 sec; followed by 94°C for 45 sec; 45°C for 45 sec; 72°C for 2 min; final extension at 72°C for 10 min, and held at 4°C, using the Programmable Thermal Controller (PTC-100™, MJ Research Inc., USA). PCR products were electrophoresed on a 1% agarose gel in 0.5X TBE at 100 V for 1 hr. The amplified products were visualized under UV light and photographed. Figure 3.2 shows the PCR amplified leptin receptor cDNA, named ObR403 based on its expected size.

![Figure 3.2](image_url)

**Figure 3.2.** RT-PCR product of the hypothalamic leptin receptor. Leptin receptor was amplified from the cDNA obtained from total hypothalamus RNA. Reverse transcription was performed using hexamers [lanes 2-5] and oligo(dT)₁₂₋₁₈ primers [lanes 6-9]. Lane 1 is a control without reverse transcriptase. M = 100 base-pair DNA ladder. PCR products were fractionated in a 1.5% agarose gel for 1.5 hr.

♦ Cloning of ObR403 cDNA

The amplified ObR403 cDNA was cloned by T-A cloning method using pGEM®-T Easy vector systems according to the manufacturer's instructions (Promega Corp., Madison, WI). Briefly, the ObR403 cDNA was mixed with 50 ng pGEM®-T Easy vector (3:1 and 1:3 ratios) in a ligation reaction containing 1X T4 DNA ligase buffer and 0.3 Weiss units/µl T4 DNA ligase. The reaction was carried out overnight at 10°C, and placed on
E. coli JM 109 strain was transformed with 50 ng DNA according to supplier's instructions (Promega Corp., Madison, WI). Briefly, bacterial cells were thawed on ice. Cells were mixed with the ligated DNA and incubated on ice for 30 min. The cells were heat-shocked for 50 sec in a 42°C water bath and placed back on ice for 2 min. Sterile SOC medium, pH 7.0 [2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl] at room temperature was added and the cells were allowed to recover for 1.5 hr at 37°C with shaking at 225 rpm. Cells were diluted 1:10, spread-plated on Luria-Bertani (LB) agar plates containing 50 μg/ml ampicilin and incubated at 37°C for 16 hours.

Individual isolated colonies were picked and cultured overnight in 5 ml LB medium containing 100 μg/ml ampicilin. DNA minipreps were performed, as described below, to identify the clones that were successfully transformed with the ObR403 cDNA.

3.6 PLASMID DNA EXTRACTION PROTOCOL

Plasmid DNA was extracted using the Wizard™ Plus DNA purification system (Promega Corp., Madison, WI). Cells were pelleted by centrifugation at 10,000 xg for 10 min at 4°C using the Eppendorf microcentrifuge. The pellet was completely resuspended in 300 μl of cell resuspension solution [10 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 1 mM EDTA, 10 μg/ml lysozyme]. To the suspension, 300 μL of fresh lysis solution [0.2 N NaOH, 1% SDS] was added and the contents were mixed by inversion. Then, 300 μl of the neutralization solution [1.32 M potassium acetate, pH 5.2] was added and mixed by inversion. The lysate was centrifuged at 10,000 xg for 5 min at 4°C in a microcentrifuge. The supernatant was carefully aspirated and mixed with 1 ml of DNA purification resin. The DNA/resin mix was then transferred into the barrel of a mini-column/syringe assembly and vacuum was applied to draw the solution through the mini-column. Then 2
ml of column wash solution was added to the mini-column and vacuum was applied as above. The mini-column was dried under vacuum and transferred to an Eppendorf tube for further drying by centrifugation at 10,000 xg for 2 min. The plasmid DNA was eluted by adding 50 μl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and centrifuged at 10,000 xg for 20 sec. The plasmid DNA was subjected to restriction enzyme analyses and agarose gel electrophoresis, and aliquots were stored at -20°C until required for further analysis. **Figure 3.3** shows the gel electrophoresis profile of ObR403 cDNA cloned into pGEM-T Easy vector. The ObR403 DNA insert was then excised from the pGEM-T Easy vector using EcoR1, and subcloned into the EcoR1 site of pBluescript II SK(+/-).

![Figure 3.3](image_url)

**Figure 3.3.** Restriction endonuclease digestion of purified pZObR403 plasmid. The amplified plasmid was undigested (lane 1), insert excised with EcoR1 (lane 2) and BamH1-HindIII (lane 3), and plasmid linearized with BamH1 (lane 4) and HindIII (lane 5). Lanes M are 100-bp/1-kb DNA ladders. Fragments were fractionated on a 1% agarose gel containing ethidium bromide at 80V for 1.5 hr. Arrow indicates the position of the 403 bp fragment after EcoR1 digestion.
3.7 SEQUENCING OF CDNA CLONES

The cloned and purified plasmid DNAs for pZObR403, pSOCS-3 and pSTAT-3 were sequenced using T7 universal primers. The plasmid DNA inserts were PCR amplified using T7 primers with conditions as follows: denaturation at 95°C for 4 min, and 30 cycles of denaturation at 95°C for 30 sec, synthesis at 56°C for 1 min and extension at 70°C for 30 sec, and final extension at 70°C for 5 min. The inserts were sequenced by using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Foster City, CA). Sequencing reaction products were purified using 96-well gel filtration kit for Big Dye Terminator clean-up, and loaded in a 6% FS automated polyacrylamide sequencing gel in a 373A automated DNA sequencer (Perkin Elmer, Applied Biosystems, Foster City, CA). Figure 3.4 shows an electrophoretogram of the ObR403 sequence.

3.8 GEL PURIFICATION OF cDNA INSERTS

Purified plasmid DNA was digested overnight with specific restriction enzymes as indicated by the restriction maps and on the legends to the figures. Restriction fragments were fractionated by 1% agarose gel electrophoresis in 0.5X TBE (Tris-Borate-EDTA) buffer at 100 V for 1.5 hr. The fragments were visualized under UV light and photographed. The band of interest was excised from the gel, minced in 200 µl TE buffer and transferred into the nebulizer and Microcon concentrating system (Amicon Inc., Beverly, MA). The unit was centrifuged at 10,000 xg for 5 min. The cDNA was recovered by centrifuging the Microcon in an inverted position as above for 30 s. The DNA was quantified by 1% agarose gel electrophoresis using the high or low DNA Mass ladder (GIBCO BRL, Grand Island, NY).
Figure 3.4. An electrophoretogram of pZObR403 plasmid DNA sequence obtained by sequencing using the ABI PRISM® BigDye™ terminator cycle sequencing ready reaction kit after purification of the PCR products. Samples were loaded onto a 36 cm gel containing 5% LongRanger gel, and run on an ABI PRISM® 373 automated DNA sequencer. A BLAST search (www.ncbi.nlm.nih.gov/BLAST) identified nucleotides 4-69 as pBluescript II vector sequence and nucleotides 72-330 as rat longform leptin receptor gene sequence.
3.9 *IN VITRO* TRANSCRIPTION REACTION FOR PREPARATION OF RIBOPROBES

**♦ Preparation of Template**

Template DNA (50 µg) was linearized by complete restriction enzyme digestion with an enzyme that cleaves distally from the selected promoter site. An aliquot of the linearized product (0.5 µg) was analyzed by 1% agarose gel electrophoresis in 0.5X TBE at 100 V for 2 hr to determine complete digestion by restriction enzymes. The linearized template DNA was then incubated with 100 µg/ml Proteinase K in 10 mM Tris-HCl (pH 8.0), 0.5% SDS and 2 mM CaCl₂ at 50°C for 30 min. The mixture was extracted once with phenol/chloroform (1:1) to remove any contaminating proteins. The aqueous phase was carefully transferred to a new tube and the linearized DNA was precipitated with 0.1 volume of 5M ammonium acetate and 2 volumes of ethanol at -20°C for 1 hr. The template DNA was recovered by centrifugation at 14,000 rpm for 15 min at 4°C. The pellet was washed with 70% ethanol, air-dried and resuspended in nuclease-free water to 0.5 µg/µl. The quality of template DNA was assessed by 1% agarose gel electrophoresis and the concentration was estimated by comparison to the DNA mass ladder.

**♦ Assembly of the Transcription Reaction**

The transcription reaction was carried out according to the manufacturer’s instructions using the RPA III kit (Ambion Inc., Austin, TX). Reagents were added at room temperature. One µg of the linearized DNA template, transcription buffer, ribonucleotides (NTPs), [α-³²P]UTP and RNA polymerase (T3 or T7) were mixed in a final volume of 10 µl and incubated at 37°C for 1 hr. The template DNA was removed by digestion with 0.5 µl of RNase-free DNase I (2U/µL) at 37°C for 15 min. The reaction
was terminated by adding 0.5 µL of 0.5 M EDTA. An equal volume of gel loading buffer was added to the reaction, heated at 95°C for 5 min, and then chilled on ice for 5 min. One µl aliquot was removed and added to 10 ml of EcoLite (Amersham, Buckinghamshire, UK) scintillation liquid and radioactivity was counted using the Beckman LS6000 scintillation counter (Beckman Instruments Inc., Fullerton, CA). The remaining aliquot was heated at 95°C for 3 min, loaded onto a 5% acrylamide/8M urea gel and electrophoresed at 300 V for 2.5 hr. After electrophoresis, the gel was exposed to X-ray film (Eastman Kodak Co., Rochester, NY) for 1-5 min in the dark. The film was developed using the Kodak X-OMAT automatic developer. The full-length labeled transcript was precisely localized and excised with a razor blade. The gel piece was transferred into an Eppendorf tube containing 350 µL of Elution Buffer, vortexed and centrifuged briefly. The elution was allowed to proceed overnight at 37°C. One µl aliquot of the eluted probe was removed to count radioactivity as described above.

♦ Ribonuclease Protection Assay (RPA)

Ribonuclease protection assay (RPA) was carried out essentially as described by the manufacturer (Ambion Inc., Austin, TX). Routinely, 20 or 40 µg total RNA was mixed with antisense radiolabeled riboprobe (300,000 cpm), and the mixture was precipitated with 0.1 volumes of ammonium acetate and 2.5 volumes of absolute ethanol at —20°C for 15 min. Tubes were centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was completely aspirated. The pellet was dissolved in hybridization solution and the tubes were heated at 95°C for 4 min and vortexed vigorously. The tubes were incubated in a 45°C water bath for 18 hours to allow hybridization of probe and complementary mRNA. After hybridization, a diluted mixture of RNase A+T1 solution was added and the tubes
were incubated at 37°C for 30 min to digest the unprotected single-stranded RNA. RNases were inactivated by adding the RNase inactivating solution, and the protected RNA was precipitated at -20°C for 15 min. The tubes were centrifuged as before and the supernatant was discarded. The pellets were resuspended in gel loading buffer and heated at 95°C for 4 min to solubilize and denature the RNA. The protected fragments were resolved on a 5% denaturing polyacrylamide gel at 250 V for 3 hr in 1X TBE. The gels were carefully transferred onto a Whatman 3M filter paper and dried at 80°C under vacuum. The dried gels were exposed to a phosphoscreen overnight. Signals were quantified using the Phosphoimager and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Figure 3.5 shows a typical RPA gel showing the hypothalamic and liver leptin receptor protected fragments. The long form ObR, represented by the 403-bp protected fragment, was evident only in the hypothalamus and none was seen in the liver. The short isoform, 270-bp, of ObR was present in both tissues.

3.10 PREPARATION OF TOTAL PROTEIN LYSATES

Total protein lysates were prepared according to O’Donnell, et al., (1995). Briefly, frozen hypothalamic tissue was placed directly into a 2 ml Eppendorf tube containing 5 volumes of ice-cold TEGMD buffer [TEGMD = 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% Glycerol, 10 mM Sodium Molybdate, 1 mM Dithiothreitol], and homogenized on ice using a sonicator (Ultrasonic Homogenizer, 4710 series, Cole-Parmer) at output 6 for 2 sec to disrupt the tissue and cells. The sonication was repeated 5 more times on ice. The resulting homogenate was centrifuged at 105,000 xg for 45 min at 4°C in a Beckman L8-80 ultracentrifuge. The supernatant, containing the total protein fraction, was collected and aliquoted into 1.5 ml Eppendorf tubes.
Figure 3.5. Leptin receptor ribonuclease protection assay of total RNA from the hypothalamus and liver. Increasing concentration of total RNA from the hypothalamus and liver were hybridized overnight with $^{32}$P-labeled antisense ObR403 and $\beta$-actin RNA riboprobes. Samples were processed as detailed in the preceding section and fractionated on a 5% sequencing gel. M is the century RNA molecular marker. The protected fragments, 403 and 270 bp, represent the long and short isoforms of the leptin receptor. The other bands could represent other ObR transcripts that we did not study very closely. The $\beta$-actin protected fragment was used as an internal standard to ensure linearity.
Protein concentration was determined by using the BCA assay (Pierce Chemical Co., Rockford, IL). The tubes were snap-frozen in liquid nitrogen and stored at -80°C until needed for analysis.

3.11 PREPARATION OF HYPOTHALAMIC NUCLEAR EXTRACTS

Nuclear extracts from hypothalami were prepared according to the method of Vaisse, et al. (1996) with modifications. The hypothalami were cleanly dissected and frozen in liquid nitrogen. Frozen hypothalami were homogenized in 0.5 mL of buffer A [10 mM Hepes, pH 7.9; 10 mM KCl; 1.5 mM MgCl₂; 1 mM DTT; 1 mM Na₃VO₄; 1 mM EDTA; 1 mM EGTA; 10% glycerol; 0.5 mM PMSF; 10 mM NaF; 1 mM Benzamidine; 1 mM sodium pyrophosphate; 1 µg/ml each of leupeptin, aprotinin, pepstatin], five strokes each, in a loose-fit and a tight-fit Dounce homogenizer, respectively. Homogenates were centrifuged at 2,000 xg for 10 min at 4°C, and the supernatant (containing the cytosolic extract) was carefully removed into a new tube. The pellet was resuspended in 4 volumes of buffer A and homogenized in a tight-fit Dounce homogenizer. The homogenate was centrifuged as above and the supernatant was combined with the one obtained above. The pellet, which contained nuclei, was resuspended in 2 volumes of buffer B [20 mM Hepes, pH 7.9; 420 mM NaCl; 10 mM KCl; 20% glycerol; 1 mM DTT; 1 mM Na₃VO₄; 1 mM EDTA; 1 mM EGTA; 0.5 mM PMSF; 10 mM NaF; 1 mM Benzamidine; 1 mM sodium pyrophosphate; 1 µg/ml each of leupeptin, aprotinin, pepstatin], and the nuclear proteins were extracted for 30 min on ice, with shaking. The tubes were centrifuged at 16,000 xg for 10 min at 4°C and the supernatant (containing the nuclear proteins) was carefully removed into a new tube and the pellet was discarded. The supernatant was diluted 1:1 with buffer C [20 mM Hepes, pH 7.9; 10 mM KCl; 20% glycerol; 1 mM DTT; 1 mM
Na$_3$VO$_4$; 1 mM EDTA; 1 mM EGTA; 0.5 mM PMSF; 10 mM NaF; 1 mM Benzamidine; 1 mM sodium pyrophosphate; 1µg/ml each of leupeptin, aprotinin, pepstatin], aliquoted and snap-frozen in liquid nitrogen and then stored at −80°C. Protein concentrations were determined by using the BCA reagent (Pierce Chemical Co., Rockford, IL). The nuclear extract was used to analyze proteins by Western blotting and gel shift assays.

**3.12 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)**

Electrophoretic Mobility Shift Assay (EMSA) or gel shift assays were performed according to the method of Vaisse, et al. (1996), with modifications of Levy et al. (1989) and Gronowski et al. (1995). The Stat-3 high affinity c-fos sis-inducible element (m67-SIE) probe was used to determine the binding capacity of nuclear proteins in this study. The pair of m67-SIE oligonucleotide sequences used were m67-SIE sense, 5' -CGCTCCATTTCCCGTAAATCAT-3', and m67-SIE antisense, 5' -CGCTCATGATTTCAGGGAATG-3'.

The double-stranded DNA was generated by annealing equimolar concentrations of the oligonucleotides in annealing buffer [10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA]. The mixture was heated to 95°C for 5 min and allowed to cool slowly at room temperature for 2 hr. The annealed m67-SIE was diluted to 6 and 20 ng/µL with nuclease-free water. For use in EMSA studies, 20 ng of m67-SIE DNA was end-labeled using 10 µCi [γ-$^{32}$P]ATP and T4 polynucleotide kinase at 37°C for 30 min. The reaction was stopped by addition of sterile water and the probe was purified by using the Bio-Gel 30 spin-columns at 1,000 xg for 4 min. Radioactivity was counted and the probe was diluted to 35,000 cpm/µL, which represents a final probe concentration of 0.06 ng/µL. For competition assays in EMSA, a 100-fold excess of cold m-67 (6 ng) was added in the
reaction. All EMSA incubations were carried out at room temperature. One to 5 μg hypothalamic nuclear extract was incubated with 2 μL of 1 μg/μL poly(dI-dC)-poly(dI-dC) (Pharmacia Biotech Inc., Piscataway, NJ), in EMSA buffer [20 mM Hepes, pH 7.9, 40 mM KCl, 10 mM MgCl₂, 10 mM NaCl, 0.2 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 1 μL of 20 mg/ml BSA, and 4% Ficoll] in a final volume of 24 μL. For nonspecific binding, 2 μL of unrelated sheared E. coli B DNA was added to one tube, and for antibody supershift, 3 μL of Stat-3 was added. The mixture was incubated for 10 min and the ³²P-labeled m67-SIE (3 x 10⁴ cpm) was added. The mixture was further incubated for 15 min, and thereafter 3 μL of 10X gel retardation loading buffer [1X = Tris-HCl, pH 7.5; 0.4% glycerol; 0.002% bromophenol blue]. The samples were resolved on a pre-run (100 V, 30 min) 4% native PAGE at 250 V for 1-2 hours in 0.5X TBE. The gel was fixed in 10% acetic acid solution for 15 min and vacuum dried at 80°C for 1 hr. The dry gel was exposed to the phosphoscreen overnight and the bands were analyzed using the PhosphoImager and quantified using the ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

3.13 IMMUNOPRECIPITATION OF CYTOSOLIC AND NUCLEAR PROTEINS

Generally, 100 to 300 μg of hypothalamic nuclear or cytosol extracts were used for immunoprecipitation. Proteins were mixed with immunoprecipitation buffer [150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, fresh 0.2 mM Na₃VO₄, fresh 0.2 mM PMSF] and 10 μL of monoclonal or polyclonal antibody (as indicated in the respective legends to figures on protein assays) was added and incubated for 2 hr at 4°C with shaking at 120 rpm. Thereafter, 20 μL of 50% slurry Protein-A agarose (Pierce Chemical Co., Rockford, IL) was added and further
incubated for 2 hr at 4°C, with agitation. The mixture was centrifuged at 16,000 xg for 4 min at 4°C. The beads were washed three times with immunoprecipitation buffer. The beads were resuspended in an equal volume of 2X Laemmli sample buffer and proteins were eluted by boiling for 5 min. The tubes were centrifuged at 16,000 xg for 4 min and the eluates were analyzed on 8 or 10% SDS-PAGE. After electrophoresis, the proteins were transferred overnight with cold transfer buffer [25 mM Tris base, 192 mM Glycine, 15% methanol] onto PVDF nylon membranes (Boehringer-Mannheim, GmbH, Germany) using the Bio-Rad Trans-blot apparatus (Bio-Rad Laboratories, Hercules, CA). Western blotting was carried out using the Renaissance® chemiluminescence reagent plus method (NEN Inc., Boston, MA). Briefly, membranes were blocked for 1 hr with 5% nonfat dry milk in TBS-T [Tris-buffered saline with 0.05% Tween-20]. After blocking, the membrane was washed with TBS-T three times for 10 min each wash. After washing, the membrane was incubated for 1 hr with diluted primary antibody in 1% nonfat dry milk in TBS-T. The membrane was washed as above and incubated for 1 hr with an appropriate HRP-conjugated secondary antibody diluted 1:2000 in 1% nonfat dry milk in TBS-T. The membrane was washed as above. Depending on the membrane size, equal volumes of reagents A and B were mixed together and incubated for exactly 1 min with the membrane. After 1 min, the reagent was discarded. The membrane was placed in a bag and exposed to X ray film. After processing, the membrane was stripped using the stripping buffer [62.5 mM Tris-HCl, 2% SDS, 100 mM β-mercaptoethanol], washed and reprobed with other antibodies.
3.14 RT-PCR FOR SOCS-3 AND STAT-3 mRNA EXPRESSION

Five µg of total RNA from individual hypothalami was reverse-transcribed by oligo(dT) priming and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) using oligo(dT)15-18. mRNA expression was determined by PCR using the following primer sequences: 5'-ACCAGCGCCACTTCTTCACA-3' and 5'-GTGGAGCATCATCTGGTCC-3' to amplify a 450-bp DNA fragment of SOCS-3 (GenBank AF075383, Bjorbaek et al. (1999)), 5'-AAGGACATCAGTGGCAAG-3' and 5'-ACAGGCCGACAGAAACATAG-3' to amplify a 715-bp DNA fragment of STAT-3 (GenBank X91810) and 5'-GACAAAGTTCTCAAGACAGCAGAAAAAC-3' and 5'-ACTTCAGTGAGGAGCAGATTACAGGG-3' were used to amplify a 528-bp DNA fragment of cyclophilin (GenBank M19533). PCR products were then cloned directly into pCR2.1 T-A cloning system (Invitrogen, Carlsbad, CA), amplified and restriction mapped as shown in Figures 3.6 and 3.8 for SOCS-3 and STAT-3, respectively. The identity of each cloned product was confirmed by sequencing using the ABI PRISM 373 Big Dye terminator sequencing kit, as shown in Figure 3.7 and 3.9 for SOCS-3 and STAT-3, respectively. Quantitation of mRNA expression was performed by quantitative RT-PCR and ethidium bromide staining as described by Emilsson et al. (1999). The intensities of the bands were quantified using the ChemiImager™ 4000 (Alpha Innotech Corp., San Leandro, CA). The bands were quantified relative to cyclophilin as an internal standard.
Figure 3.6. Restriction endonuclease digestion of purified pSOCS-3 plasmid. The amplified plasmid was undigested (lane 6), 250-bp insert excised with KpnI (lane 1), ~460-bp SOCS-3 insert excised with EcoR1 (lane 2), HindIII-XhoI cut plasmid (lane 3), HindIII-linearized plasmid (lane 4) and XhoI-linearized plasmid (lane 5). Lanes M are 100-bp/1-kb DNA ladders. Fragments were fractionated on a 1% agarose gel containing ethidium bromide at 80V for 1.5 hr. Arrow indicates the position of the SOCS-3 fragment after EcoR1 digestion.
Figure 3.7. An electrophoretogram of pSOCS-3 plasmid DNA sequence obtained by sequencing using the ABI PRISM® BigDye™ terminator cycle sequencing ready reaction kit after purification of the PCR products. Samples were loaded onto a 36 cm gel containing 5% LongRanger gel, and run on an ABI PRISM® 373 automated DNA sequencer. A BLAST search (www.ncbi.nlm.nih.gov/blast) identified nucleotides 10-80 as pCR2.1/TOPO vector sequence and nucleotides 82-470 as rat SOCS-3 gene sequence.
Figure 3.8. Restriction endonuclease digestion of purified pSTAT-3 plasmid. The amplified plasmid was undigested (lanes 3), digested with EcoR1 (lanes 1, 4, 5) and AvaI (lane 2). The arrow indicates the 730-bp STAT-3 insert. Lane M is the 100-bp/1-kb DNA ladder. Fragments were fractionated on a 1% agarose gel containing ethidium bromide at 80V for 1.5 hr. Lanes 4 and 5 show EcoR1-digested STAT-3 fragment from large-scale preparations.
Figure 3.9. An electrophoretogram of pSTAT-3 plasmid DNA sequence obtained by sequencing using the ABI PRISM® BigDye™ terminator cycle sequencing ready reaction kit after purification of the PCR products. Samples were loaded onto a 36 cm gel containing 5% LongRanger gel, and run on an ABI PRISM® 373 automated DNA sequencer. A BLAST search (www.ncbi.nlm.nih.gov/blast) identified nucleotides 60-120 as pCR2.1/TOPO vector sequence and nucleotides 121-390 as rat Stat3 gene sequence.
CHAPTER FOUR

LEPTIN EFFECTS ON LIVER GENE EXPRESSION AND METABOLISM

4.1 INTRODUCTION

The liver is one of the most complex organs in the body, carrying out a multitude of different processes. Metabolism of glucose, fatty acids and protein occur in the liver. The liver synthesizes and breaks down glucose by the processes of gluconeogenesis and glycolysis, respectively. Glycogen is also synthesized and broken down in the liver by processes of glycogenesis and glycogenolysis. Hepatic protein synthesis is involved in enzyme regulation and in the production of other circulating factors. Among all these functions, a change in liver glucoregulatory function is a characteristic response to feeding, fasting, and to glucocorticoids. Liver glucose regulatory responses are mediated by the balance of glucose uptake into the liver and the endogenous glucose release from the liver into circulation. Glucose uptake into the liver is an insulin independent process carried out by glucose transporters, Glut-1 and Glut-2 (Zierler, 1999). The balance between glycolysis and gluconeogenesis and the ratio of glycogenesis and glycogenolysis determines the rate of endogenous production of glucose from the liver.

Gluconeogenesis is an energy-dependent process whereby lactate, pyruvate, glycerol, and some amino acids are converted to glucose. The most important function of gluconeogenesis is the maintenance of blood glucose levels during times of low food supply and/or low glycogen stores. Hepatic gluconeogenesis and glycogenolysis are alternative sources of the glucose produced by the liver. Hepatic gluconeogenesis is affected by many modulators, the most important of which include insulin, glucagon, glucocorticoids and catecholamines (Chu, et al., 1997). Insulin suppresses endogenous
glucose output by inhibiting glucagon release from the pancreas (McCall, et al., 1998; Sindelar, et al., 1997), and also directly inhibits gluconeogenesis and glycogenolysis (Giacca, et al., 1997; Sindelar, et al., 1998).

Regulatory mechanisms of enzymes involved in hepatic gluconeogenesis have been extensively studied (Pilkis and Granner, 1992). Both transcriptional and post-transcriptional regulatory mechanisms are involved. A number of hormones regulating the expression of genes that encode regulatory enzymes have been studied. Insulin induces mRNAs that encode glycolytic enzymes and represses mRNAs that encode gluconeogenic enzymes, whereas cAMP has opposite effects. Glucose also regulates the expression of these hepatic enzymes (Kahn, 1997; Rencurel and Girard, 1998). One of the extensively studied regulatory liver enzymes is phosphoenolpyruvate carboxykinase (PEPCK). PEPCK is the rate-limiting enzyme in gluconeogenesis and its activity is solely determined by gene regulation, and not by allosteric effectors. PEPCK gene is regulated by a variety of dietary and hormonal signals, which result in an alteration of the synthesis of the enzyme, both by transcription and by translation. Major factors that increase PEPCK gene expression include cAMP, glucocorticoids, and thyroid hormone, whereas insulin inhibits this process (Pilkis & Granner, 1992). Rose and Heath (1986) reported that corticosterone or sympathetic activation stimulate PEPCK synthesis by increasing glucagon and hepatic cAMP, despite the increase in portal insulin levels. Regulatory elements that control PEPCK gene transcription in liver, kidney and adipose tissue have been delineated, and many of the transcription factors that bind to these elements have been identified (Hanson & Reshef, 1997).
Genetic studies in rodents have revealed leptin and its receptor as important factors in the regulation of both appetite suppression and energy expenditure. Obesity had been largely related to insulin resistance, and the discovery of leptin has shed new insight on the regulation of body weight and energy metabolism. Evidence that leptin exerts adipose-reducing effects in rodents was obtained from pair-feeding and Zucker rats studies (Levin, et al., 1996; Unger, 1997). This suggested a significant metabolic regulatory role for leptin, in addition to appetite suppression. Moreover, the expression of the leptin receptors in peripheral tissues, such as liver, muscle and adipose tissue (Ghilardi, et al., 1996; Luoh, et al., 1997; Cohen, et al., 1996), further supported this metabolic role of leptin. Based on these findings, it is possible that leptin interferes with glucose metabolism by acting directly on the liver. In fact, leptin was reported to exert a direct effect on glucose metabolism in skeletal muscle (Ceddia, et al., 1999) and isolated adipocytes (Ceddia, et al., 1998).

In vitro studies with a human hepatic cell line, HepG2 cells, and the rat hepatoma cell line, H4-II-E, indicated that leptin causes attenuation of several insulin-induced effects (Cohen, et al., 1996). In contrast, Wang, et al. (1997), showed that in rat (H-35) and human (HepG2) hepatoma cell lines expressing the long form of the leptin receptor, leptin did not cause any significant alteration in insulin effects. They therefore argued that, in this instance, leptin will not contribute to the diabetic symptoms associated with obesity.

In vivo studies by Rossetti and colleagues (1997) demonstrated that an acute increase in plasma leptin enhances the inhibitory effect of insulin on hepatic glucose production, induces redistribution of glucose fluxes within the liver and induces changes
in the expression of genes encoding hepatic enzymes that closely resemble those of fasting. It is evident that these studies focused on the effects of leptin on insulin actions and hepatic glucose production, and generated conflicting results regarding the role of leptin in liver glucose metabolism. Recently, Nemecz, et al. (1999) reported that in the presence of leptin, adrenaline-stimulated hepatic glucose release was suppressed in perfused livers. These studies performed in isolated perfused rat liver indicated that leptin directly and acutely modulated hepatic glucose flux.

In the present study, I investigated whether leptin modulates expression of the PEPCK gene in rat hepatocytes. Using Northern blotting, the time course of expression of PEPCK mRNA in untreated hepatocytes, and in those treated with either leptin, dexamethasone, or insulin was determined.

4.2 EXPERIMENTAL PROTOCOLS

Animals

Adult, eight-week-old male lean and obese Zucker rats and 10-week-old Sprague-Dawley rats were used in this study. They were housed individually in hanging wire-mesh cages attached to an automated watering system in a room with a 12 hour light/dark (7:00 AM to 7:00 PM) cycle at a temperature of 22-23°C. All rats consumed nonpurified chow diet (Rodent chow 5001, Purina Mills, St. Louis, MO, USA). All protocols used were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee (IACUC). Hepatocytes were prepared by the in situ collagenase perfusion method as described in Chapter three.
Experiment 4.1  **Time course of PEPCK mRNA expression in hepatocytes from Sprague Dawley rats**

This experiment investigated the effects of leptin, dexamethasone and insulin on the expression of PEPCK in hepatocytes obtained from Sprague Dawley rats. Two adult, 10 week old male Sprague Dawley rats were used per experiment. The rats were *ad libitum* fed before use. Hepatocytes were prepared in the morning and plated before 12:00 noon. Cells were allowed to attach to the substratum for 4 hr, and thereafter incubated in WE medium containing 5% fetal calf serum. The spent medium was changed the next day and cells were incubated in serum-free medium supplemented with 10 mM glutamine containing either 100 ng/ml leptin, 100 nM dexamethasone or 100 nM insulin. Cells were treated for the times indicated in the figure legends. Total RNA was isolated as described in Chapter three.

Experiment 4.2  **The time course of PEPCK mRNA expression in hepatocytes from lean and obese Zucker rats**

This experiment investigated the effects of leptin, dexamethasone and insulin on the expression of PEPCK in hepatocytes obtained from lean and obese Zucker rats. Two lean and two obese 8-week-old Zucker rats were used per experiment. The rats were either fed *ad libitum* or were food deprived a day before use. Hepatocytes were prepared in the morning and plated before noon. Cells were allowed to attach to the substratum and thereafter incubated in WE medium containing 5% fetal calf serum. The spent medium was changed the next day and cells were incubated in serum-free medium supplemented with 10 mM glutamine containing either 100 ng/ml leptin, 100 nM dexamethasone or 100
nM insulin. Cells were treated for the times indicated in the figure legends. Total RNA was isolated as described in Chapter three.

Experiment 4.3 **Hormone concentration effect on PEPCK mRNA expression in hepatocytes from lean and obese Zucker rats**

This experiment determined the effects of increasing concentrations of leptin, dexamethasone and insulin on the expression of PEPCK mRNA in hepatocytes obtained from lean and obese Zucker rats. Hepatocyte cells were handled as described above and were incubated with increasing concentrations of the hormones for 3 hr in serum-free medium. After 3 hr treatment, spent medium was aspirated and cells were harvested by lysis in solution D and RNA was extracted as described in Chapter three.

Experiment 4.4 **The time course of glucose production from hepatocytes from lean Zucker rats**

This experiment determined the time course of glucose production induced by leptin, dexamethasone and insulin from hepatocytes isolated from either lean Zucker or Sprague Dawley rats. Hepatocytes were isolated from lean Zucker rats and plated as above. After adherence to the plate, cells were incubated in serum-free WE medium containing 1 ng/ml dexamethasone and 1 ng/ml insulin. After overnight incubation, spent medium was aspirated and cells were treated with either leptin, dexamethasone or insulin in 2 ml of serum-free and glucose-, L-glutamine-, and sodium pyruvate-free DMEM. At each time point, the medium was aspirated and frozen for use in glucose analysis studies. Cells were harvested by washing with ice-cold PBS and total RNA was extracted as described in Chapter three.
4.3 RESULTS

Experiment 4.1

This experiment was designed to investigate whether leptin affects the expression of PEPCK mRNA in hepatocytes. As shown in Figure 4.1, leptin induced the PEPCK gene expression in Sprague Dawley rat hepatocytes after 3 hr incubation. Compared with serum-free medium control, leptin at concentrations greater than 3 ng/ml increased the levels of PEPCK mRNA expression. Incubation of hepatocytes with fetal bovine serum (FBS) also increased the expression of PEPCK mRNA compared to control. This data showed that FBS may contain factors that can induce the expression of PEPCK mRNA, with leptin being one of those factors.

Figure 4.2 shows the effects of leptin, dexamethasone, or both on PEPCK mRNA expression. Leptin at 30 and 100 ng/ml induced the expression of PEPCK mRNA compared to serum-free control hepatocytes. Dexamethasone at 100 nM increased PEPCK mRNA expression compared to control. A higher concentration of dexamethasone (1 μM) was cytotoxic to cells and induced PEPCK mRNA levels lower than at the 100 nM dose. The induction of PEPCK mRNA was, however, still greater than that of the untreated control. Treatment of hepatocytes with a combination of 30 ng/ml leptin and 100 nM dexamethasone showed a slight, but statistically not significant, increase in PEPCK mRNA expression, which was reduced when the leptin concentration was increased to 100 ng/ml. The cytotoxicity observed in 1 μM dexamethasone-treated hepatocytes was reduced by co-treatment with leptin. This was accompanied by a significant increase in PEPCK mRNA expression compared to untreated control and 1 μM dexamethasone-treated cells.
Figure 4.1 Effects of leptin concentrations on PEPCK gene expression in hepatocytes treated with media alone, FBS or various concentrations of leptin. Hepatocytes from ad libitum fed Sprague Dawley rats were incubated for 3 hr with different concentrations of leptin. Total RNA was extracted and analyzed by Northern blot. (A) Northern blot analyses of PEPCK and β-actin mRNA. (B) Histogram showing the quantification of PEPCK mRNA using ImageQuant software. * p < 0.05 from zero leptin group (2 observations in triplicate per group).
Figure 4.2 Effects of leptin and dexamethasone on PEPCK mRNA expression in hepatocyte from Sprague Dawley rats. Hepatocytes from ad libitum fed Sprague Dawley rats were incubated for 3 hr with either leptin (30 and 100 ng/ml) or dexamethasone (0.1 and 1.0 µM) or the combinations of leptin and dexamethasone. Total RNA was extracted and analyzed by Northern blot. Relative expression of PEPCK mRNA was determined by Northern blot analysis and bands were quantified using the PhosphorImager ImageQuant software. Data expressed as mean ± SEM for samples in triplicates. * p < 0.05 compared to control group and NS indicates not statistically significant.
Experiment 4.2

This experiment was designed to determine the time course of PEPCK gene expression in hepatocytes from lean (Figure 4.3) and obese (Figure 4.4) Zucker rats treated with leptin, dexamethasone and insulin. As shown in Figure 4.3, leptin treatment of hepatocytes from lean rats caused an increase in PEPCK mRNA expression at the 3 hr time point and this was followed by a decline in mRNA expression. Dexamethasone did not increase PEPCK gene expression until after 12 hours of incubation, which is consistent with the literature. Insulin, had no effect on the expression of PEPCK mRNA.

The results of the time course of PEPCK mRNA expression in hepatocytes from obese Zucker rats are shown in Figure 4.4. In these cells, untreated obese Zucker rat hepatocytes showed a rapid decrease in PEPCK mRNA expression. This decrease was also evident in all other treatments, with leptin, dexamethasone and insulin. These hepatocytes showed no response to treatment with any of the hormones.

The effect of leptin and dexamethasone on the production of glucose from the gluconeogenic substrates, pyruvate and glutamine, was also determined, as shown in Figure 4.5. Hepatocytes from lean Zucker rats were incubated in serum-, glucose-, L-glutamine-, and sodium pyruvate-free DMEM medium supplemented with 10 mM pyruvate and 10 mM glutamine. As shown in this figure, control cells produced on average 10 mg/dL glucose in the medium, except after 24 hr where the levels of glucose in the medium rose to over 120 mg/dL. Leptin, on the other hand, progressively increased the production of glucose from 10 mg/dL to about 150 mg/dL after 24 hr in culture. Dexamethasone only effected changes in gluconeogenic glucose after 24 hr in culture.
Figure 4.3 Time course of PEPCK gene expression in hepatocytes from lean Zucker rats treated with leptin, dexamethasone and insulin. Hepatocytes were cultured for the indicated times in serum-free medium containing either 100 ng/ml leptin, 100 nM insulin or 100 nM dexamethasone (DEX). Relative expression of PEPCK mRNA was determined by Northern blot analysis and bands were quantified using the PhosphorImager ImageQuant software. β-actin mRNA shown is that of the leptin treated samples. Data expressed as mean % of control at 0 hr ± SEM for samples in triplicates. * p < 0.05 compared to control time value (n = 3 observations per group).
Figure 4.4 Time course of PEPCK gene expression in hepatocytes from obese Zucker rats treated with leptin, dexamethasone and insulin. Hepatocytes were cultured for the indicated times in serum-free medium containing either 100 ng/ml leptin, 100 nM insulin or 100 nM dexamethasone (DEX). Relative expression of PEPCK mRNA was determined by Northern blot analysis (A) and bands were quantified using the PhosphorImager ImageQuant software (B). Three different blots for leptin dexamethasone and insulin treatment, and β-actin shown is for insulin treatment. Data is expressed as mean % of control ± SEM for samples in triplicate.
Figure 4.5 Effects of leptin (100 ng/ml) and dexamethasone (100 nM) on glucose production of hepatocytes from lean Zucker rats. Hepatocytes were cultured for the indicated times in serum-free DMEM medium supplemented with 10 mM glutamine and 10 mM pyruvate and treated with either 100 ng/ml leptin or 100 nM dexamethasone (DEX). Glucose produced in the medium was assayed using the glucose kit from Sigma. Data expressed as mean ± SEM for samples in triplicates. * p < 0.05 compared to control values at the same time.
Experiment 4.3

This experiment was undertaken to determine the effects of leptin and dexamethasone on the expression of PEPCK mRNA in lean and obese Zucker rats after 3 hr in culture. In this study, as shown in Figure 4.6, PEPCK mRNA expression was ten-fold higher in hepatocytes from obese Zucker rats compared to those from the lean rats. Treatment with 0.1 μM dexamethasone increased PEPCK gene expression. Again, treatment with 1 μM dexamethasone decreased the expression of PEPCK mRNA compared to 0.1 μM dexamethasone treatment. Surprisingly, no effect of PEPCK mRNA expression was observed with leptin treatment of hepatocytes from lean Zucker rats. In hepatocytes from obese Zucker rats, dexamethasone and leptin treatment did not affect the expression of PEPCK mRNA.

4.4 DISCUSSION

The objective of experiments described here was to investigate the direct effects of leptin, dexamethasone and insulin on the gene expression of the liver cytosolic form of PEPCK and to determine whether changes in gene expression would account for the changes in sensitivity to these hormones in both lean and obese Zucker rats. It has been proposed that leptin may play a role in carbohydrate metabolism and insulin action (Flier, 1997; Cohen, et al., 1996; Kolaczynski, et al., 1996). Conversely, leptin treatment of \( ob/ob \) mice decreased both plasma insulin and glucose concentrations, suggesting that leptin may directly or indirectly improve \( in \) vivo insulin action (Pellemounter, et al., 1995; Weigle, et al., 1995; Schwartz, et al., 1996; Halaas, et al., 1995). Recent work in cultured hepatocytes and human hepatoma cells indicated that leptin may regulate gene expression of a key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase and also
Figure 4.6 Effects of leptin and dexamethasone on PEPCK mRNA expression in hepatocyte from lean (A) and obese (B) Zucker rats. Hepatocytes from ad libitum fed lean and obese Zucker rats were incubated for 3 hr with either leptin (10 and 100 ng/ml) or dexamethasone (0.1 and 1.0 μM). Total RNA was extracted and analyzed by Northern blot. Relative expression of PEPCK mRNA was determined by Northern blot analysis and bands were quantified using the PhosphorImager ImageQuant software. Data expressed as mean ± SEM for samples in triplicates.
antagonize insulin action (Cohen et al., 1996). In the studies described in this dissertation, leptin was shown to increase the expression of PEPCK mRNA in hepatocytes from Sprague Dawley and lean Zucker rats, but not obese Zucker rats. The effect of leptin was neither synergistic nor additive when combined with dexamethasone, although leptin seemed to have a protective effect against cytotoxicity induced by high dexamethasone concentrations.

Leptin increased the expression of PEPCK mRNA after 3 hr in culture of hepatocytes from lean Zucker rats, and this was accompanied by a steady increase in glucose production from gluconeogenic substrates. In contrast, no effect of either leptin, dexamethasone or insulin was observed in the expression of PEPCK mRNA from hepatocytes isolated from obese Zucker rats. The amount of glucose produced was insignificant and below detection limits in hepatocytes from obese Zucker rats. This could, of course, be due to either the quick utilization of glucose by these cells for glycolytic purposes to supply energy to the cell or that no glucose was produced in the presence of gluconeogenic substrates, pyruvate and glutamine. The latter could be due to the observed decrease in PEPCK mRNA levels, resulting in the shut off of gluconeogenesis. Alternatively, the high intracellular levels of glucose in these cells may repress the PEPCK mRNA and consequently inhibit gluconeogenesis. These observations are consistent with the literature, where studies have shown that insulin resistance in genetically obese Zucker rats affects peripheral tissues, such as liver, muscle and adipose tissue (Czech, et al., 1978; Kemmer, et al., 1979; Terrettaz, et al., 1986). The responsiveness to insulin of basal gluconeogenesis from lactate was shown to be reduced in hepatocytes from obese Zucker rats compared to their lean littermates (Sanchez-
Gutierrez, et al., 1994; Sanchez-Gutierrez, et al., 1997). This data was consistent with the reduction of gluconeogenesis observed in studies reported herein.

Recent in vivo euglycemic hyperinsulinemic clamp studies have reported that leptin, when administered intravenously for 6 hr, enhanced the inhibition of hepatic glucose production in response to insulin. Also, leptin induced redistribution of intrahepatic glucose fluxes with a decrease in hepatic glucose production by inhibiting glycogenolysis (Rossetti, et al., 1997). In another study, leptin by itself was not capable of changing glucose production by the liver, but it markedly reduced the rate of glucagon-stimulated hepatic glucose production (Ceddia, et al., 1999). These two studies, in vivo and in vitro, respectively, showed that leptin is associated with a decrease in hepatic glycogenolysis. In the presence of gluconeogenic precursors, such as glycerol, L-lactate, L-alanine and L-glutamine, leptin reduced glucose production by isolated hepatocytes. This, however, was contradictory to the effect of leptin on PEPCK mRNA gene expression, where leptin was shown to increase PEPCK gene expression (Cohen, et al., 1996; Rossetti, et al., 1997). These studies showed an increase in PEPCK mRNA expression after intravenous leptin infusion in rats or after incubation of human hepatoma cells with leptin.

In our experiments, we used isolated rat hepatocytes and exposed them to increasing leptin concentrations (0, 3, 10, 30 and 100 ng/ml) for 3 hr. Within this incubation period, temporal changes in gene expression of PEPCK were observed. Initially, we used hepatocytes isolated from ad libitum fed rats, and could not observe any gluconeogenic responses to leptin, dexamethasone and insulin. It was reported that hepatocytes from ad libitum fed rats have increased glycolysis and glycogenolysis due to
the increase in adenosine monophosphate (AMP) levels (López, et al., 1988). AMP is a known allosteric activator of phosphofructokinase and glycogen phosphorylase. In the presence of increased AMP concentrations, activation of PEPCK and amino acid (L-glutamine)-induced gluconeogenesis could not be observed due to intensive glycolysis. This situation, therefore, altered the capability of hepatocytes to respond to gluconeogenic precursors and to leptin dexamethasone and insulin.

When we made the switch to using overnight fasted rats, the effects of these hormones became evident. In the presence of L-glutamine, the average glucose production of hepatocytes increased with time of incubation with leptin. Dexamethasone treatment only increased glucose production after 12 hr in culture, indicating the dependence of this mechanism on PEPCK gene expression. It has been reported that PEPCK gene expression in hepatocytes treated with dexamethasone does not occur until 12 hours in culture. Our studies are, therefore, consistent with the late expression of PEPCK by dexamethasone and shows that leptin induces the expression much earlier. From these studies, leptin was capable of altering the metabolic flux in hepatocytes from overnight fasted rats by increasing not only the PEPCK gene expression, but also by increasing glucose production from these cells.

Recently, it has been reported that 5 ng/ml leptin administered directly to perfused livers of ob/ob mice in vitro, caused an increase in glycogen synthesis, which was accompanied by an increase in hepatic glycogen synthase activity (Cohen, et al., 1998). This short term leptin treatment is consistent with leptin modulating pathways that control hepatic glycogen synthesis in an insulin independent manner. In our studies under fasting conditions, we assumed that glycogen stores had been depleted and that glycogen
synthesis and breakdown was at a minimum. Therefore, addition of L-glutamine to the medium could be used by hepatocytes as a source of energy for the maintenance of hepatocyte metabolism. Also assuming limited glycogenolysis due to low levels of glycogen in fasted rats, leptin caused a direct effect on gluconeogenesis in isolated hepatocytes by increasing PEPCK gene expression. The direct mechanism by which leptin promotes gluconeogenesis in the liver remains to be determined.

Various peripheral tissues or organs have detectable levels of mRNA encoding the long (ObR-L) and short (ObR-S) leptin receptor isoforms. The abundance of the long isoform in peripheral tissues is minimal (Ghilardi, et al., 1996; Luoh, et al., 1997). It remains to be investigated how leptin receptors are involved in the modulation of leptin effects in isolated hepatocytes, especially considering the known fact that the majority of the transcripts in the liver encode the short isoforms of the leptin receptor. In the current studies, we could not identify any of the long isoform of the leptin receptor in the hepatocytes. Only the short isoform of the leptin receptor was present. This observation is consistent with the literature (Hoggard, et al., 1997; Ghilardi, et al., 1996). Zhao and colleagues (2000), recently showed the presence of various short isoforms of ObR in hepatocyte extracts and only the hypothalamic extracts contained both the long and short ObR isoforms (Zhao, et al., 2000).

*In vitro* studies of Cohen and colleagues (1996), using HepG2 and H4II hepatoma cells, reported that leptin attenuated insulin-induced activities by altering the insulin signaling pathway. For example, leptin treatment reduced the tyrosine phosphorylation of insulin receptor substrate (IRS)-I and decreased the association of Grb2 with IRS-I. Recently, Nemecz et al. (1999) showed that leptin or insulin inhibited hepatic glucose
release using *in vitro* perfused livers. A combination of the two hormones had no further inhibition. They speculated that leptin could exert its inhibitory effects via the same signaling pathway as that of insulin. Also, Walder et al. (1997) have demonstrated that leptin inhibits insulin binding to its receptor in isolated adipocytes.

Recent studies have also shown that in cells expressing short forms of ObR, JAK2 and IRS-1, leptin can induce tyrosine phosphorylation of IRS-1 through the short form of the leptin receptor (Bjorbaek, et al., 1997). Also, leptin has been shown to activate PI3-kinase in primary hepatocytes, myotubes (C₂C₁₂) and pancreatic β-cells (Berti, et al., 1997; Kellerer, et al., 1997).

In non-obese humans, plasma leptin values lie between 1 and 20 ng/ml, while values up to 100 ng/ml exist in obese humans (Unger, et al., 1997). The leptin concentrations (3-100 ng/ml) used in the studies reported here were within the physiological range, inclusive of obese and non-obese ranges. It can be concluded from our data and results from short term *in vivo* leptin administration (Rossetti, et al., 1997) that leptin regulates liver glucose production through PEPCK, by antagonizing the effect of insulin on PEPCK mRNA expression and thus activating the gluconeogenic pathway. Also, our experience and data obtained with hepatocytes isolated from obese Zucker rats show that these rats have an intrinsically defective response to short term leptin, dexamethasone and insulin treatment under gluconeogenic conditions. This may arise from the in vivo impairment of the insulin-mediated reduction of cAMP in these hyperinsulinemic obese animals.
CHAPTER FIVE

EFFECTS OF GLUCOCORTICOIDS ON GENE EXPRESSION IN LEAN AND OBESE ZUCKER RATS

5.1 INTRODUCTION

The homozygous recessive trait found in Zucker fatty (fa/fa) rats is associated with massive obesity, increased total body fat, increase in fat cell size and number, increased levels of serum insulin leading to gradual development of insulin resistance, and an increase in lipoprotein lipase activity in adipose tissue (Bray & York, 1979). Compared to lean littermates, the obese Zucker rats have hyperinsulinemia, hyperlipidemia, mild hyperglycemia and abnormal glucose tolerance. *In vitro* and *in vivo* studies have demonstrated that insulin resistance in obese Zucker rats affects peripheral tissues, such as liver, muscle and adipose tissue (Czech, et al., 1978; Crettaz, et al., 1980; Terrettaz & Jeanrenaud, 1983; Terrettaz, et al., 1986). In the liver glucose output was not blocked by the basal hyperinsulinemia, indicating insulin resistance (Terrettaz & Jeanrenaud, 1983). In contrast, hepatic glycolysis in obese Zucker rats does not show insulin resistance, as this metabolic pathway is continuously overstimulated by the hyperinsulinemia present in these animals (Jeanrenaud, 1988).

Genetically obese rodents and women with abdominal obesity have slightly elevated basal corticosterone/cortisol secretion during the diurnal trough (Walker, et al., 1992; Pasquali, et al., 1993). Altered circadian rhythms have been described in the Zucker rat (Prins, et al., 1986; Fukagawa, et al., 1992; Martin, et al., 1979; Murakami, et al., 1995). These altered phases of daily rhythms may also characterize obesity in humans (Belisle, et al., 1988; Fricker, et al., 1990). The contribution of the circadian rhythm to the development and maintenance of excessive weight gain and obesity is unknown.
In general, glucocorticoids increase muscle protein breakdown and gluconeogenesis in stress and starvation to maintain the glucose supply for the brain (Steele, 1975). The clinical syndrome of glucocorticoid excess (Cushing's syndrome) is associated with glucose intolerance, obesity and hypertension (Andrews & Walker, 1999). By opposing the actions of insulin, glucocorticoids could contribute to insulin resistance and obesity.

Adrenalectomy (ADX) was shown to slow the weight gain in both obese Zucker rats and in their lean littermates, with the greater effect of ADX in the obese rats (Bray, et al., 1992). ADX also produces a number of profound effects on other animals with recessive inherited obesity. In the ob/ob mouse, ADX reduced food intake (Saito & Bray, 1984), slowed down body weight gain (Bray, 1982; Yukimura & Bray, 1978b), increased energy expenditure (Vander Tuig, et al., 1984), restored norepinephrine turnover to normal in BAT and heart (Vander Tuig, et al., 1984), abolished insulin resistance in muscle (Oshima, et al., 1984), lowers circulating insulin levels (Yukimura & Bray, 1978a) and normalized glucose tolerance (Bailey, et al., 1986; Solomon, et al., 1977). Similar changes have been shown for the db/db mouse upon ADX (Bray, 1982). In the obese Zucker (fa/fa) rat, ADX also produced decrease in weight gain, decreased food intake and increased energy expenditure due to enhanced thermogenesis in BAT (Yukimura, et al., 1978b; Marchington, et al., 1983). ADX also changed the characteristics of the adipose tissue. The fat pad weights of epididymal, inguinal and retroperitoneal WAT were decreased by ADX. This decrease was associated with a decrease in fat cell size in the epididymal and retroperitoneal fat pads, and a decrease in the LPL activity of WAT of obese Zucker rats and not the lean (Bray, et al., 1992). These
studies suggested that ADX normalizes anomalies associated with genetic obesity, but does not completely reverse the genetic defect of the fatty Zucker rat (Bray, et al., 1989).

Increased \textit{ob} mRNA expression has been reported for all forms of obesity, namely genetic, hypothalamic and dietary (Zhang, et al., 1994; Trayhurn, et al., 1995a; Maffei, et al., 1995; Frederich, et al., 1995a; Funahashi, et al., 1995). This suggested that obesity may be associated with increased expression of the \textit{ob} gene. However, \textit{ob/ob} mice do not produce normal leptin. Synthesis and release of leptin is regulated by neuroendocrine, endocrine and paracrine signals that impinge on the adipocyte. Fasting, \(\beta_2\)- and \(\beta_3\)-adrenergic receptor agonists, cAMP and thyroid hormones decrease the level of \textit{ob} mRNA expression and leptin production (Trayhurn, et al., 1995a; Frederich, et al., 1995; Hardie, et al., 1996; Ahima, et al., 1996; MacDougald, et al., 1995; Saladin, et al., 1995; Fain, et al., 1997; Escobar-Morreale, et al., 1997; Valvaci, et al., 1997; Yoshida, et al., 1997; Slieker, et al., 1996; Mantzoros, et al., 1996; Trayhurn, 1996). Glucocorticoids, cytokines and insulin increase the \textit{ob} mRNA expression (De Vos, et al., 1995; Slieker, et al., 1996; Wabitsch, et al., 1996; Considine et al, 1997; Wabitsch, et al., 1996; Grunfeld, et al., 1996; Sarraf, et al., 1997) and circulating protein levels (Malmström, et al., 1996).

Leptin signaling is mediated by the leptin receptor (OBR) (Tartaglia, et al., 1995; Lee, et al., 1996; Chen, et al., 1996). In humans and rodents, two major isoforms of OBR, long and short, are detected, which differ in the intracellular domains that represent products of alternative splicing. Despite normal ligand binding activity, OBR-S has been described as being incapable of signaling (Ghilardi, et al., 1996; Baumann, et al., 1996). It has, however, been recently shown that the short forms of OBR carry out limited signaling function in peripheral tissues (Bjorbaek, et al., 1997; Murakami, et al., 1997;
For example, in human HepG2 cells and lean rat hepatocytes, it has been shown that leptin enhances PEPCK mRNA levels (Cohen, et al., 1996; Chapter 3 of this dissertation). These effects of leptin were attributed to the action of leptin on OBR-S because OBR-L could not be detected in either study. In contrast, the OBR-L isoform, which is primarily expressed in the hypothalamus, is considered to be the signaling-competent receptor (Vaisse, et al., 1996; Ghilardi, et al., 1996; Baumann, et al., 1996). The obese Zucker (fa/fa) rats carry a mutation (Gln269Pro) of the OBR in the extracellular domain, whereas the db/db mouse has a mutation in the intracellular domain due to splicing defect (Chua, et al., 1996a; Chua, et al., 1996b; Truett, et al., 1991). The mutation in the Zucker rat has been shown to reduce leptin receptor affinity and decreased signaling capacity of the OBR (Crouse, et al., 1998, Yamashita, et al., 1998), and the splicing defect in the db/db mouse produces receptors that are signaling incompetent (Chua, et al., 1996b).

To gain insight into the effects of glucocorticoids on gene expression in the liver and hypothalamus of lean and obese Zucker rats, I studied 1) the effect of adrenalectomy on the liver PEPCK mRNA expression, 2) the effect of adrenalectomy on the leptin receptor mRNA expression in the hypothalamus and liver, and 3) the effect of physiological changes of corticosterone and leptin on the leptin receptor mRNA expression, in lean and obese Zucker rats. I assessed changes in serum hormone levels and modulations of the mRNA expressions, in an attempt to test the hypotheses that glucocorticoid excess in obesity 1) causes hyperglycemia due to dysregulation of PEPCK, and 2) leads to hyperleptinemia, which down-regulates the leptin receptor mRNA expression, and leads to a reduced response to endogenous leptin.
5.2 MATERIALS AND METHODS

Animals

Age-matched, eight to ten-week-old male or female lean and obese Zucker rats, were used in these studies. All protocols used were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee (IACUC). The animals were housed individually in shoe-box cages in a temperature-controlled room with a 12-hour light/dark cycle. All rats consumed nonpurified chow diet (Rodent Chow 5001, Purina Mills, St. Louis, MO, USA) ad libitum throughout the experiment, and water or saline (where indicated) was provided in water bottles.

Experiment 5.1 Effects of adrenalectomy on liver PEPCK mRNA expression in lean and obese Zucker rats

Age-matched lean and obese Zucker rats were bilaterally adrenalectomized via a dorsal approach under isoflurane anesthesia. One group of adrenalectomized rats were subcutaneous implanted with 3 week-slow release 50 mg corticosterone pellets (Innovative Research of America, Sarasota, FL). Adrenalectomized rats were provided with 0.9% saline drinking water and allowed to recover for a week. Body weights were monitored daily after adrenalectomy. After recovery, rats were killed by decapitation to allow collection of trunk blood and preparation of serum. Liver, epididymal adipose tissues, hypothalamic and hippocampal tissues were dissected, weighed and snap-frozen in liquid nitrogen and stored at −80°C until used for RNA extractions. Serum samples were stored at −80°C before being used for hormone assays. Serum insulin and leptin were measured using commercially available RIA kits (Linco, St. Charles, MO, USA) based on rat standards according to the supplier’s instructions. Serum corticosterone was
assayed using a commercial corticosterone RIA kit (ICN Pharmaceuticals, Costa Mesa, CA). Total RNA was extracted from liver by the modified acid-phenol guanidinium-isothiocyanate and Northern analyses (PEPCK, β-actin) were performed as described in general methods. The intensities of the signals were analyzed relative to β-actin using the PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA) and quantified using ImageQuant software.

Experiment 5.2  **The effect of adrenalectomy on liver leptin receptor (OBR) mRNA expression in lean and obese Zucker rats**

Liver total RNA was obtained from Experiment 4.1. Semi-quantitative RT-PCR and ribonuclease protection assays for OBR-S and β-actin were carried out as described in the General Methods section. The intensities of the signals were analyzed relative to β-actin using the PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA) and quantified using ImageQuant software.

Experiment 5.3  **The effect of adrenalectomy on hypothalamic OBR, NPY-Y1, NPY-Y5 and CRH mRNA expression in lean and obese Zucker rats**

This experiment investigated the effects of adrenalectomy on the mRNA expressions of OBR and genes associated with regulation of food intake, namely NPY-Y1 and Y5 receptors, and CRH, in lean and obese Zucker rats. Total RNA was extracted from hypothalami of lean and obese Zucker rats by the TRIzol method as described in the general methods. RPAs for OBR, CRH, NPY-Y1, NPY-Y5 and β-actin were carried out as described in general methods. The intensities of the signals were analyzed relative to β-actin using the PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software.
The effect of corticosterone diurnal rhythm on liver and hypothalamic OBR mRNA expressions in lean and obese Zucker rats

This experiment investigated the relationship of diurnal changes in corticosterone and leptin to the diurnal variation in the expression of OBR in lean and obese Zucker rats.

Eight-week-old, weight-matched lean and obese Zucker rats were used in this experiment. Rats were adapted to their living environment with a 12 hr light/dark cycle (6:00 am-6:00 pm), and food and water was provided ad libitum. After adaptation, the first group of rats was sacrificed at 4:00 p.m., the second at 9:00 pm and the last group at 8:00 a.m. the next day. Trunk blood was collected for serum preparation. Liver, epididymal adipose tissues, hypothalamic and hippocampal tissues were dissected, weighed and snap-frozen in liquid nitrogen and stored at —80°C until used for RNA extractions. Serum samples were stored at -80°C before being used for hormone assays. Serum corticosterone, insulin and leptin were measured as described above. Total RNA was extracted from liver by the TRIzol method as detailed in the general methods. RPAs for OBR and β-actin were carried out as described in general methods, and OBR signal intensities were analyzed relative to β-actin using the PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA) and quantified using ImageQuant software.

Statistics

Analyses of plasma corticosterone, leptin and insulin data as well as body and tissue weights were performed using a two-way analysis of variance (ANOVA). Analysis of gene expression was done by Student’s t test.

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5.3 RESULTS

Experiment 5.1 Effects of adrenalectomy on liver PEPCK mRNA expression in lean and obese Zucker rats

In all experiments, age-matched obese rats were significantly heavier than lean rats. Changes in body and tissue weights and serum hormones caused by adrenalectomy are summarized in Table 5.1. At the end of the experiments, obese sham-operated rats had significantly higher body weights compared to lean rats. The increase in body weight was associated with an increase in mass of epididymal white adipose tissue and the liver. Adrenalectomy attenuated the increase in body weight in obese rats, and this decline was associated with decreased epididymal fat and liver weights compared with the sham-operated rats. Corticosterone replacement restored the obesity syndrome in adrenalectomized rats, increasing the body weights parallel to those of sham-operated rats. Sham-operated obese Zucker rats had higher serum corticosterone, insulin and leptin levels than the lean Zucker rats (Table 5.1). Adrenalectomy reduced the levels of corticosterone to low levels in both the lean and obese rats, and also decreased the levels of circulating leptin and insulin in lean and obese. Corticosterone replacement in lean rats increased the levels of circulating corticosterone, leptin and insulin to the levels approaching or comparable to those found in sham-operated rats, whereas in obese rats the hormone levels remained below those of the sham-operated rats.

The results for the PEPCK mRNA expression in lean and obese Zucker rats of various corticosterone status are shown in Figure 5.1. Sham-operated obese Zucker rats had significantly higher levels of PEPCK mRNA expressed compared to sham-operated lean rats. Adrenalectomy significantly reduced the PEPCK mRNA levels of obese Zucker
### Table 5.1. Body and epididymal WAT weights, and serum hormones of adrenalectomized, adrenalectomized and steroid replaced, and sham-operated lean (Fa/?) and obese (fa/fa) Zucker rats

<table>
<thead>
<tr>
<th></th>
<th>LEAN ZUCKER RAT</th>
<th>OBESE ZUCKER RAT</th>
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<tbody>
<tr>
<td></td>
<td>ADX</td>
<td>ADX+R</td>
</tr>
<tr>
<td>Initial Body weight (g)</td>
<td>196.7 ± 11.7</td>
<td>203.2 ± 13.7</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>206.9 ± 12.7</td>
<td>215.5 ± 15.9</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>10.2 ± 3.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.3 ± 4.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymal WAT (g)</td>
<td>1.15 ± 0.13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.83 ± 0.23&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>6.1 ± 2.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>103.3 ± 8.76&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.3 ± 0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.56 ± 0.59&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>0.96 ± 0.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.2 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
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Values are means ± SEM. There were significant differences in body weight gain ($F_{5,67} = 9.78$, $P < 0.0001$), epididymal WAT ($F_{5,46} = 93.05$, $P < 0.0001$), serum corticosterone ($F_{5,86} = 65.79$, $P < 0.0001$), serum insulin ($F_{5,86} = 34.71$, $P < 0.0001$), and serum leptin ($F_{5,86} = 57.01$, $P < 0.0001$) levels between lean and obese Zucker rats. Significant difference between groups is indicated by the differences in letter symbols, with $P < 0.05$. 
rats compared with sham-operated obese rats. In contrast, adrenalectomy had no significant effect on the PEPCK mRNA levels of lean rats compared with the sham-operated lean rats. Corticosterone replacement of obese adrenalectomized rats did not return the PEPCK mRNA levels to that of sham-operated rats. Corticosterone replacement did not have any effect on PEPCK mRNA expression in adrenalectomized and steroid replaced lean rats.

**Experiment 5.2 The effect of adrenalectomy on liver leptin receptor (OBR) mRNA expression in lean and obese Zucker rats**

In this experiment we determined the effects of adrenalectomy on the expression of liver OBR mRNA in lean and obese Zucker rats. The expression of liver OBR mRNA was examined by semi-quantitative RT-PCR and by RPA. The OBR-S mRNA expression and quantification by RT-PCR are shown in Figure 5.2. Adrenalectomy significantly increased OBR-S mRNA level in livers of obese Zucker rats compared with sham-operated obese rats. Corticosterone treatment of obese adrenalectomized rats decreased the levels of OBR-S mRNA to that comparable to sham-operated obese rats. In contrast, adrenalectomy and corticosterone replacement had no significant effects on the OBR-S mRNA expression in livers of lean Zucker rats. Similar observations were obtained in a separate experiment using RPA to measure OBR-S (Figure 5.3). Again, a statistically significant increase in OBR-S mRNA expression was obtained in adrenalectomized obese Zucker rats compared to sham-operated obese rats. Adrenalectomy did not have any effect on the OBR-S mRNA expression from lean Zucker rats. However, corticosterone replacement did not show any effect on OBR-S mRNA expression in either the lean or obese rats in this experiment.
Experiment 5.3

This experiment was designed to determine the effects of ADX on the expression of OBR-L mRNAs in the hypothalamus of lean and obese Zucker rats. The OBR mRNA expression was examined by semi-quantitative RT-PCR and RPA. Figure 5.4 shows the effect of ADX on OBR-L mRNA expression in lean and obese Zucker rats. ADX caused a significant increase in OBR-L mRNA expression in obese Zucker rats compared to sham-operated rats. ADX did not have any effect on the OBR-L mRNA expression in lean rats compared to sham-operated rats. The effect of corticosterone replacement was not evaluated in this study.

Figure 5.5 shows the effects of ADX on OBR mRNA expression in the hypothalamus of lean and obese Zucker rats as determined by RPA. Unexpectedly, ADX did not have any significant effect on the expression of either the long and short isoforms of OBR in lean and obese Zucker rats. We also determined the effect of ADX on the expression of NPY receptors and CRH mRNA levels. Figure 5.6 shows the RPA autoradiograms for NPY-Y1, NPY-Y5, CRH and β-actin for lean (A) and obese Zucker rats. In all experimental groups, the NPY-Y1, NPY-Y5 receptors and CRH were significantly higher in lean Zucker rats compared to obese Zucker rats. ADX did not have any effects on the mRNA levels of NPY-Y1, NPY-Y5 receptors and CRH.

Experiment 5.4

This experiment was designed to determine the effects of basal physiological changes in corticosterone during the diurnal rhythm, on the expression of hypothalamic OBR-S and OBR-L mRNAs in lean and obese Zucker rats. Figure 5.7 shows the changes in basal levels of corticosterone and leptin in lean and obese Zucker rats. In lean rats,
**Figure 5.1.** Effects of adrenalectomy on liver PEPCK mRNA expression in lean and obese Zucker rats. (A) A representative Northern blot of PEPCK and β-actin mRNA. (B) Histogram showing the quantification of PEPCK mRNA relative to β-actin. Total liver RNA (40 μg/lane) was analyzed by Northern blotting. Relative expression of PEPCK mRNA was determined by hybridization with $^{32}$P-labeled PEPCK and β-actin cDNAs. Lanes 1,3,13,15 = lean-ADX, lanes 2,4,14,16 = obese-ADX, lanes 5,7,17,19 = lean-ADX+R, lanes 6,8,18,20 = obese-ADX+R, lanes 9,11,21,23 = lean-sham, and lanes 10,1222,24 = obese-sham. Bands were quantified using the ImageQuant software. Data are expressed as means ± SEM. This figure represents typical data obtained from 5 separate experiments. * $p < 0.05$ compared to lean sham-operated rats, ** $p < 0.005$ compared to sham-operated obese rats.
Figure 5.2. Effects of adrenalectomy on liver OBR-S mRNA expression in lean and obese Zucker rats. (A) A representative semi-quantitative RT-PCR for OBR-S and β-actin mRNA. Lanes 1, 5, 9 = lean-sham, lanes 2, 6, 10 = lean-ADX, lanes 3, 7, 11 = obese-sham and lanes 4, 8, 12 = obese-ADX. (B) Histogram showing the quantification of OBR-S mRNA relative to β-actin. Total RNA was extracted and analyzed by RT-PCR. 10 µg of total RNA was reverse transcribed and amplified by PCR in the presence of [α-32P]dCTP (10 µCi was used for whole experiment). cDNA (5 µL) was mixed with sequencing stop solution and fractionated on a sequencing gel. The gel was dried and exposed to phosphoscreen. Expression of OBR-S mRNA was determined relative to β-actin. Bands were quantified using the ImageQuant software. Data is expressed as means ± SEM for 3 rats in each group. * p < 0.05 compared to obese sham-operated rats.
Figure 5.3. Ribonuclease protection assay to determine the effect of ADX on liver leptin receptor mRNA expression. Total RNA was extracted from livers of ADX, ADX+R and sham-operated lean (A) and obese (B) Zucker rats. RNA was hybridized with $^{32}$P-labeled antisense probes for OBR, GR and $\beta$-actin and processed as described in the general methods. Representative RPA autoradiograms for short leptin receptor isoforms, and histograms showing the quantification of the OBR-S mRNAs. The arrow shows the 270-bp protected fragment for OBR-S, and the * arrow shows GR protected fragment. P is undigested probe for OBR (top band) and GR (bottom band). Data are presented as means ± SEM. * p < 0.05 compared to obese sham-operated rats.
Figure 5.4. Effects of adrenalectomy on hypothalamic OBR-L mRNA expression in lean and obese Zucker rats. (A) A representative semi-quantitative RT-PCR for OBR and β-actin mRNA. Lanes 1, 5, 9 = lean-sham, lanes 2, 6, 10 = lean-ADX, lanes 3, 7, 11 = obese-sham and lanes 4, 8, 12 = obese-ADX. (B) Histogram showing the quantification of OBR-L mRNA relative to β-actin. Total RNA was extracted and analyzed by RT-PCR. 10 μg of total RNA was reverse transcribed and amplified by PCR in the presence of [α-32P]dCTP (10 μCi was used for whole experiment). cDNA (5 μL) was mixed with sequencing stop solution and fractionated on a sequencing gel. The gel was dried and exposed to phosphoscreen. Expression of OBR-L mRNA was determined relative to β-actin. Bands were quantified using the ImageQuant software. Data is expressed as means ± SEM for samples in triplicate. * p < 0.05 compared to obese sham-operated rats.
corticosterone were high 2 hr before lights off and slightly, but significantly \((p < 0.05)\), decreased after feeding. The levels of corticosterone further decreased significantly \((p < 0.005)\) the following morning [**Figure 5.7 (A)**]. The levels of corticosterone was also elevated in obese rats before dark-onset. After feeding, the corticosterone levels decreased significantly \((p < 0.05)\). The levels of corticosterone in the obese rats did not decrease any further in the morning compared to the lean rats. Serum leptin levels in lean rats showed some rhythm [**Figure 5.7 (B)**]. Leptin levels in these rats were lower before dark-onset and significantly \((p < 0.005)\) increased after feeding. In the morning, leptin levels were significantly \((p < 0.005)\) reduced compared to those observed after feeding. In contrast, no leptin rhythm was observed in obese rats, even though there was a change in corticosterone levels in these rats. **Figures 5.8 and 5.9** indicates the effect of leptin and corticosterone rhythms on the liver and hypothalamic OBR-S and hypothalamic OBR-L mRNA expressions in lean and obese Zucker rats. There were no significant effects of leptin or corticosterone rhythms on OBR mRNA expression.

### 5.4 DISCUSSION

This study was undertaken to investigate the role of glucocorticoids on the regulation of gene expression of PEPCK and OBR mRNAs in lean and obese Zucker rats. Data presented in this dissertation provide evidence for a role of glucocorticoids in modulating PEPCK and OBR mRNA, particularly in obese animals. Adrenalectomy reduced circulating serum corticosterone, insulin and leptin levels in both lean and obese rats. The decrease in serum hormones was accompanied by an increase in the hypothalamic OBR-L and OBR-S mRNA, and liver OBR-S mRNA in obese Zucker rats.
Figure 5.5. Ribonuclease protection assay to determine the effect of ADX on leptin receptor mRNA expression. Total RNA was extracted from individual hypothalami of ADX, ADX+R and sham-operated lean and obese Zucker rats. RNA was hybridized with $^{32}$P-labeled antisense probes for OBR and β-actin and processed as described in the general methods. A) A representative RPA autoradiogram for leptin receptor isoforms, and B) histograms showing the quantification of the OBR-L and OBR-S mRNAs, where the open-bars are lean and dark-bars are obese samples. Data are presented as means ± SEM. There were no statistically significant differences between all groups.
Figure 5.6. Ribonuclease protection assay to determine the effects of adrenalectomy on NPY receptors mRNA expression in the hypothalamus of lean and obese Zucker rats. Total RNA was extracted from individual hypothalami of ADX, ADX+R and sham-operated lean and obese Zucker rats. RNA was hybridized with $^{32}$P-labeled antisense probes for NPY-Y1, NPY-Y5, CRH and β-actin and processed as described in the general methods. Representative RPA autoradiograms for NPY-Y1, NPY-Y5, CRH, and β-actin in lean (A) and obese (B) Zucker rats.
Figure 5.6. (cont.). Histograms showing the quantification of the NPY-Y1, NPY-Y5 and CRH mRNA relative to β-actin, where the open-bars are lean and dark-bars are obese samples. Data are presented as means ± SEM. There were no statistically significant differences caused by ADX between all groups.
The increase in the mRNA expressions was concomitant with the correction of the hyperleptinemia by adrenalectomy in obese Zucker rats.

An increase in hepatic gluconeogenesis is believed to be an important factor responsible for the fasting hyperglycemia detected in patients with NIDDM (Valera, et al., 1994). The presence of mild hyperglycemia has been reported in obese Zucker rats compared to lean rats, and this was more pronounced in males than in females (Triscari, et al., 1979; observation in this study). This hyperglycemia was associated with elevated liver and kidney PEPCK activity and kidney glucose production in obese compared to lean rats. Furthermore, total liver glycogen levels and rates of glycogen synthesis were increased significantly in obese compared to lean rats. The authors concluded that the mild hyperglycemia present in obese Zucker rats was not associated with delayed disappearance of intravenously administered glucose, but could be due to the increased production of glucose by whole kidney and liver (Triscari, et al., 1979).

Recently, Friedman and colleagues (1997), using transgenic mice expressing the intact PEPCK(460)-C-reactive protein transgene, showed that these mice were obese, hyperinsulinemic, and developed fasting hyperglycemia with age. Levels of CRP reporter gene expression were increased 2-fold despite severe hyperinsulinemia compared with non-diabetic non-obese transgenic mice, and treatment of obese diabetic db/db transgenic mice with the glucocorticoid receptor blocker RU 486 decreased plasma glucose by 50% and reduced PEPCK, GLUT2, glucose-6-phosphatase, tyrosine aminotransferase and CRP reporter gene expression to levels similar to those of non-obese normoglycemic transgenic mice. These results showed that -460 bp of 5'-flanking sequence is sufficient to mediate the induction of PEPCK gene transcription in genetically obese db/db mice.
Figure 5.7. Serum corticosterone and leptin rhythms in lean and obese Zucker rats. Serum was obtained from lean and obese Zucker rats killed by decapitation at 4:00 pm (open bars), 9:00 pm (slashed bars) and 8:00 am (black bars). Corticosterone (A) and leptin (B) were measured by RIA kits. The insert is an enlargement of the serum leptin levels in lean Zucker rats to indicate the rhythm in leptin. Data is expressed as mean ± SEM.
Figure 5.8. The effect of leptin and corticosterone rhythms on liver OBR-S mRNA expression. Livers were dissected from lean and obese Zucker rats killed by decapitation. Histogram showing the quantification of OBR-S mRNA relative to β-actin. Total RNA was extracted and analyzed by RPA. 40 μg of total RNA was hybridized to 32P-labelled antisense ObR403 probe. Expression of OBR-S mRNA was determined relative to β-actin. Bands were quantified using the ImageQuant software. Data is expressed as means ± SEM for samples in triplicate.
Figure 5.9. The effect of leptin and corticosterone rhythms on hypothalamic OBR-S and OBR-L mRNA expression. Hypothalami were dissected from lean and obese Zucker rats killed by decapitation. Histograms showing the quantification of OBR-S and OBR-L mRNA relative to β-actin. Total RNA was extracted and analyzed by RPA. 40 μg of total RNA was hybridized to 32P-labelled antisense ObR403 probe. Expressions of OBR-S and OBR-L mRNA were determined relative to β-actin. Bands were quantified using the ImageQuant software. Data is expressed as means ± SEM for samples in triplicate.
during the development of hyperglycemia, demonstrating that the mechanism underlying increased expression of gluconeogenic enzymes in the \textit{db/db} mouse requires the action of glucocorticoids (Friedman, et al., 1997). Sharma and Patnaik (1983, 1984) have shown that the activity of PEPCK is highest in livers of adult rats and that adrenalectomy decreases significantly the activity of PEPCK of livers of rats of all the ages. Administration of hydrocortisone to adrenalectomized rats increased the activity of PEPCK in the liver of young and adult rats but not in the old rats. Our studies indicate a significant induction of liver PEPCK mRNA in obese Zucker rats, and that this increase can be reduced by adrenalectomy. PEPCK mRNA transcription is negatively regulated by insulin. The obese Zucker rat is hyperinsulinemic, but this high endogenous insulin concentration cannot inhibit PEPCK gene transcription. Rosella and colleagues (1995) recently described an overexpressed, non-insulin-responsive gluconeogenic PEPCK. This leads us to speculate that obese Zucker rats express this type of PEPCK mRNA, which is mainly under regulation by glucocorticoids. Data presented in this study is consistent with previous reports which showed an increased \textit{ob} gene expression and leptin levels in adipose tissue from obese Zucker rats compared to lean littermates (Murakami \& Shima, 1995). Maffei and colleagues (1995) reported a 50-fold increase in circulating leptin in obese Zucker rats, which was quantitatively comparable to our results. They also showed that an increased expression of the \textit{ob} gene is common to other animal genetic obesity models and diet-induced obesity (Maffei, et al., 1995).

The beneficial effects of adrenalectomy, such as decreases in food intake, body weight gain, plasma insulin, triglycerides, fat cell size and LPL activity, have been shown to be reversed by the replacement of corticosterone (Castonguay, et al., 1986; Freedman,
et al., 1986; Bray & York, 1979). However, the mechanisms by which the loss of glucocorticoids prevents the phenotypic expression of obesity in the obese Zucker rat are still unknown. This study provides data consistent with the possibility that expression of the leptin receptor is regulated by glucocorticoids, whereby glucocorticoids increase leptin gene expression and protein levels which then causes downregulation of OBR-L and OBR-S. This suggests that corticosterone, directly or indirectly, is an important factor in the expression of obesity in fa/fa rats. It is possible that glucocorticoid hormones have a direct effect to regulate transcription of the OBR gene. Indirectly, corticosterone increases ob gene expression and secretion, and this increase in leptin may lead to downregulation of the leptin receptor isoforms. Alternatively, it is also possible that glucocorticoids modulate other genes, the products of which regulate leptin receptor or leptin gene expression. Obese (fa/qa) Zucker rats carry a mutation (Gln269Pro) on the cytokine-binding domain of the leptin receptor (Chua, et al., 1996), and this mutation has been shown to reduce leptin receptor signaling (Chen, et al., 1996). As adrenalectomy prevented further weight gain in fa/qa rats, it suggests that the effects of adrenal steroids are independent of the changes observed in the levels of the mutated receptor. The increase in leptin expression in fa/qa rats could result from a number of mechanisms. It could reflect the absence of an appropriate leptin feedback onto the mutated leptin receptor. Alternatively, it is possible that increased hyperinsulinemia may stimulate increased synthesis and secretion of leptin in obese Zucker rats. This was recently supported by the observation that ob gene expression was not increased in preweaning obese rats, whereas it increased with increase in insulin after weaning and in adult animals (Cusin, et al., 1995). In contrast, basal leptin was not further increased by
hyperleptinemia in obese rats despite the higher plasma insulin concentration reached in obese animals (Pagano, et al., 1997). This could indicate a possible alternative mechanism whereby the ob gene is not under the control of insulin in obese fa/fa rats, but under the control of glucocorticoids. Our data supports the latter mechanism, which is further supported by the observation that fasting, which reduces plasma insulin and increases glucocorticoids both in lean and obese Zucker rats, inhibits ob gene expression only in lean rats (Cusin, et al., 1995). This indicates that increase in already high glucocorticoids in obese rats during fasting increased ob gene expression.

It is still unclear whether hyperleptinemia in obesity results from the increased size of the white adipose tissue, or if other hormonal, metabolic and neuroendocrine factors may also play a role. Our results suggest that glucocorticoids are important regulators of leptin and leptin receptor mRNA expression, because adrenalectomy was effective in decreasing the former and increasing the latter. This reciprocal modulation of the ligand and its receptor could suggest that the receptor is down-regulated in the presence of circulating high ligand, in this case with leptin down regulating the receptor in the presence of high glucocorticoids. This also indicates that, in the absence of glucocorticoids, leptin secretion by white adipose tissue is reduced. This could reflect a direct effect of the absence of adrenal glucocorticoids on leptin gene expression or it could be due to decreased fat pad size induced by removal of adrenal glucocorticoids. However, the effect of insulin on our system cannot be entirely discounted, even though a role of insulin in influencing leptin concentration in humans does not support this. The size of the white adipose tissue was shown to be a relevant determinant of circulating leptin (Considine, et al., 1996). Our results also indicate that glucocorticoids do influence
leptin secretion by adipose tissue, since leptin secretion was increased by the replacement with corticosterone in adrenalectomized rats.

NPY is the most potent orexigenic agent known, and icv administration thereof causes hyperphagia, hyperinsulinemia and obesity. However, the mechanism by which NPY causes obesity development remains to be defined. Much research is now focused on which NPY receptor might mediate the potent NPY induced feeding response. Several pieces of evidence pointed to NPY-Y1 and NPY-Y5 receptor subtypes to be the candidates for this action. Glucocorticoids play an important role in energy balance, as excessive corticosterone promotes obesity and increase NPY synthesis and NPY-Y1 receptor expression in the ARC. ADX reduces hyperphagia and body weight in obese Zucker rats, and prevents obesity induced by central icv NPY infusion. Larsen et al. (1994) reported that ADX does not alter NPY-Y1 mRNA expression in the ARC. Our data is consistent with this observation, because ADX did not influence the mRNA levels of NPY-Y1 and NPY-Y5. In contrast, Wisialowski and colleagues (2000) recently showed that ADX decreases NPY-Y1 and NPY-Y5 receptor mRNA in the VMH. It is possible that significant localized changes in NPY receptor expression within nuclei occur, but overall there seems to be no changes in these receptor mRNAs in our studies of the whole hypothalamus. However, these observations could imply that NPY receptors are modulated by glucocorticoids. It is also possible that glucocorticoids act by regulating the levels of CRH, which has been shown to decrease food intake and inhibit NPY synthesis and release. The decrease in NPY expression in ADX rats was proposed to be a result of increased CRH activity as well as decreased corticosterone levels. In this study,
however, no changes in CRH mRNA were observed, but we cannot rule out changes occurring within the PVN, the site of glucocorticoid effects on CRH.

Glucocorticoids, at physiological concentrations, stimulate leptin secretion by enhancing the pre-translational machinery in human visceral fat. This effect was more pronounced in obese subjects due to a greater responsiveness of the ob gene and could contribute to the metabolic abnormalities associated with central obesity by paracrine or endocrine actions of hyperleptinemia on adipocytes and liver (Halleux, et al., 1998). Unlike dexamethasone, insulin had no direct stimulatory effect on ob gene expression and leptin secretion, and even prevented the positive response to dexamethasone by a cAMP-independent mechanism that remained functional despite insulin resistance (Halleux, et al., 1998). A similar argument can be presented in this study, where insulin, per se, may not have any effect on the expression and secretion of leptin, but that the observed effects were exclusively under the influence of glucocorticoids.

The existence of abnormal regulation of the HPA axis in genetically obese Zucker rats has been reported (Guillaume-Gentil, et al., 1990). Previous data have shown increased morning plasma corticosterone in obese animals (White, et al., 1988). Some results have described the absence of a circadian rhythm of the corticosterone in obese rats (Martin, et al., 1978; Gibson & Krieger, 1981). In the present study, basal measurement of corticosterone and leptin were determined. Morning serum corticosterone levels of obese Zucker rats are significantly higher than those of their lean littermates. Afternoon corticosterone levels are significantly higher than the morning levels in lean rats, and this level is decreased 3 hr after meal time. In contrast, corticosterone levels in obese rats decreased after meal time, but did not decrease to
lower levels in the morning. This data could be in keeping with the preservation of a “partial” circadian rhythm of corticosterone in the obese rats, even though the trough will not be as deep as it could be in the lean rats. This data supports that of Yukimura and colleagues (1978). This data is also in keeping with the study that showed an increased daily urinary corticosterone output in obese Zucker rats in comparison to lean littermates (Cunningham, et al., 1986). Furthermore, our results may indicate that there is an increased activity of the HPA axis in the obese Zucker rats compared to the lean rats. In contrast to the partial diurnal rhythm of corticosterone in obese Zucker rats, no leptin rhythm was observed in these rats. Lean Zucker rats showed a rhythm where leptin was reduced before dark-onset, increased 2 hr after a meal and decreased during the lights on. This rhythm could be a functional regulator of the leptin receptor in lean rats, with the receptor decreasing during high leptin and increasing during the low levels. This, however, would be a lost function in obese Zucker rats that are hyperleptinemic and have no rhythm of the circulating leptin. This effect could induce down-regulation of the leptin receptor, which may in turn be responsible for potential leptin resistance. Even though we observed leptin rhythm in lean Zucker rats, no rhythm of OBR was seen in these rats. This could suggest that leptin does not regulate OBR or that the time of sampling for OBR was inappropriate because we did not allow a lag time from leptin measurements.

The presence of glucocorticoids is necessary for the development and maintenance of obesity (Fletcher, 1986), and the administration of corticosterone to normal rodents causes hyperinsulinemia (Diamant & Shafrir, 1975) and increased fat deposition and hyperphagia (Bray, et al., 1990). Furthermore, adrenalectomy of underweight rats blunted hyperphagic response associated with free access to food, and
this defect was normalized by central administration of glucocorticoids (Green, et al., 1992). These observations suggested that hypercorticosteronemia observed in the obese rat contributes to several aspects of its metabolic abnormalities (Jeanrenaud, et al., 1985). This is validated by reports that administration of cortisol to normal humans produced insulin resistance (Rizza, et al., 1982), a similar feature of Zucker rats (Jeanrenaud, et al., 1985). The increased HPA activity of the obese Zucker rats has been shown to be associated with an abnormal (increased) regulation of CRF release (Bestetti, et al., 1990). Adrenalectomy may therefore, help restore the hypothalamic CRF content and normalize abnormalities of obesity via restoration of the autonomic nervous system (Rohner-Jeanrenaud, et al., 1989).

While we could demonstrate effects of adrenalectomy on the levels of OBR mRNA in obese rats, the lack of difference in OBR mRNA between lean and obese rats and the lack of a diurnal variation in OBR mRNA suggest that corticosterone does not regulate OBR at physiological concentrations. However, the dramatic effect of adrenalectomy might be interpreted to suggest that there is a permissive requirement for glucocorticoids for the downregulation of the leptin receptor.
CHAPTER SIX

EFFECTS OF ADRENALECTOMY ON THE LEPTIN RECEPTOR SIGNALING SYSTEM

6.1 INTRODUCTION

Adrenalectomy prevents the development of all forms of rodent obesity, including those that have a defect in the leptin signaling pathway. The mechanism through which this response is mediated remains unclear, but is related primarily to the removal of adrenal glucocorticoids because glucocorticoid replacement of adrenalectomized animals restores fat deposition and obesity (Bray, et al., 1990a; Bray, et al., 1990b; Saito & Bray, 1984; Bruce, et al., 1982; Freedman, et al., 1986). Also, blockage of type II GR with the antagonist RU486, inhibits development of obesity induced by the overactivity of the GR receptor (Langley & York, 1990; Okada, et al., 1990). Leptin, a hormone secreted by the adipose tissue, plays an important role in the regulation of energy balance, providing a signal to the central nervous system on the levels of triglyceride stores (Halaas, et al. 1995; Friedman & Halaas, 1998; Friedman, 1999). Glucocorticoids and insulin stimulate leptin gene expression in the adipose tissue and leptin protein secretion into the circulation (De Vos, et al., 1995; Slieker, et al., 1996; Wabitsch, et al., 1996; Malmström, et al., 1996). Administration of glucocorticoids increases food intake in rodents, overriding the effect of increased endogenous leptin expression and secretion. These contrasting effects of glucocorticoids may suggest that glucocorticoids either reduce sensitivity to leptin or oppose leptin action through independent pathways. Indeed, an increase in response to leptin has been described in adrenalectomized rats by Zakrzewska and colleagues (1997). Corticotropin-releasing hormone (CRH), which suppresses food intake, is negatively regulated by glucocorticoids. However, CRH deficiency after ADX
did not affect food intake and body weight, indicating that factors other than or in addition to CRH are important in mediating food intake responses after ADX (Jacobson, 1999).

The effects of leptin are impaired when receptors of neurotransmitters implicated in feeding, namely GLP-1, MC-4, and NPY, are either knocked out or blocked by specific antagonists (Marsh, et al., 1999; Hollopeter, et al., 1998b; Scrocchi, et al., 1997; Goldstone, et al., 1997). These observations led to the conclusion that leptin’s actions to inhibit food intake may not be direct, but mediated or modified by the action of other neurotransmitters. Figure 6.1 shows some of the suggested possible pathways that leptin uses to regulate feeding. Evidence supporting an interaction between leptin and NPY came from studies showing that the NPY mRNA expression in the ARC is increased in animal models with leptin signaling abnormalities (Schwartz, et al., 1996a). Peripheral injection of leptin to fasted rats and ob/ob mice normalized the overexpression of NPY in these situations (Stephens, et al., 1995; Schwartz, et al., 1996a; Wang, et al., 1997; Schwartz, et al., 1996b). More evidence for the interaction between leptin and NPY was shown by the presence of leptin receptors on NPY neurons (Håkansson, et al., 1996; Baskin, et al., 1999a; Baskin, et al., 1999b). NPY, which is modulated by leptin and glucocorticoids, is an important mediator of leptin’s action in the CNS (Rohner-Jeanrenaud, et al., 1996), since recombinant leptin inhibits, and glucocorticoids enhance, hypothalamic NPY gene expression and secretion (Stephens, et al., 1995; Schwartz, et al., 1996).

Neurons of the ARC also synthesize POMC, which is enzymatically cleaved into melanocyte stimulating hormones (α, β, and γ) and β-endorphin. α-MSH has been shown
to affect food intake by activating post-synaptic receptors, especially the melanocortin-4 (MC-4) receptor subtype (Zemel & Shi H, 2000; York, 1999; Kask, et al., 1998). Targeted disruption of this receptor subtype causes obesity (Huszar, et al. 1997). POMC neurons in the ARC express leptin receptor mRNA, suggesting that leptin may bind to neurons in this brain region to influence the production of melanocortins (Cheung, et al., 1997). Injection of leptin to fasted animals and ob/ob mice increased POMC mRNA expression, but this effect was not observed in db/db mice which lack the capacity for leptin signaling (Mizuno, et al., 1998; Schwartz, et al., 1996b).

**Figure 6.1.** Possible pathways downstream of leptin receptor to control feeding. Corticotropin releasing hormone (CRH), pro-opiomelanocortin (POMC) and cocaine-amphetamine related transcript (CART) decrease food intake, whereas neuropeptide Y (NPY), orexin, melanin-concentrating hormone (MCH) and agouti-related protein (AGRP) increase food intake. Agouti-related protein (AGRP) is increased by leptin and competes with α-MSH for the MC-4 receptor to decrease food intake.
CRH is released in the median eminence, where it induces ACTH release from the anterior pituitary via the hypothalamic pituitary portal circulation. In addition to its ability to activate the HPA axis, CRH also regulates food intake and energy balance (Arase, et al., 1989). Loss of the negative feedback from glucocorticoids to the brain following ADX acts to increase CRH mRNA and content in the PVN (Jacobson, et al., 1990).

The leptin receptor (OBR) is a single membrane-spanning receptor that is similar to the class I cytokine receptor family (Tartaglia, et al., 1995). The majority of the transcripts in most tissues are those encoding the short forms of the receptor (Ghilardi, et al., 1996), and the transcript that encodes the long form is less abundant except in the hypothalamus. Within the hypothalamus, the long OBR isoform is expressed in regions that are thought to control food intake and body weight, namely, the arcuate nuclei (ARC), ventromedial nuclei (VMN) and paraventricular nuclei (PVN) (Mercer, et al., 1996). The functions of the long and short intracellular domains of the leptin receptor are currently being defined. The short isoforms play a major role in transporting leptin from the blood into the brain or out of the brain into the cerebrospinal fluid (CSF) for clearance (Cumin, et al., 1996; Cumin, et al., 1997; Esler, et al., 1998). The long OB-R isoform provides intracellular signaling by acting through the JAK-STAT pathway as illustrated in Figure 2.5, particularly the STAT-3 protein in the hypothalamus (Vaisse, et al., 1996). The JAK proteins are associated with the receptor intracellular domain, where they phosphorylate tyrosine residues of the receptor upon ligand binding. The phosphorylated receptor then provides the docking site for STAT proteins, which are also tyrosine phosphorylated upon binding the phosphorylated receptor. The activated STAT proteins
then dimerize and translocate to the nucleus to stimulate gene transcription (Darnell, et al., 1994; Darnell, 1996; Darnell, 1997).

Recently, a family of cytokine-induced cytokine signaling inhibitors has been described. It includes eight members, namely the cytokine-inducible SH2 proteins (CIS) and suppressor of cytokine signaling (SOCS) 1 to 7 (Hilton, et al., 1998). Structurally, the SOCS proteins are composed of an N-terminal region of variable length and amino acid composition, a central SH2 domain, and a previously unrecognized C-terminal motif called the SOCS box. By using the SOCS box amino acid sequence consensus, 16 other proteins that contain this motif were identified. These proteins fall into five classes based on the protein motifs found N-terminal of the SOCS box. In addition to four new SOCS proteins (SOCS-4 to SOCS-7) containing an SH2 domain and a SOCS box, three new families of proteins that contain either WD-40 repeats (WSB-1 and -2), SPRY domains (SSB-1 to -3) or ankyrin repeats (ASB-1 to -3) N-terminal of the SOCS box were identified (Hilton, et al., 1998).

The expression of SOCS proteins is induced by various cytokines, and once expressed, SOCS proteins down-regulate JAK/STAT pathways and hence modulate the biological response. Peripheral administration of leptin to ob/ob, but not db/db mice, rapidly induced SOCS-3 mRNA in hypothalamus (Bjorbaek, et al., 1998; Emilsson, et al., 1999). This leptin-dependent increase of SOCS-3 mRNA was seen in areas of the hypothalamus expressing high levels of the long form OBR. Furthermore, SOCS-3 was shown to block leptin-induced signal transduction in mammalian cell lines. The expression of SOCS-3 mRNA in the ARC and DMN is increased in agouti mice, an obesity model of leptin-resistance. This indicated that SOCS-3 is a leptin-inducible
inhibitor of leptin signaling, and suggested SOCS-3 to be a potential mediator of leptin resistance in obesity (Bjorbaek, et al., 1998). Furthermore, Auernhammer and colleagues (1998), showed an inhibition of LIF-stimulated STAT-3 phosphorylation in SOCS-3 overexpressing AtT-20 cells, and concluded that SOCS-3 inhibits the JAK-STAT pathway.

Adrenalectomy has been shown to increase sensitivity to leptin (Zakrzewska, et al., 1997). We hypothesize that this leptin sensitivity upon adrenalectomy could be through several mechanisms, including direct action on expression of the leptin receptor, changes in activation of the JAK/STAT pathway or expression of the suppressors of cytokine signaling or through direct effects on the neuropeptide genes that modulate the effects of leptin on energy balance. To determine if glucocorticoids impair the activity of the leptin receptor signaling pathway, we studied the effects of adrenalectomy and corticosterone replacement on components of the leptin receptor signaling pathway, including the hypothalamic leptin receptors, SOCS-3, STAT-3 and NPY.

6.2 EXPERIMENTAL PROTOCOLS

Animals

Ten-week-old male Sprague Dawley rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). They were housed individually in hanging wire-mesh cages attached to an automated watering system in a room with a 12 hour light/dark (7:00 AM to 7:00 PM) cycle at a temperature of 22-23°C. All rats consumed nonpurified chow diet (Rodent chow 5001, Purina Mills, St. Louis, MO, USA). Food was available ad libitum throughout the experiment. All protocols used in these studies were reviewed and approved by the Pennington Center’s Institutional Animal Care and Use Committee.
Intracerebroventricular (icv) Cannulation

Rats weighing 270-300 g were anesthetized by intraperitoneal (ip) injection with 0.125ml anesthesia per 100g body weight [anesthesia = mixture of Ketamine (80mg/ml); Ace Promazine (1.6mg/ml) and Xylazine (5mg/ml)]. They were stereotaxically implanted with a stainless steel guide cannula (25 gauge, 14-mm long) into the third cerebral ventricle. The coordinates were: 2.8 mm posterior to bregma, 0.0 mm lateral to midsagittal, and 8.1 mm ventral to the dura according to the brain atlas of Paxinos and Watson (1982). Cannulas were secured in place with anchor screws and dental acrylic and occluded with 30-gauge wire stylet. The injector (31-gauge) was projected 0.5 mm longer beyond the tip of the guide cannula. Body weights were monitored daily during the 7 days recovery period.

Adrenalectomy

Seven days after recovery from cannula placement surgery, rats were bilaterally adrenalectomized via a dorsal approach under isoflurane anesthesia. One group of adrenalectomized rats were subcutaneous implanted with 3 weeks-release 100 mg corticosterone pellets (Innovative Research of America, Sarasota, FL). Adrenalectomized rats were provided with 0.9% saline drinking water and allowed to recover for 7 days before leptin treatment. Body weights were monitored daily after adrenalectomy.

In vivo experiments

Intracerebroventricular microinfusions (2.0 µl leptin/rat) in unrestrained rats were at the rate of 1µl/min using an infusion pump (Harvard Apparatus, South Natick, MA). Each animal was infused between 3:00 and 3:30 pm (4 hours before dark onset). Rats received either vehicle (sterile physiological saline, 2 µl/rat) or 2.5 µg/rat recombinant
leptin (R&D Systems, Inc., Minneapolis, MN) dissolved in 2 μL saline vehicle. Twenty-four (24) h food intake and body weight were monitored. After 24 hours, rats were given second icv injections of saline or 2.5 μg leptin. Two hours later, rats were killed by decapitation. Trunk blood was collected for the determination of plasma hormones. Hypothalami, epididymal fat pads and livers were dissected out, weighed and snap-frozen in liquid nitrogen and stored at −80°C until used for total RNA and protein analyses. The dose of leptin chosen for these studies was based on our lab’s previous studies that determined the concentration-dependence of leptin effects on food intake (Lin, et al., 1999). The time selected for tissue sampling (2 h after icv administration and 2 h before dark onset) was also based on the food intake profiles exhibited by rats receiving leptin.

RNA analysis

Total RNA was extracted from hypothalamus and liver tissues by the modified guanidinium-isothiocyanate method (Chomczynski and Sacchi, 1987) using TRizol reagent (Life Technologies, GIBCO BRL, Gaithesburg, MD), with modifications as described in the General Methods section. RPA (OBR, β-actin), semi-quantitative RT-PCR (SOCS-3, STAT-3, cyclophilin) and Northern (NPY, β-actin) analyses were carried out as described in the General Methods section. The intensities of the signals were analyzed relative to appropriate internal standard using the PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software.

Protein analysis

Total lysates, nuclear and cytosolic fractions were prepared from hypothalami according to the method of Vaisse, et al. (1996) as described in the General Methods section. Nuclear STAT-3 protein was immunoprecipitated with anti-STAT-3 antibody
(Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and sequentially immunoblotted with anti-phosphotyrosine (pY20), and anti-STAT-3 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). SOCS-3 and SHPTP-2 proteins were immunoprecipitated and immunoblotted from the cytosolic fraction using their respective antibodies purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Phosphorylation of SHPTP-2 was determined by immunoblotting with anti-phosphotyrosine (clone 4G10) antibody (Upstate Biotechnology, Lake Placid, NY).

Electrophoretic mobility shift assay

DNA binding studies were performed using the c-fos sis-inducible element (m67-SIE) which specifically binds STAT-3 protein dimers, as described in the General Methods section. Nuclear extracts were incubated with radiolabeled double-stranded probe and the products were fractionated on a 5% non-denaturing PAGE. The intensities of the bands were analyzed using the PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software.

Serum assays

Plasma corticosterone, insulin and leptin were measured with radioimmunoassay (RIA) kits (Linco Research, Inc., St. Charles, MO) based on rat standards according to the supplier’s instructions. Nonesterified fatty acids (NEFAs) from serum were quantified using a colorimetric in vitro enzymatic assay according to the manufacturer’s specifications (Wako Pure Chemical Industries Ltd., Richmond, VA). The blood glucose levels were measured with a blood glucose meter (Glucometer Elite, Bayer Corp., Elkhart, IN).
Statistics

Data are presented as means ± SEM. Statistical analysis were performed using the Student’s *t* test, and two-way analysis of variance (ANOVA). *Post hoc* analysis was performed using the Duncan multiple range test method at *p* < 0.05.

6.3 RESULTS

Adrenalectomy attenuates the development of obesity and leptin reduces food intake and body fat in rodents. To test the hypothesis that adrenalectomy increases the sensitivity to leptin, we adrenalectomized and steroid-replaced rats with corticosterone pellets and injected them icv with either saline vehicle or 2.5 μg leptin. Figure 6.2 shows the effects of adrenalectomy and leptin treatment on food intake. In saline treated rats, ADX reduced 24-h food intake by 35% in ADX rats compared to sham-operated rats. Leptin treatment of sham-operated rats significantly decreased 24-h food intake by 43% compared to saline treated rats. Corticosterone replacement of saline-treated ADX rats returned the food intake to the level equivalent to that of sham-operated rats, indicating an inhibition of ADX-induced decrease in food intake. Treatment of ADX rats with leptin significantly decreased food intake by 73% compared to saline treated ADX rats. When corticosterone replaced rats were treated with leptin, leptin significantly decreased food intake, however corticosterone reduced the sensitivity of leptin-induced decrease in food intake by 50% compared with leptin treated ADX rats. Table 6.1 shows the data obtained on body and tissue weights, serum hormones, glucose and NEFAs. ADX significantly reduced the epididymal fat pad weight compared to sham-operated rats. Leptin treatment of ADX rats decreased epididymal fat pad weight by 32% compared to ADX-saline rats, however this decrease was not significant.

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Figure 6.2. Effects of adrenalectomy and leptin treatment on the 24-hr food intake. Sprague-Dawley rats received an injection of 2 μL saline vehicle or 2.5 μg leptin in 2 μL icv before dark-onset, and food intake was measured after 24-hr. Results are expressed as means ± SEM. Significant differences between groups are indicated by the differences in letter symbols. ANOVA showed that there is significant differences in food intake (F_{5,42} = 22.92, p = 0.0001).

Corticosterone-replacement in ADX rats attenuated leptin-induced fat pad weight loss, and permitted weight gain to the level of saline-treated corticosterone-replaced ADX rats. As with the slight body weight change, corticosterone-replaced ADX rats had similar epididymal fat pad weights regardless of leptin treatment. In sham-operated rats, however, leptin treatment significantly reduced the weight of the liver compared to saline. Furthermore, leptin treatment consistently caused a decrease in the liver weights in all groups, but the decreases were not significant.

To determine the effects of ADX and leptin treatment on serum hormones, changes in serum insulin, corticosterone, leptin, glucose and NEFA levels in ADX,
Table 6.1. Body weight, liver and epididymal WAT weights and serum hormone levels of adrenalectomized, adrenalectomized and steroid replaced, and sham-operated lean Sprague Dawley rats

<table>
<thead>
<tr>
<th></th>
<th>ADX</th>
<th>ADX+R</th>
<th>SHAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Leptin</td>
<td>Saline</td>
</tr>
<tr>
<td>Initial Body weight (g)</td>
<td>277.6 ± 25.5</td>
<td>316.2 ± 4.4</td>
<td>244.7 ± 11.6</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>275.2 ± 24.1</td>
<td>295.1 ± 3.4</td>
<td>239.1 ± 10.8</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>-2.5 ± 1.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>-21.1 ± 1.2&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>-5.6 ± 1.1&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymal WAT (g)</td>
<td>2.42 ± 0.18&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.64 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.03 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>9.95 ± 0.76&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>8.81 ± 0.95&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>9.56 ± 0.38&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>18.7 ± 18.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>29.2 ± 19.4&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>304.4 ± 37.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.56 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.95 ± 0.17&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>0.55 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.53 ± 0.81&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.42 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>114 ± 14.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>130.6 ± 5.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>129.8 ± 3.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NEFAs (mmol/L)</td>
<td>0.26 ± 0.04&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.31 ± 0.05&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.21 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Values are means ± SEM. Significant difference between groups is indicated by the differences in letter symbols, with P < 0.05. There were 6-8 animals in each experimental group. ANOVA showed significant differences in body weight change (F<sub>5,42</sub> = 6.16, p = 0.002), epididymal WAT (F<sub>5,42</sub> = 3.84, p = 0.005), liver (F<sub>5,42</sub> = 3.84, p = 0.02), corticosterone (F<sub>5,42</sub> = 21.17, p = 0.0001), leptin levels (F<sub>5,42</sub> = 4.03, p = 0.01), and glucose (F<sub>5,42</sub> = 2.56, p = 0.01), but not in insulin (F<sub>5,42</sub> = 2.37, p = 0.07), and NEFAs (F<sub>5,42</sub> = 2.37, p = 0.06). 

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[a,b] Significant difference between groups.
[c] Significant difference between groups.
[d] Significant difference between groups.
[e] Significant difference between groups.
steroid-replaced and sham-operated rats were measured (Table 6.1). In sham-operated rats, leptin treatment reduced plasma insulin levels by almost 50% compared to saline treated sham-operated rats. ADX significantly reduced plasma insulin levels compared to sham-operated rats. However, leptin treatment of ADX rats had no further effects on serum insulin. Corticosterone-replacement showed a tendency to increase plasma insulin levels, even though they did not increase to the level of sham-operated rats. In ADX and replaced rats, plasma corticosterone levels were equivalent regardless of leptin treatment. ADX rats had very low corticosterone levels. Sham-operated and ADX-replaced rats had higher levels of corticosterone, which were also equivalent regardless of leptin treatment. Replacement of ADX rats with corticosterone increased serum corticosterone to levels that were approximately 50% of those seen in the sham-operated rats. Plasma leptin levels varied not only as a result of leptin infusion, but also in association with corticosterone depletion and replacement. ADX significantly reduced the levels of plasma leptin. However, replacement with corticosterone did not elevate plasma leptin levels in saline treated rats. Plasma leptin was increased in ADX rats infused with leptin and replacement with corticosterone also showed an increase in plasma leptin levels. The increment in leptin levels in leptin-treated rats was far greater in sham-operated rats compared to ADX rats, with the replaced rats showing intermediate levels.

To determine the effects of ADX and leptin treatment on the hypothalamic leptin receptor levels, we used a ribonuclease protection assay to measure mRNA expression for the leptin receptor isoforms in ADX, steroid-replaced and sham-operated rats (Figure 6.3). ADX significantly increased ObR-L mRNA levels compared to sham-operated rats (0.104 ± 0.02 vs 0.051 ± 0.009, p < 0.05). Corticosterone replacement of ADX rats did
not have an effect on the ObR-L mRNA expression compared to ADX rats. Leptin treatment had no effect on the levels of ObR-L mRNA in any experimental groups. ADX significantly increased ObR-S mRNA levels compared to sham-ADX rats (0.078 ± 0.012 vs 0.047 ± 0.008, p < 0.05). Corticosterone replacement did not alter these ADX-induced increases in ObR-L and ObR-S mRNA levels. In sham-operated rats, leptin treatment did not have an effect on the ObR-S mRNA expression compared to saline treated rats (0.048 ± 0.008 vs 0.038 ± 0.003, p > 0.05). Leptin treatment had no effect on the levels of ObR-S mRNA in any experimental groups.

To assess the effects of ADX and leptin treatment on hypothalamic STAT-3 mRNA levels, semi-quantitative RT-PCR was used to measure STAT-3 mRNA expression (Figure 6.4). The hypothalamic STAT-3 mRNA levels were increased following ADX compared to sham-operated rats (1.30 ± 0.05 vs 0.78 ± 0.10, p < 0.001). Glucocorticoid-replacement of ADX-saline treated rats reduced the levels of STAT-3 mRNA (0.99 ± 0.08 vs 1.30 ± 0.05, p< 0.05). Leptin treatment of ADX rats had no further effect on the STAT-3 mRNA expression compared to saline treatment (1.28 ± 0.05 vs 1.30 ± 0.05). Leptin treatment of sham-operated rats reduced STAT-3 mRNA expression compared to saline treated rats (0.56 ± 0.05 vs 0.78 ± 0.08, p< 0.05).

To assess the effects of ADX and leptin treatment on hypothalamic STAT-3 total protein levels, immunoprecipitation and Western blotting was used to measure STAT-3 protein levels in whole hypothalamic lysates (Figure 6.5). ADX increased the levels of total STAT-3 protein levels compared to sham-operated rats. Leptin treatment of ADX rats increased the levels of STAT-3 compared to saline treated rats. In steroid-replaced ADX rats, the levels of STAT-3 protein were variable, but replacement with
corticosterone caused a decrease in STAT-3 protein in 75% of the replaced samples. Unlike the effects of leptin on the STAT-3 mRNA levels, leptin treatment had no effect on the levels of STAT-3 protein in sham-operated rats. This discrepancy may be due to differences in the times of sampling for the mRNA and protein, where the rate of transcription would have been different between these groups at the time of sampling.

We then investigated whether the increase in STAT-3 protein levels due to ADX was associated with increased STAT-3 activity. Therefore, nuclear protein extracts were immunoprecipitated with STAT-3 polyclonal antibody to measure phosphorylation of STAT-3 protein (Figure 6.6). Leptin treatment increased STAT-3 protein phosphorylation in the hypothalamus of sham-operated rats compared to saline-treated rats. ADX induced constitutive STAT-3 protein tyrosine phosphorylation compared to sham-operated saline-treated rats. Leptin treatment of ADX rats further increased nuclear STAT-3 protein phosphorylation compared to leptin-treated sham operated rats. Steroid replacement of adrenalectomized rats returned the levels of STAT-3 protein phosphorylation to that of sham-operated saline treated rats. However, leptin effects on STAT-3 protein phosphorylation were abolished. In order to make sure that equal levels of protein were loaded on the gel and that efficiency of transfer was optimum, membranes were stripped and re-blotted with the STAT-3 polyclonal antibody. Western blotting with STAT-3 antibody verified that the tyrosine phosphorylated 92-kDa protein was STAT-3 and that each lane contained the same amounts of the STAT-3 protein. This, therefore, showed that ADX and leptin treatment induced phosphorylation of STAT-3, which is then translocated to the nucleus to affect transcription of STAT-3-dependent genes.
Figure 6.3. Ribonuclease protection assay to determine the effect of ADX and leptin treatment on leptin receptor mRNA expression. Total RNA was extracted from individual hypothalami of rats treated icv with either saline vehicle or 2.5 µg leptin. RNA was hybridized with $^{32}$P-labeled antisense probes for ObR and β-actin and processed as described in the general methods. A) A representative RPA for leptin receptor isoforms, and B) histograms showing the quantification of the ObR-L and ObR-S mRNAs, where the open-bars are saline-treated and dark-bars are leptin-treated samples. M is century RNA molecular markers. Data are presented as means ± SEM. * $p < 0.05$ compared to sham-operated rats.
Figure 6.4. Effects of ADX and leptin treatment on the hypothalamic STAT-3 mRNA expression. Total RNA was extracted from hypothalami of ADX, ADX-steroid replaced and sham-operated rats. RNA was reverse transcribed and PCR amplified using STAT-3 specific primers. PCR products were fractionated in a 1.5% agarose gel for 2 hr. A) A representative agarose gel showing the 715-bp STAT-3 PCR product and cyclophilin as internal standard, and B) histogram showing the quantification of the STAT-3 mRNA. Open-bars are saline-treated and dark-bars are leptin-treated samples. Data are presented as means ± SEM for two independent observations. * p < 0.05, ** p < 0.001 and p < 0.0001 compared between indicated groups.
Figure 6.5. Effects of ADX and leptin treatment on hypothalamic STAT-3 protein levels. Total STAT-3 protein levels were analyzed by immunoprecipitating STAT-3 from hypothalamic lysates of rats treated with either saline vehicle or 2.5 μg leptin using STAT-3 antibody, followed by Western blotting and analysis with STAT-3 antibody. A) Immunoblot of STAT-3. B) Densitometric values of the immunoblot. Open-bars are saline-treated and dark-bars are leptin-treated samples. Data are presented as means ± SEM. * p < 0.05 compared to sham-operated rats.
Figure 6.6. Effects of ADX and leptin treatment on tyrosine phosphorylation of STAT-3 protein. Tyrosine phosphorylation of STAT-3 was analyzed by immunoprecipitating STAT-3 from hypothalamic nuclear extracts obtained from ADX, ADX-replaced and sham-operated rats treated with either saline vehicle or 2.5 μg leptin using STAT-3 antibody, followed by Western blotting and analysis with antiphosphotyrosine (pY20) antibody. The membranes were stripped and reprobed with STAT-3 antibody. A) Immunoblot of phosphotyrosine and STAT-3. B) Densitometric values of the immunoblot. Open-bars are saline-treated and dark-bars are leptin-treated samples. Data are means ± SEM. * p < 0.05 compared between indicated groups.
Nuclear extracts were also used to examine the effects of ADX and leptin treatment on STAT-3 DNA binding activity on the sis-inducible element (SIE) from a c-fos promoter (m67-SIE) by using gel shift assays (Figure 6.7). The DNA-protein complex was inhibited by an excess of unlabeled m67-SIE oligonucleotide. Supershift analysis showed that the complexes bound to the m67-SIE in response to ADX and leptin treatment were composed of STAT-3. Leptin treatment of sham operated rats enhanced the activation of STAT-3 compared to saline treated rats. STAT-3 phosphorylation was already enhanced in ADX rats compared to sham-operated saline treated rats, which suggests constitutive DNA binding activity. This STAT-3 DNA binding activity was not further upregulated by leptin treatment, but rather decreased. Corticosterone replacement of ADX rats did not affect DNA binding activity, but again leptin treatment reduced DNA binding activity.

SOCS proteins represent a family of negative regulators of cytokine signaling that switch off the cytokine signal by binding to the SH2 domain of JAK proteins, thereby inhibiting the activation of STAT proteins. This family consists of eight known members, namely, the cytokine-inducible SH2 proteins (CIS) and the suppressor of cytokine signaling 1 to 7 (SOCS-1-7). Recently, peripheral administration of leptin to ob/ob mice was shown to rapidly induce SOCS-3 mRNA in the hypothalamus, but this effect was absent in db/db mice (Bjorbaek, et al., 1998; Emilsson, et al., 1999). The effects of ADX and leptin treatment on SOCS-3 mRNA expression (Figure 6.8) and protein levels (Figure 6.9) were examined by using semi-quantitative RT-PCR and Western blot analysis of immunoprecipitated SOCS-3, respectively. Leptin treatment of sham-operated
Figure 6.7. Effects of ADX and leptin treatment on the DNA binding activity of STAT-3 protein. Nuclear extracts were incubated with $^{32}$P-labeled m67-SIE and fractionated on a 5% non-denaturing PAGE. A) A representative EMSA gel, and B) densitometric quantification of the STAT-3-DNA complex. Open bars are saline treated and dark bars are leptin treated samples. Lane 1 = DNA only, Lane 2 = DNA and unrelated DNA, lane 3 = competition with 100-fold molar excess of cold m67-SIE, lane 4 and 5 = STAT-3 antibody competition. Data are presented as means ± SEM. * p < 0.05 and ** p < 0.005 compared to indicated groups.
rats had no effect on SOCS-3 mRNA expression, but increased SOCS-3 protein levels. ADX decreased the SOCS-3 mRNA expression and protein levels compared to sham-operated rats. Glucocorticoid-replacement of ADX rats returned the levels of SOCS-3 mRNA and protein to that of sham-operated rats. Leptin treatment of ADX or ADX-replaced rats had no significant effects on SOCS-3 mRNA, but increased protein levels.

Activation of OBR-L by leptin treatment has recently been shown to induce tyrosine phosphorylation of the SH2-containing phosphotyrosine phosphatase 2 (SHP-2 or SHP-2) and its recruitment to the cytoplasmic domain of OBR-L (Carpenter, et al., 1998). SHP-2 has also been shown to negatively regulate STAT3-mediated gene induction after activation of OBR-L (Carpenter, et al., 1998). Therefore, the effects of ADX and leptin treatment on the protein levels and phosphorylation of SHP-2 were examined (Figure 6.10). ADX had no effect on the levels and phosphorylation of SHP-2 protein in all experimental groups. However, leptin treatment showed some increase in tyrosine phosphorylation of SHP-2 protein.

NPY, a potent stimulator of food intake that is down-regulated by leptin and glucocorticoids, mediates leptin's action in the CNS (Rohner-Jeanrenaud, et al., 1996). The effects of ADX and leptin treatment on the expression of hypothalamic NPY mRNA were determined by using Northern blot analysis (Figure 6.11), adrenalectomy significantly reduced NPY mRNA levels and this effect was reversed by corticosterone replacement. Leptin treatment of sham-operated and corticosterone replaced adrenalectomized rats also significantly reduced NPY mRNA expression compared to saline-infused controls. However, no further decrease in NPY mRNA expression was observed in ADX rats treated with leptin.
Figure 6.8. Effects of ADX and leptin treatment on the hypothalamic SOCS-3 mRNA expression. RNA was reverse transcribed and PCR amplified using SOCS-3 specific primers. PCR products were fractionated in a 1.5% agarose gel for 2 hr. A) A representative agarose gel showing the 450-bp SOCS-3 PCR product and cyclophilin as internal standard, and B) histogram showing the quantification of the SOCS-3 mRNA. Open-bars are saline-treated and dark-bars are leptin-treated samples. Data are presented as means ± SEM for two independent observations. * p < 0.05 and ** p < 0.001 between indicated groups.
Figure 6.9. Effects of ADX and leptin treatment on SOCS-3 protein levels. SOCS-3 protein levels were analyzed by immunoprecipitating SOCS-3 from hypothalamic cytosol extracts obtained from ADX, ADX-replaced and sham-operated rats treated with either saline vehicle or 2.5 µg leptin using SOCS-3 antibody (gift of Dr. Jeffrey Flier, Harvard Medical School, Boston, MA), followed by Western blotting and analysis with SOCS-3 antibody (Santa Cruz Biotechnology, Inc.). A) A representative immunoblot of SOCS-3 protein, and B) densitometric quantification of the immunoblot. Open-bars are saline-treated and dark-bars are leptin-treated samples. Data are presented as mean ± SD. * p < 0.05 and ** p < 0.005 compared between indicated groups.
**Figure 6.10.** Effects of ADX and leptin treatment on SH-PTP2 protein phosphorylation and levels. Tyrosine phosphorylation of SH-PTP2 was analyzed by immunoprecipitating SH-PTP2 from hypothalamic cytosol extracts obtained from ADX, ADX-replaced and sham-operated rats treated with either saline vehicle or 2.5 μg leptin using SH-PTP2 antibody, followed by Western blotting and analysis with antiphosphotyrosine (4G10) antibody. The membranes were stripped and reprobed with SH-PTP2 antibody. A) Representative immunoblots of phosphotyrosine and SH-PTP2 protein, and B) densitometric quantification of the immunoblots. Open-bars are saline-treated and dark-bars are leptin-treated samples. Data are presented as mean ± SEM. No statistical significance was found between the groups.
Figure 6.11. Effects of ADX and leptin treatment on NPY gene expression. A) representative Northern blots of NPY and β-actin mRNA. (B) Histogram showing the quantification of NPY mRNA relative to β-actin. Total hypothalamic RNA (20 μg/lane) was analyzed by Northern blotting. Relative expression of NPY mRNA was determined by hybridization with 32P-labeled NPY and β-actin cDNAs. Bands were quantified using the ImageQuant software. Data are expressed as means ± SEM. * p < 0.05 compared to indicated groups.
6.4 DISCUSSION

The key findings of this study were: 1) that the feeding response to leptin is sensitive to adrenal glucocorticoids, their absence enhancing the anorectic response to leptin; 2) that mRNA expression of the leptin receptor isoforms is increased by adrenalectomy; 3) that adrenalectomy increased STAT-3 mRNA expression and total protein levels; 4) that leptin treatment of sham-operated rats induced phosphorylation of STAT-3, and adrenalectomy induced constitutive phosphorylation of STAT-3 protein; 5) that adrenalectomy and leptin treatment induced STAT-3 DNA binding activity, but there was no synergy between adrenalectomy and leptin treatment; 6) that adrenalectomy decreased the expression of SOCS-3 mRNA and protein levels, whereas leptin treatment increased SOCS-3 protein levels; and 7) that the increased sensitivity to leptin upon adrenalectomy is not due to further changes in NPY mRNA expression.

Our original observations that adrenalectomy increases the expression of both the long-form and short-form leptin receptors in the hypothalamus of obese Zucker rats suggested that adrenal glucocorticoids might modulate their effect on leptin response through modulation of leptin receptor activity (Madiehe, et al., 1999). Leptin affects food intake and body weight by actions on the receptors in the hypothalamus, and adrenalectomy has been shown to reduce food intake and stop the development of obesity. Our study is consistent with the published data (Zakrzewska, et al., 1997) which showed that adrenalectomized rats have increased sensitivity to leptin treatment. The mechanism by which adrenalectomy increases leptin sensitivity was the subject of this study. We hypothesized that glucocorticoids modulate the leptin receptor expression and activity in the hypothalamus. To identify the components of the leptin signal transduction...
pathway modulated by glucocorticoids, we investigated the effects of adrenalectomy on either gene expression, protein phosphorylation, or DNA binding activity of several candidate proteins, which included leptin receptor, STAT-3, SOCS-3, SHP-2 and NPY.

In this dissertation, we show that adrenalectomy caused an increase in the levels of mRNA for the long and short forms of the receptor. This was associated with a reduction in circulating leptin levels, which may suggest that the change in receptor expression may help to enhance the effects of leptin on food intake when leptin is administered directly into the brain. The data could also be interpreted as reflecting that leptin can modulate the expression of its own receptors, with receptor gene expression increasing when leptin levels decrease. However, if this is the case it appears to be a delayed response in the absence of glucocorticoids, since acute treatment of adrenalectomized rats with leptin, did not reduce the levels of either ObR-L or ObR-S mRNA. The increase in leptin receptor population in the hypothalamus may lead to increased receptor activity and thereby cause pronounced effects of leptin action.

Leptin receptors have been identified in NPY/AGRP- and POMC/CART-containing neurons of the ventromedial and ventrolateral ARC, respectively (Elias, et al, 1998; Hakansson & Meister, 1998), and in MCH- and orexin-containing neurons of the lateral hypothalamus (Elias, et al., 1998; Elmquist, et al., 1997; Elmquist, et al., 1998). This suggested that these messengers are mediators of the actions of leptin in the hypothalamus. Recent functional studies showed that NPY, AGRP, POMC-derived peptides, CART, MCH and orexins are all important regulators of food intake (Sahu, 1998; Marsh, et al., 1999; Hollopeter, et al., 1998b; Scrocchi, et al., 1997; Goldstone, et
al., 1997). The exact mechanism by which leptin activates these targets is relatively unknown.

Leptin has been shown to control insulin secretion from pancreatic β-cells (Kieffer, et al., 1997; Ookuma, et al., 1998; Poitout, et al., 1998; Pallett, et al., 1997). Furthermore, central administration of leptin inhibits insulin secretion and increases insulin sensitivity of peripheral tissues directly (Kamohara, et al., 1997; Cusin, et al., 1998; Minokoshi, et al., 1999; Haque, et al., 1999). Leptin receptors were identified on pancreatic β-cells (Kieffer, et al., 1996; Islam, et al., 1997; Emilsson, et al., 1997), and it was later showed that leptin-mediated inhibition of insulin secretion was by activation of ATP-sensitive K⁺ channels (Kieffer, et al., 1997). Seufert and colleagues (1999), showed that leptin inhibited transcription of the preproinsulin by altering transcription factor binding to sequences upstream from the elements that confer glucose responsivity to the rat insulin I gene promoter. Therefore, leptin exerts inhibitory effects on both insulin secretion and insulin gene expression in pancreatic β-cells. In this study, leptin decreased insulin levels in sham-operated rats, but not in ADX rats. This may suggest that leptin effects on insulin secretion may require circulating glucocorticoids.

Leptin regulates food intake and body weight via interactions with hypothalamic neuronal circuits expressing leptin receptors. The long isoform of ObR acts through the JAK-STAT pathway of signal transduction. Recent evidence suggests that STAT-3 transcription factor mediates leptin’s action in the hypothalamus (Vaisse, et al., 1996; Rosenblum, et al., 1996; Baumann, et al., 1996; McCowen, et al., 1998; Hakansson & Meister, 1998). In our study, immunoblotting analysis of hypothalamic nuclear extracts with pY20 antibody demonstrated leptin-induced STAT-3 phosphorylation and DNA
binding activity, consistent with the literature (Vaisse, et al., 1996; McCowen, et al., 1998). ADX induced a similar, but constitutive phosphorylation of STAT-3 and DNA binding activity. This constitutive phosphorylation of STAT-3, but not the DNA binding activity, was increased by leptin treatment. It was rather puzzling that constitutive STAT-3 tyrosine phosphorylation after ADX and leptin treatment did not translate into increased DNA binding. This finding might be explained if the excess phosphorylated STAT-3 was bound by PIAS3, a recently described protein inhibitor of activated STAT-3 (Chung, et al., 1997). PIAS3 blocks the DNA binding activity of STAT-3, thus inhibiting STAT-3 mediated gene activation. We did not study other STAT proteins, especially STAT-1, because McCowen and colleagues (1998) showed that leptin did not increase the phosphorylation of STAT-1 and STAT-5, despite abundant expression of these signaling molecules in the hypothalamus.

Cytokines are secreted proteins that regulate important cellular responses, with well defined key events in cytokine signal transduction. Cytokines induce receptor dimerization, leading to activation of members of the JAK family of cytoplasmic tyrosine kinases. In turn, members of the STAT family of transcription factors are recruited and phosphorylated, dimerize and increase the transcription of genes with STAT recognition sites in their promoters. These cellular responses to cytokines are tightly controlled, but little is known about how cytokine signal transduction is switched off. Few molecules have now been identified which are able to switch these signals off. The suppressors of cytokine signaling (SOCS) proteins are a new family of negative regulators of cytokine signal transduction. The expression of SOCS proteins is induced by cytokines, including leptin (Bjorbaek, et al., 1998). Once expressed, SOCS down-regulate JAK/STAT
pathways and hence modulate the biological response. The leptin-dependent increase of SOCS-3 mRNA was seen in areas of the hypothalamus expressing high levels of the long form OB-R (Bjorbaek et al., 1998; Emilsson et al., 1999). Furthermore, SOCS-3 was shown to block leptin-induced signal transduction in mammalian cell lines, suggesting that SOCS-3 is a leptin-inducible inhibitor of leptin signaling, and suggested SOCS-3 to be a potential mediator of leptin resistance in obesity (Bjorbaek et al., 1998). In our study, leptin treatment of sham-operated rats increased SOCS-3 protein levels in the hypothalamus, which is consistent with the literature (Bjorbaek et al., 1998; Emilsson et al., 1999). In contrast, adrenalectomy decreased SOCS-3 mRNA expression and SOCS-3 protein levels. This decrease in SOCS-3 potentially provides a mechanism by which leptin resistance could be alleviated.

In support of this proposal for downregulation of SOCS-3 after adrenalectomy, we searched the rat SOCS-3 (GenBank AJ249240) upstream region and identified a putative glucocorticoid response element (GRE) with a TGAACCAGGCA sequence. This confirms the possibility that SOCS-3 can be upregulated by glucocorticoids. This upstream region also contains two putative STAT-3 binding elements that have been characterized (Auernhammer et al., 1999), and showed to regulate SOCS-3 expression induced by LIF. Our studies show that, in the absence of glucocorticoids, leptin activation of STAT-3 led to a moderate increase in SOCS-3 protein levels, whereas sham-operated rats showed a much greater stimulation of SOCS-3 after leptin treatment. This could suggest that STAT-3 activation and glucocorticoids act in a concerted manner to regulate SOCS-3.
In trying to establish the mechanism of regulation of SOCS-3 expression by leptin and the mechanism by which SOCS-3 inhibits leptin action, Bjorbaek, et al. (1999) stably transfected CHO cells with the long form of the leptin receptor. In these cells, leptin induced transient expression of endogenous SOCS-3 mRNA and protein. Leptin signaling was blocked by pretreatment of cells with leptin, which correlated with increased SOCS-3 expression. Forced expression of SOCS-3 in OBR-L transfected COS cells resulted in inhibition of leptin-induced tyrosine phosphorylation of JAK2, providing further evidence that SOCS-3 is a leptin-regulated inhibitor of leptin signaling in vivo (Bjorbaek, et al., 1999).

Activation of OBR-L has been reported to induce the tyrosine phosphorylation of the tyrosine phosphatase SH2-containing phosphatase 2 (SH-PTP2 or SHP-2) and that SHP-2 binds to OBR. Mutation of Tyr986 to Phe of OBR-L was shown to abrogate SHP-2 phosphorylation and binding to the receptor, but this mutation led to dramatically increased gene induction mediated by STAT3. Carpenter and colleagues (1998) indicated that SHP-2 is a negative regulator of STAT3-mediated gene induction after activation of OBR, raising the possibility that blocking the interaction of SHP-2 with OBR could overcome leptin resistance and boost leptin's weight-reducing effects in obese individuals (Carpenter, et al., 1998). In our study, adrenalectomy did not have any effect on SHP-2 protein phosphorylation and levels. This suggests that, upon activation of OBR-L by ADX, SHP-2 is inactivated and its interaction with the leptin receptor is reduced. This would lead to increased STAT-3 protein activation, consistent with the notion that inhibition of SHP-2 phosphorylation will lead to increased leptin action through STAT-3.
Li and Friedman (1999) cotransfected OBR-L, JAK2 and SHP-2 into 293T cells to study the mechanism by which SHP-2 inhibits leptin induced STAT-3 activation. They showed that leptin treatment resulted in direct binding of SHP-2 to OBR-L and that the bound SHP-2 is itself tyrosine phosphorylated after leptin treatment. In the absence of SHP-2 phosphorylation, the level of JAK2 phosphorylation was increased. Tyrosine phosphorylation of the OBR-L and STAT3 were not affected by phosphorylation of SHP-2, suggesting that activation of SHP-2 by the leptin receptor results in a decreased phosphorylation of JAK2 and may act to attenuate leptin signal transduction (Li and Friedman, 1999). This could explain, at least in part, how ADX induced increased STAT-3 phosphorylation compared to sham-operated rats. During adrenalectomy, no increased phosphorylation of the SHP-2 was observed, indicating that ADX does not modulate tyrosine phosphorylation of SHP-2. Leptin treatment increased SHP-2 tyrosine phosphorylation, and ADX caused the constitutive phosphorylation of STAT-3. The latter could be the driving force in the failure of SHP-2 to attenuate leptin signaling.

Initially, among several mediators of leptin action within the hypothalamus, NPY was the most compelling (Erickson et al., 1996). Several properties suggested that it might be an essential conduit for the leptin signal. Injection of NPY centrally evokes virtually all of the features of leptin deficiency, including hyperphagia, decreased BAT thermogenesis, and hyperinsulinemic insulin resistance. Repetitive injection causes obesity. Arcuate nucleus NPY is increased by starvation and is elevated in the ob/ob mouse, both of which are restored to normal by leptin treatment. Recently, leptin receptors were found in cells of the ARC containing NPY mRNA or POMC mRNA (Mercer, et al., 1996; Meister, 2000). Following leptin administration, NPY cells in the
ARC did not express Fos but expressed SOCS-3 mRNA. In contrast, leptin induced both Fos and SOCS-3 expression in POMC neurons, many of which also innervated the LHA. These findings suggested that leptin directly and differentially engages NPY and POMC neurons that project to the LHA, linking circulating leptin and neurons that regulate feeding behavior and body weight homeostasis (Thornton, et al., 1997; Elias, et al., 1999). Recent studies have shown that α-MSH, produced from POMC precursors, decreases food intake (Brown, et al., 1998). In the arcuate nucleus, which also expresses NPY, approximately 30% of the POMC neurons express the mRNA for the leptin receptor long form, and arcuate POMC mRNA expression is regulated positively by leptin (Thornton, et al., 1997; Mercer, et al., 1996). Thus, one can envision a loop in which rising leptin drives the increase in arcuate POMC expression, which then projects α-MSH containing axons to cell bodies elsewhere in the hypothalamus, causing decreased food intake. Both ADX and leptin administration directly into the brain decrease NPY gene expression and release. Treatment of ADX rats, which already have low NPY levels, with leptin can further decrease orexigenic neuropeptides (NPY and AGRP) and increase anorexigenic ones (POMC and CART) by acting directly on the leptin receptors found in these neurons.

Leptin is reported to have effects in peripheral tissues that are independent of its central effects on food intake and body weight. ADX causes a decrease in white adipose tissue depots and size (Edens, et al., 1999), whereas corticosterone replacement increases white adipose tissue weight gain. Leptin treatment, probably through sympathetic stimulation of white adipose tissue, causes fat loss which is modulated by the inhibitory effect of corticosterone on sympathetic outflow. This increased sympathetic outflow to
these depots may further be increased by ADX, and therefore have an increased sensitivity to leptin in the absence of glucocorticoids. Furthermore, leptin has been shown to decrease lipid synthesis (Edens, et al., 1999). ADX could enhance the adipocyte response to leptin on lipid synthesis and fat mobilization. Moreover, Sarmiento and colleagues (1997) showed that the peripheral effects of leptin include increased thermogenesis and lipid oxidation in brown fat coupled with increased lipolysis and decreased fat synthesis in white and brown fat, which lead to a rapid reduction in the body weight and adiposity of mice (Sarmiento, et al., 1997). It is, therefore, conceivable that the sensitivity to leptin after ADX, could be due to the combined inhibitory effects of leptin on WAT leptin production and the disappearance of WAT, as induced by both ADX and leptin, but it may also be a consequence of the energy imbalance that is induced by leptin in the absence of glucocorticoids.

The induction of SOCS genes, by STAT elements present in the SOCS promoter, has so far been restricted to activators of the cytokine receptor family, such as LIF, IL-6, interferon γ, GH and leptin. Although STAT activation is the hallmark of cytokine action, they can be activated by other agents, such as angiotensin II, EGF and PDGF. Recent evidence, therefore, indicates that STAT proteins can be activated by a variety of receptor and non-receptor protein-tyrosine kinases (Ihle, 1996). Unlike cytokine-induced activation of STATs, where JAKs are known to play a pivotal role in phosphorylating STATs, the mechanism for receptor protein-tyrosine kinase-mediated activation of STATs remains elusive. Insulin modulates cellular metabolism by modifying the activity and intracellular localization of several proteins, and by modulating transcription (O’Brien & Granner, 1996). Recently, insulin has been shown to induce tyrosine

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phosphorylation and DNA binding activity of STAT-5B in a perfused rat liver (Chen, et al., 1997), showing its involvement in the regulation of transcription by STAT factors.

Moreover, insulin via its receptor with intrinsic tyrosine kinase activity has now been shown to specifically induce SOCS-3 mRNA expression and protein translocation from the cytoplasm to the cell membrane in 3T3-L1 adipocytes (Emanuelli, et al., 2000). This expression of SOCS-3 was potentiated by STAT-5B activation, but the authors did not rule out the possibility that STAT-3 or other transcription factors stimulated by insulin might play a role in this mechanism. Caldenhoven and colleagues (1996) showed that insulin induced SOCS-3 expression in COS-7 cells that were not transfected with STAT-5B, indicating that the induction could result from low levels of endogenous STAT-3 found in these cells. SOCS proteins generally inhibit cytokine signaling through inhibition of JAK kinase, but insulin receptor kinase has been shown to activate STAT-5B independent of JAK (Chen, et al., 1997). Emanuelli and colleagues (2000), therefore, proposed that inhibition of STAT-5B occurs through competition between SOCS-3 and STAT-5B for binding to the insulin receptor, and not through inhibition of JAK kinase. This is quite intriguing for two reasons. First, not only leptin- and CNTF-treatments induce SOCS-3, but insulin does as well, and all three of these molecules are recognized as satiety factors (Woods, et al., 1998). This insulin effect, however, could provide an explanation for the paradox of increased levels of SOCS-3 in leptin-deficient ob/ob mice, which are hyperinsulinemic. It remains to be evaluated how insulin mediates its effects on SOCS-3 expression in vivo. Second, leptin and CNTF control feeding behavior and inhibit insulin release from pancreatic β-cells (Harvey, et al., 1997; Kieffer, et al., 1997; Gloaguen, et al., 1997). Therefore, if insulin induces SOCS-3 expression in vivo, leptin-
or CNTF-induced inhibition of insulin release should cause a decrease in SOCS-3 expression. But, this is not the case. On the other hand, insulin stimulates the production of leptin. It is therefore, conceivable that insulin administration in these studies increased the endogenous levels of leptin, which in turn increased the expression of SOCS-3. Alternatively, one can foresee a situation under hyperinsulinemic conditions, in which insulin induces the expression of SOCS-3 which can then negatively regulate the cytokine signal transduction of leptin. In order for leptin to overcome the inhibitory effects of SOCS-3, more and more endogenous leptin is released, and this consequently leads to leptin resistance.

In studies described in this dissertation, leptin treatment of sham-operated rats caused a decrease in serum insulin levels, but an increase in SOCS-3 mRNA expression as well as an increase in SOCS-3 protein levels. Furthermore, leptin treatment of ADX rats significantly increased SOCS-3 protein levels despite the low levels of insulin in these animals. Chavez and colleagues (1997) showed that ADX also increased sensitivity to central insulin administration, similar to that observed with leptin treatment of ADX rats in studies described in this dissertation and those of Zakrzewska and colleagues (1997). Whether the insulin sensitivity of ADX rats is due to increased STAT activation remains to be investigated. The three studies suggest that the absence of glucocorticoids increases the brain's sensitivity to leptin and insulin, and that these hormones act to lower food intake and body weight through a glucocorticoid-sensitive mechanism, making glucocorticoids the more important in the regulation of obesity.

Central administration of NPY causes hyperphagia, hyperinsulinemia, and obesity, a response that is prevented by prior adrenalectomy in rats (Stanley, et al. 1989).
NPY-induced food intake was similar in ADX and control rats after acute icv injection of NPY. Injection of NPY caused a significant increase in plasma insulin in control rats, but this effect was absent in ADX rats in which basal plasma insulin levels were also lower than controls. This suggested that glucocorticoids are necessary for acute NPY-mediated insulin release which are absent in ADX rats. Again, it stands to reason that NPY-induced insulin release leads to increase in leptin gene expression and secretion which may increase the expression of SOCS-3 to inhibit cytokine signaling, but not insulin itself inducing the expression of SOCS-3.

In contrast to the work of Emanuelli and colleagues (2000), treatment of rat hepatoma cells with insulin attenuated the IL-6 stimulation of acute phase protein genes (Campos, et al., 1996). This decrease in IL-6 stimulation by insulin was accompanied by insulin dose-dependent reduction in STAT-3 gene transcription, mRNA accumulation, protein concentration, and IL-6-inducible DNA binding activity. Insulin mediated a similar reduction in the mRNA encoding the IL-6 receptor alpha subunit and IL-6 binding activity. These effects of insulin contribute to the strongly suppressed transcriptional induction of the IL-6-responsive acute phase plasma protein genes (Campos, et al., 1996).

Using the insulin-like growth factor I receptor (IGF-IR) in vitro and in vivo to assess the role of JAKs in the process of STAT activation, Zong and colleagues (2000) found that STAT-3, but not STAT-5, was activated in response to IGF-I in 293T cells cotransfected with IGF-IR and STAT expression vectors. Moreover, tyrosine phosphorylation of STAT-3, JAK1, and JAK2 was increased upon IGF-I stimulation of endogenous IGF-IR in 293T cells transfected with the respective STAT or JAK
expression vector. Endogenous STAT-3 was tyrosine-phosphorylated upon IGF-I stimulation in the muscle cell line C2C12 as well as in various embryonic and adult mouse organs, which supported the observation in 293T cells. They also showed that SOCS-1 and SOCS-3 proteins inhibited the IGF-I-induced STAT-3 activation. This inhibition of STAT-3 activation by SOCS was overcome by overexpression of native JAK1 and JAK2 indicating that IGF-I/IGF-IR mediated activation of STAT3 in vitro and in vivo and that JAKs are essential for the process of activation (Zong, et al., 2000).

Kim and colleagues (2000) studied the leptin signal transduction directly in insulin-sensitive tissues in vivo to examine the ability of iv leptin to acutely stimulate phosphorylation of STAT-3, STAT-1, and MAPK. Both leptin and insulin stimulated tyrosine phosphorylation of STAT-3 and STAT-1 in adipose tissue, but leptin increased STAT-3 phosphorylation in liver and muscle. Both hormones also increased MAPK phosphorylation, but leptin was less effective than insulin at stimulating IRS pathways. They also showed that leptin stimulated STAT-3 phosphorylation in fat and that these effects appear to be direct. Furthermore, leptin activated STAT-3 and MAPK in adipose tissue ex vivo and in 3T3-L1 adipocytes. This activation of STAT-3 by leptin is in contrast to the effects of insulin on SOCS-3 expression described by Emanuelli and colleagues (2000). It would be interesting to determine whether the activation of STAT-3 in 3T3-L1 adipocytes is associated with SOCS-3 expression and whether SOCS-3 affects the DNA binding activity of the activated STAT-3. In their studies, Kim et al., (2000) showed that leptin rapidly activates signaling pathways directly at the level of insulin sensitive tissues presumably through the long-form leptin receptor, and this pathway overlaps with, but is distinct from, that engaged by insulin.
Figure 6.12. A proposed model of the effects of ADX and leptin treatment on the leptin receptor signaling system. In a sham-operated rat treated with leptin (A), leptin activates OBR-L and JAK, which phosphorylates STAT-3. STAT-3 dimerizes and translocates to the nucleus to influence transcription of STAT-3-dependent genes, like SOCS-3. Glucocorticoids (GC) bind to glucocorticoid receptor (GR) and causes translocation of GR, which binds to GRE regions to influence transcription. Leptin activation of STAT-3 in the presence of GC, leads to high levels of SOCS-3 gene expression and protein. SOCS-3 then binds to JAK to inhibit leptin-induced signaling. In an ADX rat treated with leptin (B), the GR cannot be translocated in the absence of GC, ADX induces constitutive activation of STAT-3. Leptin activates OBR-L and JAK, which phosphorylates STAT-3. Activated STAT-3 translocates to the nucleus and bind to STAT-3 dependent genes to influence transcription. In the absence of GC, STAT-3 induces low transcription of SOCS-3 and protein. SOCS-3 protein cannot inhibit JAK, and this leads to constitutive activation of STAT-3, which may influence expression of neuropeptide genes involved in food intake. In ADX rats treated with leptin, decrease in STAT-3 DNA binding could be due to inhibition by PIAS-3.
Another interesting observation is one in which leptin activates STAT-3 signalling mechanism in pancreatic islets and in a rat model of the pancreatic β-cell, RINm5F. Activation of STAT-3 in RINm5F and isolated rat islets by leptin induced DNA binding in a manner consistent with STAT3 activation. Conditions that mimic increased metabolic activity resulted in attenuation of leptin-mediated STAT DNA binding but had no significant effect on STAT-3 tyrosine phosphorylation in RINm5F cells (Morton, et al., 1999).

In conclusion, we have showed that ADX induces increased leptin receptor mRNA expression, a constitutive STAT-3 activation, and increases sensitivity to leptin by activating the JAK-STAT signaling pathway through activation of STAT-3 and reducing the cytokine inhibitor, SOCS-3. Our studies are consistent with those of Solano and Jacobson (1999), who recently showed that ADX-induced sensitivity to leptin is not due to further decrease in NPY. Reduced DNA binding of phosphorylated STAT-3, observed in the stimulation of STAT-3 phosphorylation by leptin treatment of ADX rats, is consistent with the work of Scarpace and colleagues (2000), where leptin treatment of old rats increased the levels of phosphorylated STAT-3 but had no effect on STAT-3 DNA binding activity compared to young rats. Rothwell et al. (1984) report that ADX of older rats produced characteristics similar to those of young rats becomes fascinating. It will be interesting, therefore, to compare the effects of ADX on the STAT-3 activation between young and old rats, for a possible involvement of this pathway in aging. The study of the involvement of glucocorticoids in leptin-induced SOCS-3 expression will certainly provide useful information for the understanding of the circuitry of various signaling networks involved in the development of obesity caused by glucocorticoids.
CHAPTER SEVEN
DIFFERENTIAL EXPRESSION OF LEPTIN RECEPTOR IN HIGH- AND LOW-
FAT FED OSBORNE-MENDEL AND S5B/PI RATS*

7.1 INTRODUCTION

The susceptibility of different rat strains to become obese when exposed to dietary manipulations has been previously reported (Schemmel, et al., 1970; Shier & Schemmel, 1975). Osborne-Mendel (OM) rats become obese when fed a high-fat (HF) diet, but the S5B/PI rat is highly resistant to the development of obesity when fed the same diet (Fisler & Bray, 1990). These two rat strains show differing preferences for dietary fat when given a choice of macronutrient sources, with OM rats consuming more calories from fat than carbohydrate, whereas the opposite is true for S5B/PI rats (Okada, et al., 1992). Furthermore, a variety of physiological differences have been described in these two rat strains. The OM rat is more insulin resistant compared to the S5B/PI rat (Buchanan, et al., 1992) and has an attenuated activity of the fatty acid sensitive K+ channel in their taste buds (Gilbertson, et al., 1998). In addition, numerous differences in their response to feeding modulators have been reported (Singer, et al., 1997; Nagase, et al., 1996; Arase, et al., 1988; Okada, et al., 1992). These have suggested the involvement of hypothalamic areas in the sensitivity of OM rats for the HF diet induced obesity. Lin, et al., (1996), while investigating the feeding response to various orexigenic agents, showed a differential feeding response of OM and S5B/PI rats to galanin, neuropeptide Y (NPY), enterostatin and β-casomorphin-1-7 but a similar inhibitory response to corticotropin releasing hormone (CRH). Enterostatin, galanin and β-casomorphin have been implicated

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in regulation of fat intake (Smith, et al., 1997; Leibowitz, et al., 1998; Lin, et al., 1998). Differences in the levels of NPY, NPY Y1 and Y5 receptor mRNAs between OM and S5B/P1 rats have also been described (Schaffhauser, et al., 1999) and may be related to the susceptibility of OM rats to develop obesity when fed a HF diet.

Leptin is a protein released from adipose tissue that enables the brain to adjust energy intake and thermogenesis in relation to the size of energy reserves (Zhang, et al., 1994; Tritos & Mantzoros, 1997; Considine & Caro, 1997; Caro, et al., 1996; Collins, et al., 1996). The action of leptin on food intake and weight loss is mediated by interaction of the hormone with its hypothalamic receptor (Chua, et al., 1996). The responsiveness of the hypothalamus to the inhibitory effects of leptin on food intake and body weight is influenced by multiple factors, including deficiency of either leptin (Campfield, et al., 1996) or leptin receptor (OBR) (Baskin, et al., 1998). OBR-L mRNA in the hypothalamus is sensitive to genetic and physiological interventions that change circulating leptin levels indicating that overexpression of OBR-L in the hypothalamus may contribute to increased leptin sensitivity. It has also been shown that rats made obese by feeding a high-calorie diet override the normal satiety effects of leptin (Widdowson, et al., 1997). When they are returned to a normal laboratory diet, they reduce their calorie intake, possibly as a result of a restoration of the satiety effects of endogenous leptin (Widdowson, et al., 1997). Also, the hypophagic response to exogenous leptin is impaired in these rats, indicative of a resistance to the satiety signal. Other studies suggest that HF diet changes the sensitivity to leptin by evoking a sustained increase in circulating leptin (Frederich, et al., 1995). Therefore, despite increased leptin levels, animals fed a high-fat diet became obese without decreasing their caloric intake.
(Frederich, et al., 1995). Leptin resistance may be caused by a variety of factors including changes in leptin transport into the brain, altered receptor population or sensitivity, changes in the JAK-STAT signaling pathway or induction of a cytokine inhibitory protein SOCS-3 (Widdowson, et al., 1997).

The present study provides evidence that OM and S5B/P1 rats differentially express leptin receptor mRNA encoding the long form of the receptor and suggest that diet-induced hyperleptinemia does not down regulate expression of the receptor gene.

### 7.2 EXPERIMENTAL PROTOCOLS

#### Animals

Osborne-Mendel (OM) and S5B/P1 rats were obtained from the breeding colonies at the Pennington Biomedical Research Center. All protocols used in these studies were reviewed and approved by the Institutional Animal Care and Use Committee.

#### Diet

The high-fat (HF) diet contained 56% of energy from fat and the low-fat (LF) diet 15%. The specific composition of the diets has been previously described (Okada, et al., 1992). The protein content of both diets was identical at 24% of total energy.

#### *In vivo* studies

Forty age-matched male, eleven-week old, OM and S5B/P1 rats were used in this study. They were housed individually in hanging wire-mesh cages attached to an automated watering system in a temperature-controlled room with a 12-hour light/dark cycle. All rats consumed nonpurified chow diet (Rodent Chow 5001, Purina Mills, St. Louis, MO, USA) until the beginning of the experiment. Food was available *ad libitum* throughout the experiment. After ten days adaptation to a reversed light/dark cycle, 20
OM and 20 S5B/P1 rats were provided with either the HF or the LF diet for 14 days. Food intake and body weight were monitored daily, and diet was replaced daily. Two hours before dark onset, rats were killed by decapitation to allow collection of trunk blood and preparation of serum. Hypothalamic tissue was dissected and frozen in liquid nitrogen prior to RNA extraction. Serum samples were stored at -80°C before being used for hormone assay.

**Ex vivo studies**

**Isolation of total RNA.** Total RNA was extracted from hypothalamic tissue by the modified guanidinium-isothiocyanate method (Chomczynski & Sacchi, 1987) using TRIzol Reagent (Gibco BRL, Gaithesburg, MD, USA), as described in the General Methods section.

cDNA probes. cDNA riboprobe for the rat leptin receptor was generated from the BamH1-linearized pZObR403 plasmid as described in the General Methods section, according to Ghilardi, et al. (1996). *In vitro* transcription was performed with the MAXIscript kit (Ambion, Austin, TX, USA) using T3 RNA polymerase and [α-32P]UTP, 800 Ci/mmol (29.6 TGBq/mmol) (NEN, Boston, MA, USA). As an internal standard, a 160-bp rat β-actin riboprobe was synthesized using a cDNA template purchased from Ambion (Austin, TX, USA). Transcripts were gel purified in a 5% sequencing gel and eluted using the probe elution buffer. The ObR probe is used to detect both the short and long forms of the receptor mRNAs by using the principle that RNase A/T1 will digest all single-stranded unprotected fragments. Therefore, the long form mRNA will yield a 403-bp protected fragment and the short form mRNA will produce a 270-bp protected fragment from the same probe.
Serum hormone assays. Serum insulin and leptin were measured with double-antibody radioimmunoassay (RIA) kits (Linco, St. Charles, MO, USA) based on rat standards according to the supplier's instructions. Serum corticosterone was assayed using a commercial radioimmunoassay (ICN Pharmaceuticals, Costa Mesa, CA).

Preparation of total protein lysate. The frozen hypothalamic tissues were homogenized in 0.25 ml of ice-cold lysis buffer [50 mM Hepes, pH 7.9; 10% glycerol; 1 mM EDTA; 1 mM sodium pyrophosphate; 1 mM sodium fluoride; 1 mM sodium vanadate; 1 mM Phenylmethylsulfonyl fluoride (PMSF); 1 μg/ml each of aprotinin, leupeptin, and pepstatin] using the motor-driven Polytron PTA 20S operated at maximum speed for 30 s. Nonidet P-40 (NP-40) and Triton X-100 were each added to a final concentration of 1%. The homogenates were incubated on ice for 15 min with shaking. After centrifugation, the supernatant was carefully removed into a new tube and the debris discarded. The protein concentration was determined by the BCA assay (Pierce). The protein samples were aliquoted into 100 μg/20μl and were snap-frozen in liquid nitrogen and stored at −80°C. The procedure was carried out on ice and dithiothreitol (DTT) and sodium vanadate were added in the beginning and at the end of the experiment.

Western blotting. An equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the protein samples (100 μg/20μL), and the proteins were heated to 100°C for 5 min. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Boehringer Mannheim) with a Trans-Blot transfer cell (Bio-Rad Laboratories, Richmond, CA) in TG buffer [48 mM Tris, 39 mM Glycine] and 20% methanol. Membranes were blocked for 1 h with 5% nonfat dry
milk in TBS-T [10 mM Tris, pH 8.0 and 150 mM NaCl with 0.05% Tween-20], incubated with ObR13A primary antibody (Alpha Diagnostics, Inc.) in 1% BSA in TBS-T for 2 h, washed three times for 15 min with TBS-T, and incubated with secondary antibody for 1 h in 1% nonfat dry milk in TBS-T. After washing three times for 20 min each time in TBS-T, antibody binding was visualized using an ECL (Renaissance) detection system. Before reuse, membranes were stripped, blocked and reprobed according to the manufacturer’s instructions. Membranes were reprobed with anti-ObR (K-20) antibody (Santa Cruz Biotechnology, Co.).

Statistics

Analyses of hypothalamic gene expression, plasma corticosterone, leptin and insulin data as well as tissue weights were performed using a three-way analysis of variance (ANOVA) with the factors of strain, diet and age. Individual comparisons between means were made using Bonferroni’s post-hoc analysis.

7.3 RESULTS

Age-matched OM and S5B/Pl rats were maintained either on a HF or LF diet for 14 days. As is characteristic of OM rats on a high fat diet, they became significantly obese, attaining body weight of 392.2 ± 6.1 g compared to 355.7 ± 9.93 of OM rats on the LF diet (Table 7.1). This amounts to 20% increase in body weight from the beginning of the study. S5B/Pl rats showed a slight increase in body weight on a high fat diet (285.5 ± 8.64 versus 261.2 ± 9.41 g). Both strains showed only marginal increases in body weights on the LF diet. The effect of dietary fat on serum hormone levels is also shown in Table 7.1. OM rats had increased levels of insulin (2.76 ± 0.40 and 2.04 ± 0.22 ng/ml,
Table 7.1. The effect of diet on body weight and serum leptin and insulin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>S5B/P1</th>
<th>Osborne-Mendel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>LF</td>
<td>HF</td>
</tr>
<tr>
<td>Initial Body weight (g)</td>
<td>269.3 ± 7.07</td>
<td>261.2 ± 9.41</td>
</tr>
<tr>
<td>Final Body weight (g)</td>
<td>267.7 ± 7.58</td>
<td>285.5 ± 8.64*</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>4.84 ± 0.82</td>
<td>4.55 ± 0.51</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.60 ± 0.06</td>
<td>0.64 ± 0.06</td>
</tr>
</tbody>
</table>

Animals (10 in each group) were exposed to HF and LF diets for 14 days. This table indicates the final body weights at the time of sacrifice. Data are presented as means ± SEM. (n = 10/group) * p < 0.05 compared to LF group, H p < 0.05 compared to equivalent S5B/P1 group.
for HF and LF groups respectively) compared to S5B/Pl rats on the same diets (0.64 ± 0.05 and 0.60 ± 0.06 ng/ml respectively). The serum insulin levels in OM rats on HF diet were elevated approximately 5-fold over those of their S5B/Pl counterparts. Diet did not have any effect on the levels of insulin in either OM or S5B/Pl rats.

Serum leptin levels in OM rats fed the HF diet were elevated 2-fold over those of their S5B/Pl counterparts. OM and S5B/Pl rats had comparable leptin levels after maintenance on the LF diet (6.20 ± 0.63 and 4.81 ± 0.82 ng/ml, respectively). Furthermore, OM rats on a HF diet had higher leptin levels than those on a LF diet (9.43 ± 1.32 compared to 6.20 ± 0.63, respectively).

Using a Ribonuclease Protection Assay, we found that neither diet nor strain had any significant effect on the expression of either the short form or the long form of the leptin receptor mRNA in the hypothalamus. However, the OM rats on both diets had an increase in the long form of the leptin receptor mRNA compared to S5B/Pl rats (Figure 7.1). Western blots of total protein lysates from the hypothalami are shown in Figure 7.2. The levels of OBR-S were reduced by over 50% in both OM and S5B/Pl rats fed the HF diet but there was no intrastrain difference. Likewise, the levels of OBR-L were decreased in rats fed the HF diet in both OM and S5B/Pl rats.

7.4 DISCUSSION

The main findings of this study were; 1) that leptin levels are increased by feeding high fat diets; 2) that leptin levels of OM rats were greater than those of S5B/Pl rats fed the same diet; 3) that there were no differences in the levels of the mRNA for the short form of the leptin receptor (OBR-S mRNA) in the hypothalamus between OM and S5B/Pl rats or with dietary fat; 4) that the level of mRNA for the long form of the
Figure 7.1. A representative RPA autoradiogram of leptin receptor gene expression in OM and S5B/P1 rats fed HF and LF diets. Total hypothalamic RNA was hybridized with an antisense riboprobe for leptin receptor as described in the Methods. Arrows indicate the positions of the long form protected fragment (403-bp) and the short-form protected fragment (270-bp). Insert: Yeast controls for the leptin receptor probe. Lane M is the century marker, lanes 1 and 2 are leptin receptor probe controls in the presence and absence of RNase A/RNase T1 enzymes, respectively.
Figure 7.1 B. Histograms of leptin receptor gene expression in OM and S5B/Pl rats fed HF and LF diets. The histogram shows the relative expression of leptin receptor (short form and long form) to β-actin mRNA in OM (filled bars) and S5B/Pl (empty bars) rats fed the HF and LF diets. Data are presented as means ± SEM (n = 6-8). * indicates p < 0.005 compared to S5B/Pl group on the same diet.
Figure 7.2. Comparison of the short isoform leptin receptor protein levels in OM and S5B/P1 rats fed HF and LF diets. Total hypothalamic protein lysates were prepared as described in the general methods section. Samples prepared from individual rat hypothalami were analyzed by Western blotting with the short form specific (ObR K-20, Santa Cruz Biotechnology Co.) leptin receptor antibodies. M is protein molecular marker. The histograms show the relative optical densities of short form leptin receptor to actin protein in OM (filled bars) and S5B/P1 (empty bars) rats fed the HF and LF diets. Data are means ± SEM. * p < 0.05 compared to LF diet.
Figure 7.3. Comparison of the long isoform leptin receptor protein levels in OM and S5B/P1 rats fed HF and LF diets. Total hypothalamic protein lysates were prepared as described in the general methods section. Samples prepared from individual rat hypothalami were analyzed by Western blotting with the long form specific (ObR13A, Alpha Diagnostics, Inc.) leptin receptor antibodies. M is protein molecular marker. The histograms show the relative optical densities of long form leptin receptor to actin protein in OM (filled bars) and S5B/P1 (empty bars) rats fed the HF and LF diets. Data are means ± SEM. * p < 0.05 compared to LF diet.
receptor (OBR-L) was increased in the hypothalamus of OM rats compared to S5B/Pl rats, but was not affected by dietary fat; 5) that the protein levels of OBR-L and OBR-S were decreased in rats fed HF diets.

While obesity in mutant rodents has been associated with decreased leptin production (ob/ob mouse) (Zhang, et al., 1994) or absence of a functional receptor (db/db mouse and fa/fa rat) (Considine & Caro, 1997; Chua, et al., 1996), these abnormalities are rare in humans (Clement, et al., 1996). Like human obesity, nonmutant obese animals typically show elevated plasma leptin levels and increased expression of leptin mRNA in the adipose tissue (Lin, et al., 1998; Guerre-Millo, 1997) suggesting leptin resistance. By manipulating nutritional variables in different inbred strains, several animal models of diet-induced obesity have been developed (Schemmel, et al., 1970). In our model of dietary obesity, Osborne-Mendel (OM) and S5B/Pl rats differ in their sensitivity to develop obesity when fed a high fat (HF) diet, OM rats become obese, whereas S5B/Pl rats remain thin (Bray, et al., 1990a; Bray, et al., 1990b). Such models provide the opportunity to evaluate the interaction of diet and genetic background on the development of obesity.

The discovery of leptin has elucidated a feedback loop in the hormonal control of energy balance and body fat. Murine mutations that lack an intact ob gene or its receptors become obese (Zhang, et al., 1994; Considine & Caro, 1997; Chua, et al., 1996). In this study, the role of leptin and its receptor in high fat diet-induced obesity was investigated. We confirmed our previous result showing hyperleptinemia in OM rats in response to HF feeding for two weeks. However, we did not observe any increase in serum leptin levels in S5B/Pl rats fed the HF diet in this study. Previously (Lin, et al.,
1998) we reported that S5B/P1 rats responded to the introduction of a HF diet by an increase in leptin gene transcription and serum leptin levels after 2 days, but the magnitude of the responses was greatly decreased at 7 days and serum leptin levels were not affected by diet after 5 weeks. Thus the absence of a HF diet induced increase in serum leptin reported in this study may reflect the length of exposure (14 days) to the diet. Previous studies reporting the hyperleptinemia in obese humans and other animals seem to contradict the ability of leptin to increase energy expenditure and reduce food intake (Bray & York, 1997). Further, the differential leptin response to a high fat diet that we observed here in OM and S5B/P1 rats suggests that sensitivity to a HF diet, rather than resistance, is associated with an increase in leptin secretion. This contrasts to studies in mice in which dietary fat-induced obesity was associated with an attenuated increase in plasma leptin (Ahren, et al., 1997). Ob gene expression and leptin production by the adipose cells are under the control of various hormonal and metabolic factors. High-fat feeding increases ob gene and plasma leptin, and induces a state of leptin resistance (Lin, et al., 1998; Ahren, et al., 1997; Van Heek, et al., 1997). Two hormones, insulin and corticosterone, increase leptin production in rodent and human adipose cells (Barr, et al., 1997; Slieker, et al., 1996; Dagogo-Jack, et al., 1996; MacDougald, et al., 1995; Malmström, et al., 1996). In contrast, the activity of the sympathetic nervous system exerts an opposite effect, mainly through activation of the adipose β3-adrenergic receptors (Guerre-Millo, 1997; Lu, et al., 1998). Insulin has been postulated to be necessary stimulus for leptin release, and our data suggest that this is the case. Hyperleptinemia developed in HF-fed OM rats in parallel with their increase in insulin secretion, whereas S5B/P1 rats, which did not have increased leptin levels, did not
increase insulin levels when fed the HF diet. Devaskar, et al (1997), have shown that leptin mRNA and peptide levels are higher during consumption of a high fat milk diet. They suggested that high levels of leptin with increasing food intake and body weight gain signify hypothalamic leptin receptor resistance during the immediate postnatal period. Our laboratory has previously shown that a high fat diet significantly increased leptin mRNA and serum leptin levels (Lin, et al., 1998). However, when leptin was administered centrally, a similar dose-dependent reduction in energy intake was observed in response to leptin in both OM and S5B/Pl rats. These responses were independent of the diet. These studies suggested that the acute susceptibility of OM rats to HF diet-induced obesity was not related to either a loss of central sensitivity to leptin or a failure to enhance leptin production (Lin, et al., 1998). Similar conclusions were made from a study of HF induced obesity mice (Van Heek, et al., 1997) in which obesity was induced in two strains of mice, C57BL/6 and AKR, by exposure to HF diet. Serum leptin increased in proportion to body weight. C57BL/6 mice on the HF diet developed leptin resistance, whereas those on LF diet retained their leptin sensitivity. Intriguingly, both groups became resistant to peripherally administered leptin after long term exposure, but retained sensitivity to centrally administered leptin (Van Heek, et al., 1997).

To date there is little information on the regulation of the leptin receptor. Both nutritional status and estradiol (E2) treatments affect OBR mRNA expression (Bennett, et al., 1998). Food deprivation increased the abundance of OBR-L transcripts in the thalamus despite a decrease in total OBR and OBR-S in this area. Fasting has also been shown to up-regulate ObR mRNA expression in the Arcuate nucleus of C57 BL/6 lean (+/+) but not obese (ob/ob) mice (Lin & Huang, 1997). Obese Zucker rats also show

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different expression to lean rats with an increased expression of OBR-L transcripts in all brain areas analyzed and a decrease in total OBR gene expression.

Changes in the balance between OBR-L and OBR-S forms of the receptor, observed in these studies could alter tissue sensitivity to leptin. In the current study, we did not identify any changes in the total hypothalamic levels of mRNA for either OBR-S or OBR-L with dietary fat, but there was a significantly elevated level of OBR-L mRNA in hypothalamus of OM compared to S5B/P1 rats. In contrast, OBR-S mRNA levels were similar in both strains. The increase in OBR-L mRNA in hypothalamus of OM rats was evident despite the increase in circulating leptin and insulin in this strain. However, Western blot analyses showed that mRNA expression levels were not an index of receptor protein levels. Indeed, OBR-L protein levels were reduced in rats fed HF diets suggesting that posttranscriptional events are important for the control of receptor activity. This result is somewhat surprising since we have previously shown that there is no difference in the dose-response curve to central leptin in OM and S5B/P1 rats fed a HF diet. The possible explanation for this may be that these receptor protein changes are not uniformly spread across all hypothalamic sites and that the changes in the nuclei that regulate feeding behavior are less dramatic. In situ hybridization and immunohistochemical approaches will be needed to investigate this possibility.

The uptake of leptin in the choroid plexus appears to be saturable (Banks, et al., 1996; Karonen, et al., 1998). The possibility exists that the high fat feeding may influence the transport and, in so doing, the availability of leptin, to the centers important in regulation of food intake and energy expenditure. Leptin may gain access to the brain via receptor-mediated transport through the blood-brain barrier (BBB), and the BBB leptin
receptor (OBR) may regulate the availability of circulating leptin to brain cells. Boado et al (1998) showed that the OBR-S is the principal leptin receptor expressed at the BBB and that this BBB OBR isoform is up-regulated by a high fat diet. We were unable to confirm this in the current study in which we assayed total hypothalamic OBR-S mRNA levels. Further, we showed that OBR-S protein levels were significantly reduced by HF feeding in both rat strains suggesting that leptin transport across the BBB would be reduced by HF feeding. This is consistent with a recent report that leptin transport is decreased in mice that become fat (Banks, et al., 1999). Again the differential responses of mRNA and protein levels for OBR-S suggest regulation at a posttranscriptional level. However, despite this reduction in OBR-S, S5B/Pl rats fed a HF diet do not become obese suggesting that mechanisms other than leptin transport and leptin receptor numbers may be important to their response.

Insulin and leptin are thought to regulate feeding behavior through their abilities to regulate transcription of several neuropeptide genes including neuropeptide Y (Baskin, et al., 1999). We have recently shown that OM rats fed a high fat diet have elevated hypothalamic NPY mRNA levels (Schaffhauser, et al., 1999), suggestive of both leptin and insulin resistance since both these hormones normally decrease neuropeptide Y. As NPY activity in the PVN promotes hyperinsulinemia and hypercorticism (Rohner-Jeanrenaud & Jeanrenaud, 1997), the elevated levels of insulin and glucocorticoids would in turn enhance leptin synthesis and secretion (Barr, et al., 1997). The majority of genetically obese rodents have similar endocrine profiles in which the hyperleptinemia is associated with hyperinsulinemia and hypercorticosteronemia (Bray, et al., 1990a; Bray, et al., 1990b; Rohner-Jeanrenaud & Jeanrenaud, 1997). Our data is, therefore, consistent
with the hypothesis, that insulin promotes the observed hyperleptinemia observed in OM rats compared to S5B/Pl rats and the increase in leptin associated with feeding a high fat diet. The apparent resistance to endogenous leptin in OM rats is reflected in the increased hypothalamic NPY levels (Schaffhauser, et al., 1999). Likewise, the anorectic response to intra-cerebroventricular insulin is lost when rats are fed a HF diet implying a fat-induced central insulin resistance (Arase, et al., 1988).

The results of this current study confirm that obesity associated with consumption of a high fat diet is characterized by hyperleptinemia. Despite the high levels of the long-form of the leptin receptor mRNA in the hypothalamus of obese OM rats and the normal levels of the short form receptor mRNA, changes in the protein levels of both receptors would lead to impaired leptin transport across the BBB and impaired leptin signaling. These protein changes would be expected to promote obesity and contribute to the leptin resistance that is seen in HF fed rats.
CHAPTER EIGHT
SUMMARY AND RESEARCH DIRECTIONS

Adrenal glucocorticoids play a significant role in the control of substrate supply to tissues. They protect the body fat stores by counter-regulating the effects of lipolytic agents (Remesar, et al., 1997), and also enhance the induction of lipogenic enzymes by insulin (Wurdeman, et al., 1978). The obese often show enhanced sensitivity to glucocorticoids (Tokuyama & Himms-Hagen, 1989. Humans and rodents maintain high circulating levels of cortisol and corticosterone, respectively, due to alterations in the activity of the HPA axis (Rosmond & Björntorp, 2000). The effects of circulating glucocorticoids are mediated through the adipose tissue, the main target for lipid storage induced by insulin (Wurdeman, et al., 1978). Glucocorticoids also regulate the expression and production of leptin from the white adipose tissue, therefore regulating the expression of its counter-regulatory hormone. Adrenalectomy prevents fat accretion of the already obese rat and stops weight gain and obesity development in all forms of obesity (Bray, et al., 1990a; Bray, et al., 1990b). After adrenalectomy, a situation exists in which all the counter-regulatory mechanisms that depend on glucocorticoids are removed. For example, the integrity of fat stores is no longer protected, because ADX affects energy balance.

Leptin, whose expression and production are controlled by glucocorticoids, modulates food intake (Weigle, et al., 1995; Rentsch, et al., 1995; Campfield, et al., 1995), body weight (Halaas, et al., 1995; Pelleymonter, et al., 1995; De Vos, et al., 1995) and energy expenditure (Mistry, et al., 1997; Haynes, et al., 1997; Scarpace, et al., 1997; Himms-Hagen, 1997) through its action in the hypothalamus. It also modulates
insulin release and function (Cohen, et al., 1996; Walder, et al., 1997) and thermogenesis (Hwa, et al., 1996). Some of these effects of leptin are analogous to those produced by adrenalectomy. For example, leptin treatment and ADX both increase BAT thermogenesis, decrease food intake and body weight, increase energy expenditure, and lower circulating levels of insulin. Furthermore, Jeanrenaud’s group showed that adrenalectomy increases sensitivity to leptin (Zakrzewska, et al., 1997). Therefore, the present studies investigated the molecular bases of this sensitivity to leptin by studying the effects of adrenalectomy and leptin treatment on the leptin signal transduction pathway, to determine whether glucocorticoids are implicated in the reduced response to leptin in obesity.

In this research project, we studied leptin receptor expression and activity in the hypothalamus and liver after adrenalectomy. To confirm that ADX increases sensitivity to leptin when given icv, we measured food intake, serum hormones, gene expression and protein levels and activities of candidates in the leptin receptor signaling pathway in the hypothalamus. Since inhibition of food intake by leptin is thought to be caused, in part at least, by inhibition of NPY, its mRNA expression in the hypothalamus was also measured. The results demonstrate that leptin treatment of adrenalectomized rats caused a large decrease in food intake and body weight, consistent with data of Zakrzewska and colleagues (1997). The possibility existed that the reduced food intake and body weight in leptin-treated ADX rats may be due to activation or inhibition of central food intake related genes or neurotransmitters. We hypothesized, that removal of adrenal glucocorticoids may potentiate leptin effects by modulating the leptin receptor signaling pathway, with positive effects on genes that are negatively regulated by glucocorticoids,
and vice versa. The results from experiments described in this dissertation show that ADX increased ObR mRNA expression, constitutively enhanced STAT-3 phosphorylation and DNA binding, decreased the cytokine inhibitory SOCS-3 mRNA and protein levels. Leptin treatment of ADX produced superior effects on food intake and body weight because leptin effects could not be counteracted by glucocorticoids. The deregulating effects of leptin treatment in ADX rats were more evident in the lowering of insulin levels, and central administration of leptin has been shown to inhibit insulin secretion and increase insulin sensitivity of peripheral tissues directly (Kamohara, et al., 1997; Cusin, et al., 1998; Minokoshi, et al., 1999; Haque, et al., 1999). The results presented here strongly support the hypothesis that glucocorticoids play a key role in the modulation of leptin-induced imbalance of energy intake and expenditure. The mobilization of fat reserves, elicited by leptin treatment, is probably hampered by glucocorticoids.

Furthermore, leptin receptors are expressed in the arcuate POMC neurons (Cheung, et al., 1997), and the anorectic effects of leptin can be blocked by central MC4-R antagonist (Satoh, et al., 1998). The neuropeptides, NPY, β-endorphin, AGRP, orexin, galanin and melanin concentrating hormone, all increase food intake and reduce sympathetic activity. In contrast, peptides including CCK, CRH, enterostatin, leptin, CART and α-MSH reduce food intake and increase sympathetic activity. The melanocortin receptor system may be particularly important in modulating food intake, because a transgenic mouse which does not express melanocortin-4 receptors is massively overweight (Marsh, et al., 1999). Adrenal glucocorticoids are important in obesity since ADX prevents the development of all forms of obesity. It will therefore be
interesting to study, by immunohistochemistry, the effects of leptin and ADX on the expression of some of these central neuropeptides. The increased sensitivity to leptin on food intake, fat stores and energy expenditure may be related to changes in these neuropeptides, but, NPY was recently showed not to be influenced by a combination of leptin and ADX (Solano & Jacobson, 1999). AGRP was recently shown to be suppressed by leptin, but was not regulated by ADX (Li, et al., 2000).

The reciprocal relationship between food intake and sympathetic activity is robust, and suggests that β-receptors in the periphery and brain may be involved in the control of feeding (Bray, 2000). Haque and colleagues (1999) examined the interaction of the effects of hypothalamic leptin and insulin administered peripherally. They reported that leptin in the hypothalamus enhances glucose uptake in certain peripheral tissues through mediation of a β-adrenergic mechanism for the sympathetic nerves innervating the tissues and that central leptin and peripheral insulin have a synergistic role in augmenting tissue glucose uptake (Haque, et al., 1999). Furthermore, exercise was shown to decrease leptin mRNA expression, an action attributed to activation of the β3-adrenergic receptor, because this decrease was blocked by pretreatment with β3-adrenergic receptor antagonist, SR-59230A (Bramlett, et al., 1999). Whether the activation of β3-adrenergic receptors (receptor numbers) or the increase in its gene expression account for the increased sympathetic activity remains to be determined. Therefore, β3-receptor stimulation, like ADX, down-regulates adipose tissue leptin mRNA in vivo and increase sympathetic activity. It will be interesting to see whether the combination of a chronic β3-receptor stimulation with CL316,243 agonist and icv leptin could show a similar response to that shown in this dissertation.
We have also showed that peripheral leptin resistance was induced in OM rats fed HF diet (Lin, et al., 1998), and also that HF diet down-regulates the hypothalamic leptin receptor protein in OM and S5B/P1 rats (Madiehe, et al., 2000). Recently, Bjorbaek and colleagues (1998) showed that leptin resistance was accompanied by increase in SOCS-3, which was named a potential marker for leptin resistance. Because leptin induce SOCS-3 mRNA and protein expression, it is imperative to expand this study to determine whether HF feeding induced-leptin receptor down-regulation was associated with SOCS-3 expression. It will also be interesting to determine the potential differences in the leptin receptor signaling between OM and S5B/P1 rats due to their distinct differences in sensitivity to HF diet-induced obesity.

The identification of other genes that could be involved in the induction of leptin resistance remains a lucrative endeavor, because it offers endless opportunities toward understanding how diet-induced obesity is manifested and how certain genes within diet-sensitive and diet-resistant individual may be influenced by a conducive environment. This study reveals a need in future research to identify potential molecules that could alleviate the decrease in leptin receptor protein, or those that could influence other genes that are regulated by leptin to reduce adiposity. Our data shows consistency with the literature, and further suggests a need to address key issues associated with leptin resistance, as these will be useful in developing drugs toward the treatment of human obesity. The identity of neuropeptides and pathways required for development of obesity need to be mapped out and various interactions be resolved. An understanding of the participants and their functions will expand the knowledge that could potentially be used in treatment of obesity and/or diabetes.
Future research should continue to focus on the interaction of glucocorticoids, leptin and insulin, the major players in obesity thus far, to offer a clear connection between glucocorticoids, leptin and insulin in the development of obesity. A variety of questions to guide and focus future research arise from this study: 1) In what ways could we activate the leptin receptor or inhibit SOCS-3 expression under conditions of high corticosterone levels? Glucocorticoid receptor antagonists have been shown to decrease body weight. 2) Can GR antagonists activate leptin receptor activity or down-regulate cytokine signaling inhibitors (e.g. SOCS-3)? The analysis of SOCS knockout mice showed that SOCS proteins have physiological role in regulating responsiveness because the SOCS knockout mice die before weaning due to liver degeneration and lymphoid deficiency (Starr, et al., 1998). Alternatively, mutations that may lead to the loss of SOCS activity could give rise to increased cytokine responsiveness, which may contribute to the development of diabetes. Therefore, small molecule effectors which modify SOCS function may potentially be useful therapeutic agents for the treatment of certain diseases, including obesity. (Starr & Hilton, 1998). 3) Are there any other unknown potentially novel systems involved in the development of obesity that are regulated by high glucocorticoid levels, and what interactions do they have with other known systems? In searching for answers to these questions, we can both develop a better understanding of how obesity is manifested and also take the necessary steps toward treatment of obesity.
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APPENDIX: LETTER OF PERMISSION

Pennington Biomedical Research Center
LOUISIANA STATE UNIVERSITY

October 24, 2000

Ms. Helen Rosenhouse-Romeo, RD
Managing Editor
Obesity Research
1090 Amsterdam Avenue, Suite 14K
New York, NY 10025
Fax: (212) 523-2098

Dear Ms. Rosenhouse-Romeo

You have recently published my paper titled "Differential expression of Leptin Receptor in High- and Low-fat fed Osborne-Mendel and SSJ/Pi rats" in Obesity Research 8(6): 467-474, September 2000. This paper was part of my dissertation research work for fulfillment of my PhD requirements.

The University dissertation editor informed me today that I need to obtain permission for the use of this copyrighted material. I hereby request permission for the use of material from this published paper for my dissertation. Unless you desire otherwise, I shall reference this material using the conventional form of referencing.

As time is of the essence in this matter, I would greatly appreciate your prompt consideration of this request.

Thank you.

[Signature]

Abram Madiehe, Graduate Student

OK to use data as cited above, for one-time, non-exclusive use as part of dissertation. Full credit must be given to Obesity Research.
VITA

Mr. Abram Madiehe was born in Potchefstroom, a town 120-km south-west of Johannesburg, South Africa. He attended Lesego Combined Primary School and Tlokwe High School in Ikageng Township, Potchefstroom. After matriculation, he enrolled with the University of the North in Pietersburg, for undergraduate and postgraduate studies. In undergraduate he majored in biochemistry, microbiology and physiology. His love for science urged him to pursue postgraduate studies in biochemistry, in which he obtained a degree of bachelor of science honours, _cum laude_, the first in the biochemistry department. The title of his thesis with emphasis on anticancer drug development, was titled “Effects of Acridine and Acridine Derivatives on Growth of HL-60 Promyelocytic Leukemia Cells”. For his efforts, he was awarded the Boehringer Mannheim (S.A.) and the Foundation for Research Development (S.A.) outstanding student awards in his class.

With an honours degree in hand, his quest for scientific knowledge and some strong encouragement from his mentor, Dr Errol Tyobeka, who was on sabbatical at the Dana Farber Cancer Institute in Boston, Mr Madiehe decided to pursue a master’s degree in biochemistry with focus in the emerging field of molecular biology. He obtained his master of science degree in biochemistry, _cum laude_, with a thesis titled “Induction of Apoptosis in Lithium-treated HL-60 cells”. During his master’s studies, he was a dedicated teaching assistant in the biochemistry department.

Towards the end of his master’s degree, he was nominated and received the prestigious United States Fulbright fellowship to study biochemistry and molecular biology abroad. He came to Louisiana State University in Baton Rouge in 1995 and enrolled in the department of biochemistry. He joined the obesity biochemistry laboratory
of Dr David York at the world renowned Pennington Biomedical Research Center, where he performed his dissertation research. His dissertation is titled “Glucocorticoid Regulation of the Leptin Receptor Signaling System in the Rat”. He published several abstracts in different meetings and two papers in peer reviewed journals.

He has demonstrated good leadership skills while in South Africa and here at LSU. He served in various student and national committees in South Africa. At LSU, he served as president of the African Student Organization, the International Student Association and the International Cultural Center, and as chairman of the Governing Board of the International Cultural Center and member of the ISO Advisory Council.

He played competitive volleyball and has hardware to show for it. He coached the University of the North volleyball team. His other hobbies include, reading nonfiction, magazines, science, international politics, tutoring, playing board games, listening to classical music and singing.

His grandfather was the most influential teacher, and became the role model that Mr Madiehe modeled himself after. The influence of his early teachers, and later his academic mentors who believed in his potential and shared their knowledge, gave him the determination to seek new knowledge through research. His life would not have been complete without the endless support of his immediate and extended family, who contributed in him being a well-rounded person. His country is looking forward to his service, and with the knowledge he obtained in obesity research, it is hoped that he will make a contribution to finding solutions to curb the lurking epidemic of obesity and diabetes. He will receive his doctor of philosophy degree at the December 2000 commencement.
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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Abram Madimabe Madiehe

Major Field: Biochemistry

Title of Dissertation: Glucocorticoid Regulation of the Leptin Receptor Signaling System in the Rat

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination: October 26, 2000