THE ROLE OF DIETARY FAT IN HYPOTHALAMIC INSULIN AND LEPTIN RESISTANCE AND THE PATHOGENESIS OF OBESITY

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“Failure is only the opportunity to begin again more intelligently.”

-Henry Ford

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CHAPTER I

BACKGROUND AND SIGNIFICANCE

Obesity Epidemic

Obesity has reached epidemic proportions in the United States with approximately two-thirds of the adult population and nearly one-fifth of children and adolescents classified as overweight or obese [1]. With tremendous consequences to our personal health and society as a whole, the obesity epidemic has become one of the foremost health concerns facing our nation today. It is well established that obesity increases the risk of many diseases and health conditions including cardiovascular disease, Type 2 Diabetes Mellitus (T2DM), hypertension, dyslipidemia, liver disease, osteoarthritis, and certain forms of cancer, with risk increasing progressively as adiposity increases. Furthermore, obesity is associated with an increase in mortality such that obese individuals have a 10 to 50 percent increased risk of death from all causes compared to healthy weight individuals [2]. According to a recent study, for the first time in two centuries the current generation of children in the United States may have shorter life expectancies than their parents by as much as 5yrs, if the rapid rise in childhood obesity remains unchecked [136]. In addition to the health problems associated with the obesity epidemic, obesity also has a significant economic impact on the United States health care system. For example, medical expenses attributed to overweight and obesity accounted for 9.1 percent of total medical expenditures in the United States for 1998 according to one study [63]; a number that may be even higher today and does not include the indirect costs associated with related diseases and complications attributed to obesity. It is truly difficult to quantify the full impact of the obesity epidemic to our health, quality of life, economy, and society.

According to the National Institutes of Health, obesity and overweight together are the second leading cause of preventable death in the United States. However, no effective therapy currently exists to combat the obesity epidemic. Through understanding the causes and pathogenesis of obesity, it may be possible to design therapeutic targets aimed at both the treatment and prevention of obesity and associated complications.

Energy Homeostasis

Even more striking than the consequences of the obesity epidemic is the dramatic rate at which the prevalence of obesity has increased over the last 25 yrs in the United States [120]. This rapid increase in the prevalence of obesity could be misinterpreted as evidence that body weight is not regulated. However, abundant experimental evidence suggests that body weight is a tightly regulated variable. For example, Bernstein *et al.* [21] demonstrated this regulation in an experiment in which a group of rats were force-fed via gastric catheters to a significant level of obesity compared to freely feeding, lean rats. After several months of force-feeding the obese group, treatment was terminated and animals were allowed to feed freely. Both groups were then followed for changes in body weight. Interestingly, within a few weeks the obese group lost all the excess body weight generated by force-feeding at which point the growth rate of the obese group matched that of the lean group. The rapid weight loss observed in the obese group was primarily mediated by a reduction in voluntary food intake. However, changes in food intake alone could not account for the entire weight loss observed in the obese group suggesting energy expenditure was elevated. In addition, the “experimental obesity in man” studies describe a similar phenomenon of tight body weight regulation in humans [170]. In these studies, subjects were given a monetary incentive to consume as many calories as possible to generate a significant

amount of weight gain. After significant weight gain was achieved compared to baseline levels, the monetary incentive to over-consume was removed but the subjects still received free access to food. As observed in the rat study, the subjects lost all the excess body weight accrued during the incentivized feeding period and returned to their original body weight within a few months. During this weight loss period, food intake was drastically reduced and only returned to normal levels when body weight was restored to basal levels. In addition, it was noted that the amount of calories required to gain and subsequently maintain the elevated body weight was greater than expected based on the subjects' basal energy requirements. This suggested that over-consumption resulted in an increase in total energy expenditure. Together, the results from these studies and others demonstrate that body weight can be tightly regulated and provides evidence for the existence of a system regulating energy balance. This regulation is known as energy homeostasis.

Energy homeostasis is defined as the physiological process whereby energy intake is matched to expenditure over time to promote the stability of body fuel stored in the form of fat. Energy intake is determined by the calories consumed from food, whereas energy expenditure is determined by a variety of components including basal metabolic rate, physical activity consisting of exercise and non-exercise activity, and the thermic effect of food. When energy intake is equivalent to energy expenditure body weight is stable; termed neutral energy balance. When energy intake exceeds energy expenditure, weight gain occurs and is termed positive energy balance. Conversely, when energy expenditure exceeds energy intake, weight loss occurs and is termed negative energy balance. This concept of energy balance is illustrated in Figure 1.

However, this is a very simplistic depiction of a highly complex physiological process. Current models of energy homeostasis indicate that the regulation of body adiposity occurs via a classical endocrine negative feedback loop involving the adiposity

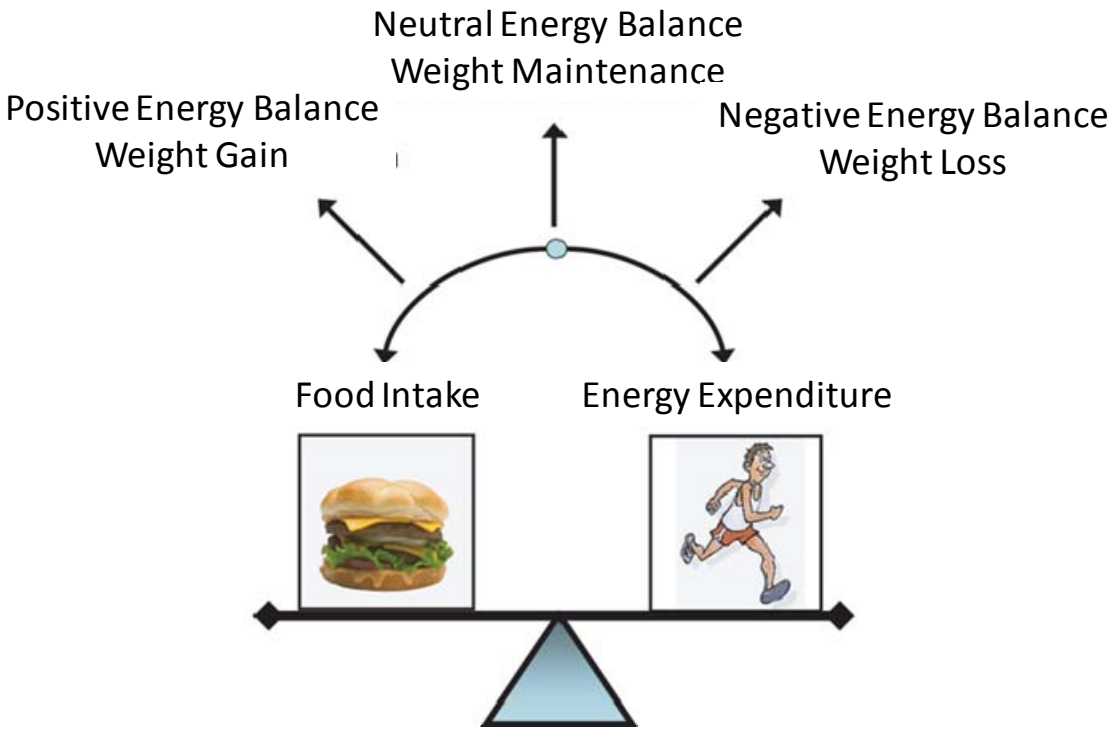


Figure 1. Diagram of Energy Balance

Changes in body weight are determined by the relative balance between food intake and energy expenditure.

signals insulin and leptin (Figure 2). In this model, insulin and leptin circulate in concentrations proportional to body fat content and act within the hypothalamus where they modulate both anabolic and catabolic neural circuits that regulate food intake and energy expenditure to maintain energy balance. For example, in response to an increase in body adiposity, elevated insulin and leptin levels repress anabolic neural circuits that stimulate food intake and inhibit energy expenditure while simultaneously activating catabolic neural circuits that inhibit food intake and increase energy expenditure. These combined effects result in negative energy balance to restore adiposity to basal levels. Conversely, circulating insulin and leptin levels decrease in response to reductions in body adiposity. In this setting, the activity of anabolic neural circuits is increased and the activity of catabolic neural circuits is decreased, ultimately resulting in a state of positive energy balance and the restoration of adiposity to basal levels.

Adiposity Signals

Conceptually an adiposity signal must meet the following criteria (reviewed in [163]); it should be secreted into the plasma in proportion to body fat stores and enter the CNS in proportion to plasma levels, receptors for a putative signal should be expressed by brain neurons involved in energy intake, central administration of a putative adiposity signal should reduce food intake, and finally, deficiency (or inhibition) of the adiposity signal should increase food intake. To date, insulin and leptin are the only known molecules to fulfill the criteria of adiposity signals.

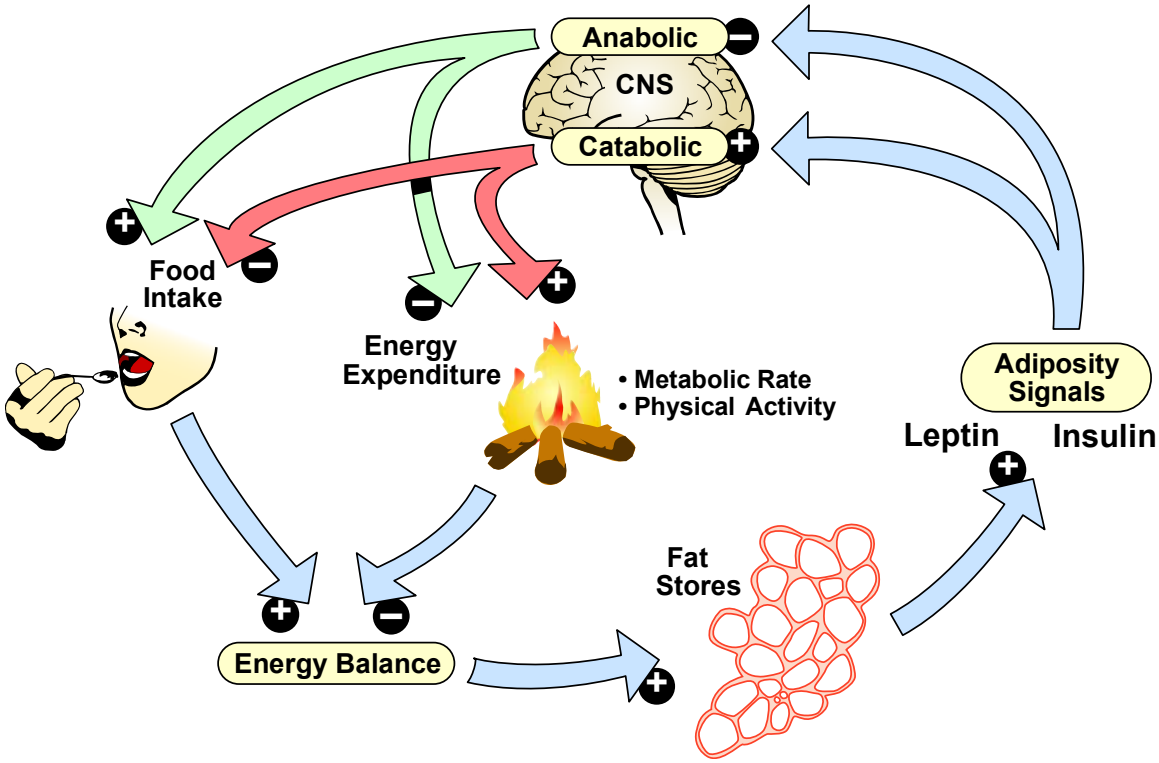


Figure 2. Model of Central Nervous System Control of Energy Homeostasis

The adiposity signals insulin and leptin circulate in concentrations proportional to body fat content and act within the hypothalamus to regulate both anabolic and catabolic neural circuits. These insulin and leptin sensitive neural pathways interact with other regulatory circuits to modulate food intake and energy expenditure, ultimately resulting in the regulation of energy balance and maintenance of adipose stores. Figure adapted from Schwartz *et al.*. [163].

Leptin

The existence of adiposity signals in the regulation of energy homeostasis was first supported by the "parabiosis" experiments conducted in the 1970s which concluded that genetically obese *ob/ob* mice lacked a circulating factor that inhibited feeding, whereas obese mice with a different gene mutation (*db/db*) were resistant to this factor [43, 44]. In 1994, Zhang and colleagues identified the adipose tissue hormone leptin as the protein encoded by the *ob* gene and which is mutated in *ob/ob* mice to induce obesity [210].

Since its discovery, leptin, the product of the *ob* gene, has been extensively studied. Leptin is a 167 amino acid hormone produced almost exclusively in adipose tissue [210] and its secretion is influenced by both the total amount of fat stores as well as short-term changes in energy status [46] [154] in which leptin production is stimulated by insulin and inhibited by fasting [110]. Consistent with its function as an adiposity signal, leptin circulates in proportion to body adiposity [46] and leptin transport across the blood-brain barrier occurs by a saturable receptor-mediated process [12]. Consistent with the entry of leptin to the CNS from the plasma is the observation that leptin concentrations in human cerebrospinal fluid directly correlate with plasma concentrations [161]. Furthermore, leptin receptors are expressed by key hypothalamic neurons involved in energy homeostasis [37, 114] and direct administration of leptin into the CNS reduces food intake and body weight in a dose dependent manner [194]. Combined with data indicating leptin deficiency (*ob/ob*) and leptin receptor mutation (*db/db*) result in severe genetic obesity, this information supports a role for leptin as an adiposity signal.

Insulin

In addition to the glucoregulatory effects of the pancreatic hormone, insulin has also been implicated in the hypothalamic control of energy homeostasis. Work conducted in the late 1970s and early 1980s first demonstrated the existence of neural circuits that respond to insulin and regulate food intake and adipose mass [202, 203]. Like leptin, the secretion of insulin is influenced by the total amount of fat stores as well as by short-term changes in energy balance [7, 28, 144]. In addition, insulin shares many of the characteristics of leptin, making it a candidate adiposity signal. Circulating insulin concentrations are proportional to adiposity and insulin enters the CNS in proportion to its plasma level [162] by a receptor mediated, saturable transport process across brain capillary endothelial cells [18]. Insulin receptors are also expressed by the same key hypothalamic neurons involved in energy homeostasis as leptin receptors [16]. In addition, central administration of insulin reduces food intake and body weight in a dose dependent manner [202] whereas neuron-specific loss of insulin receptors results in obesity [33].

The concept that insulin functions as a catabolic adiposity signal via regulation of key hypothalamic neurons is not well appreciated. Much of the reason for this is that insulin is primarily known as a prototypical anabolic hormone and for its glucoregulatory role in peripheral tissues. However, that insulin exerts opposing actions in the CNS and periphery is actually consistent with the concept of an endocrine negative feedback loop in the regulation of adipose stores [132]. As with most physiological systems, when a signaling pathway is “turned on”, a downstream signal is often generated that will “turn off” the pathway. The same is true in insulin’s regulation of energy homeostasis. The peripheral anabolic actions of insulin to regulate glucose uptake and promote energy storage are balanced by the central catabolic actions of insulin to reduce energy stores

by decreasing food intake and increasing energy expenditure. Together these opposing actions of insulin coordinately support an optimal body composition.

Central Nervous System

The hypothalamus contains numerous histologically distinct nuclei and has been shown to be an important site in the regulation of energy homeostasis [201]. Rapid progress has been made over the last several years in identifying hypothalamic neuron populations that contain specific neurotransmitters, receptors, and other factors important in the regulation of energy homeostasis. In particular, the arcuate nucleus of the hypothalamus (ARC) is uniquely positioned to receive and respond to an array of information pertaining to energy homeostasis. Located around the base of the third ventricle, it lies immediately above the median eminence. Capillaries in the underlying median eminence lack tight junctions and thus this region is thought to effectively lie outside the blood–brain barrier [32] allowing the ARC neurons ready access to information from both short-term and long-term signals of energy homeostasis. The ARC contains both “anabolic” and “catabolic” neuron populations which interact with each other and send projections to downstream neurons within the hypothalamus, the forebrain, and the motor and autonomic areas of the brainstem to regulate energy homeostasis either directly or via connections with other brain nuclei [23, 201]. Anabolic pathways are defined herein as those that promote positive energy balance by stimulating food intake and reducing energy expenditure. Conversely, catabolic pathways are defined as those that promote negative energy balance by inhibiting food intake and increasing energy expenditure.

Within the CNS, a variety of neuropeptides have been shown to either increase or decrease food intake and also influence energy expenditure (reviewed in [124]. Neurons containing neuropeptide Y (NPY; [39, 103] and agouti-related protein (AgRP;

[108, 135, 173] in the ARC were identified as anabolic effectors and neurons containing pro-opiomelanocortin (POMC; [201, 207] were identified as key catabolic effectors, both of which are responsive to insulin and leptin.

Anabolic NPY/AgRP Neurons

Since its discovery in 1982 [181], neuropeptide Y (NPY) has been identified as a major brain neuropeptide which is highly expressed in neurons of the hypothalamic arcuate nucleus [122] and is involved in the regulation of energy homoeostasis. The most well known effect of NPY, its ability to stimulate food intake, was first demonstrated by injection of the peptide into the third ventricle of rodents [39, 103]. In accordance with these observations, food-deprived rats show marked increases in NPY concentrations in the ARC [19], which are paralleled by increases in hypothalamic NPY mRNA [29]. After refeeding, increases in regional NPY concentrations and NPY release fall towards normal [155]. Furthermore, injection of NPY into the third ventricle of rodents also reduces energy expenditure [24]. This effect may be explained by a reduction in sympathetic nerve activity that stimulates heat production in brown adipose tissue [56]. NPY also acts as an insulin secretagogue via the autonomic nervous system thereby facilitating triglyceride deposition in peripheral tissues [209]. Together, these findings support a role for NPY as an anabolic hormone in the central regulation of energy homeostasis which causes a shift to positive energy expenditure.

However, studies in NPY knockout (-/-) mice suggest the presence of compensatory pathways in the control of energy homeostasis since these animals exhibit normal growth and a normal hyperphagic response to fasting [61]. One candidate is agouti-related peptide (AgRP), a molecule which is co-expressed in most NPY neurons in the ARC [201]. AgRP, like NPY, stimulates feeding when administered centrally and [135] levels are elevated in the hypothalamus of fasted rats [104]. Thus,

AgRP also appears to mediate anabolic pathways in the regulation of energy homeostasis.

In the brain, AgRP is synthesized exclusively in the ARC by neurons that project to adjacent hypothalamic areas [124] and it is an endogenous antagonist of the melanocortin 3/4 receptors in the catabolic pathway of energy homeostasis [201]. The demonstration that AgRP mRNA is abundantly co-localized with NPY identifies NPY/AgRP neurons as a unique subset that is capable of increasing food intake via two different mechanisms: by increasing NPY signaling and decreasing the catabolic melanocortin signaling pathway [72].

The primary physiological role of the anabolic NPY/AgRP neurons appears to be to sense and respond to states of negative energy balance. Accordingly, neuronal activity increases in these neurons following a critical fall in the body's energy stores and function to restore normal energy balance and body fat stores [201].

Catabolic POMC Neurons

A major effector of the catabolic pathway of energy homeostasis is pro-opiomelanocortin expressing neurons (POMC) of the hypothalamus. The POMC gene is expressed in the hypothalamic arcuate nucleus [207], and undergoes tissue-specific post-translational processing [148]. In the hypothalamus POMC is processed to produce α-melanocyte-stimulating hormone (α-MSH, [148]), which exerts a tonic inhibitory control on food intake and energy storage through its actions in the CNS. In addition, recall that the anabolic neuropeptide AgRP is an endogenous antagonist of melanocortin receptor signaling and represents one of several sites of crosstalk which exist between these two circuits. Thus, the integration of energy homeostatic signals is a complex and interconnected process such that changes in one of the effector pathways can directly or indirectly influence the opposing effector pathway.

The primary physiological role of POMC neurons appears to be to sense and respond to states of positive energy balance. These neurons are activated in response to both short- and long-term signals of nutrient excess. Overall, data indicate that POMC neurons act homeostatically to restore normal energy balance and body fat stores under conditions of energy surplus.

Hypothalamic Leptin Signaling and Action

In the ARC, both the anabolic NPY/AgRP and catabolic POMC neurons express the long form of the leptin receptor and are responsive to changes in leptin [37, 114]. Although several splice variants of the leptin receptor exist, the "long form" of the receptor (Ob-Rb) is critical to signal transduction [97] whereas the short form receptors are thought to be involved in the transport across the blood-brain barrier [186]. Importantly, the long form of the receptor is highly expressed in the hypothalamus [60, 62, 115]. The leptin signaling pathway is briefly reviewed below (Figure 3).

The leptin receptor represents a typical class-I cytokine receptor and like other class-I cytokine receptors, the leptin receptor has no intrinsic enzymatic activity [180]. Thus, propagation of downstream signaling requires the associated tyrosine kinase, janus-activated kinase-2 (Jak2)[90]. Leptin binding stimulates the activation of Jak2 via auto-phosphorylation of the kinase [5]. Once activated, Jak2 phosphorylates several tyrosine residues on the intracellular tail of the leptin receptor promoting the recruitment of downstream signaling proteins [8, 199]. The family of signal transducers and activators of transcription (STATs) are latent transcription factors recruited to activate cytokine receptor/Jak kinase complexes [158]. Leptin signaling via Jak2 leads to activation of STAT3 [187]. Tyrosine phosphorylation of STAT molecules by Jak induces dimerization, nuclear translocation, and transcriptional activation of these molecules [158]. Activation of the leptin receptor leads to its own feedback inhibition of the

signaling pathway by inducing transcription of suppressor of cytokine signaling-3 (SOCS-3) in a STAT3-dependent manner [26]. In addition to the STAT3 dependent effects of leptin, at least some of the hypothalamic effects of leptin appear to be mediated by phosphoinositide-3 kinase (PI3K) signaling. The PI3K pathway is also involved in the regulation of gene transcription and may potentially induce rapid non-genomic events affecting neuronal activity and neuropeptide release.

The leptin response in ARC neurons is coordinately regulated to maintain energy homeostasis via regulation of neuropeptide gene expression as well as neuronal activity and neuropeptide release [69, 147]. For example, leptin inhibits the anabolic NPY/AgRP neurons and suppresses expression of these orexigenic neuropeptides via both STAT3 dependent and independent mechanisms [17, 163]. Recall that NPY/AgRP neurons tonically inhibit catabolic POMC neurons via synapses with these neurons in the ARC. Thus, leptin induced hyperpolarization of NPY/AgRP neurons (i.e. inhibition), leads to a reduction in neurotransmitter release and disinhibition of POMC neurons [58] resulting in reduced food intake and increased energy expenditure. Conversely, decreased or deficient leptin activity increases food intake and reduces energy expenditure by activating anabolic NPY/AgRP neurons and inhibiting catabolic POMC neurons such that production and release of orexigenic neuropeptides is increased and production and release of anorexigenic neuropeptides is decreased [59]. In addition, leptin signaling activates and depolarizes the catabolic POMC neurons leading to an increase in POMC synthesis and neuropeptide release [48, 182] thereby resulting in reduced food intake and increased energy expenditure [35, 112]. The effect of leptin to increase energy expenditure appears to be mediated by an increase in sympathetic nervous system activity [74]. Taken together, leptin induces a spectrum of responses that leads to negative energy balance and reduced adiposity.

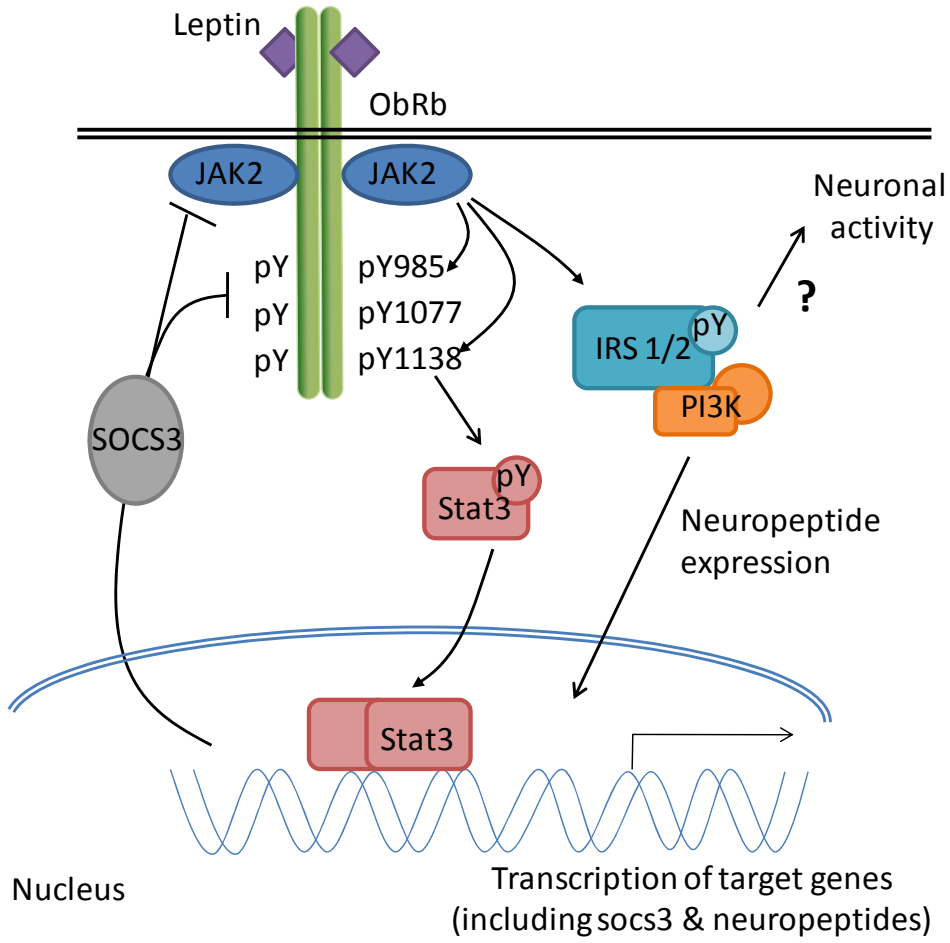


Figure 3. Neuronal Leptin Receptor Signaling Cascade

Leptin binding to ObRb leads to activation of the intracellular tyrosine kinase, Jak2, associated with the membrane-proximal regions of the receptor. Activated Jak2 then phosphorylates a number of cellular substrates including several tyrosine residues on the intracellular tail of the leptin receptor which recruits STAT3 to the receptor where it is phosphorylated by Jak2. Once phosphorylated, STAT3 molecules dimerize, translocate to the nucleus, and induce transcription of several target genes including SOCS-3. SOCS-3 expression leads to feedback inhibition of the leptin receptor signaling pathway. The PI3K pathway is also involved in the regulation of gene transcription and may potentially induce rapid non-genomic events affecting neuronal activity and neuropeptide release. Figure adapted from Bjorbaek *et al.* [25].

Hypothalamic Insulin Signaling and Action

The signaling mechanism and the biological effects of insulin have primarily been studied in classical insulin target tissues, such as skeletal muscle, fat, and liver, with respect to glucose uptake, regulation of cell proliferation, gene expression and the suppression of hepatic glucose production. However, insulin receptors are widely expressed throughout the brain, including the POMC and the NPY/AgRP neurons within the ARC [16], and recent work has focused on elucidating the signaling pathways and mechanisms underlying the anorexigenic effects of insulin within the hypothalamus.

The signaling pathway is similar in neuronal cells and is briefly reviewed here (Figure 4, detailed review in [200]). Insulin action is mediated by the insulin receptor (IR), a member of the family of tyrosine kinase receptors. Binding of insulin activates the intrinsic tyrosine kinase activity of the receptor, leading to the autophosphorylation of several tyrosine residues on the intracellular portion of the receptor. Insulin receptor substrate (IRS) proteins are then recruited to the IR and activated by IR mediated tyrosine phosphorylation. Following this phosphorylation step, IRS proteins activate PI3K, which consists of a p85 regulatory subunit and a p110 catalytic domain. Interaction between the p85 subunit and activated IRS molecule activates the p110 domain of PI3K, which catalyzes the phosphorylation of membrane bound phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 functions as an active signal transduction factor leading to the activation of several downstream signaling molecules including 3-phosphoinositide-dependent kinase-1 (PDK1). PDK1 is a serine/threonine kinase, which in turn activates protein kinase B (PKB, also called Akt). Activation of PKB mediates multiple downstream effects of insulin signaling including the phosphorylation and exclusion of the transcription factor FoxO1 from the nucleus thereby regulating neuropeptide gene

expression. For example, a recent study indicates that activation of FoxO1 promotes opposite patterns of coactivator-corepressor exchange at the AgRP and POMC promoters such that AgRP gene expression is activated and POMC gene expression is inhibited [89].

As with leptin, the energy homeostatic effects of insulin in the CNS are mediated by neuropeptide gene expression as well as neuronal activity and neuropeptide release. The anabolic NPY/AgRP neurons are inhibited by insulin and this inhibition is thought to mediate some of the anorectic actions of insulin [160]. For example, insulin was found to reduce the firing rate, and thus release of neuropeptide Y, in a population of NPY/AgRP neurons [175]. The effect of insulin to inhibit these neurons is dependent on signaling via PI3K and the opening of ATP-sensitive K^+ channels [175]. In addition, central administration of insulin reduces the expression of the orexigenic NPY gene in the ARC [160, 172]. Conversely, the catabolic POMC neurons are activated by insulin resulting in an increase in POMC gene expression and release of the anorexigenic peptide, α-MSH [20]. Upregulation of α-MSH appears to mediate at least some of the anorexigenic effects of insulin since administration of a melanocortin antagonist prevents the observed insulin-induced reduction in food intake [20]. However, central insulin administration reduces weight to a greater extent than can be accounted for by reduced caloric intake alone [204] suggesting a role for insulin in the regulation of energy expenditure. Thus insulin, like leptin, induces a spectrum of responses that lead to the loss of body fat stores.

Together, insulin and leptin interact with neural circuits within the CNS that exert potent unidirectional effects on energy balance. These neural circuits include those that stimulate food intake and reduce energy expenditure to promote weight gain (anabolic pathways) and those that reduce food intake and increase energy expenditure to promote weight loss (catabolic pathways). In response to increases in adiposity, and

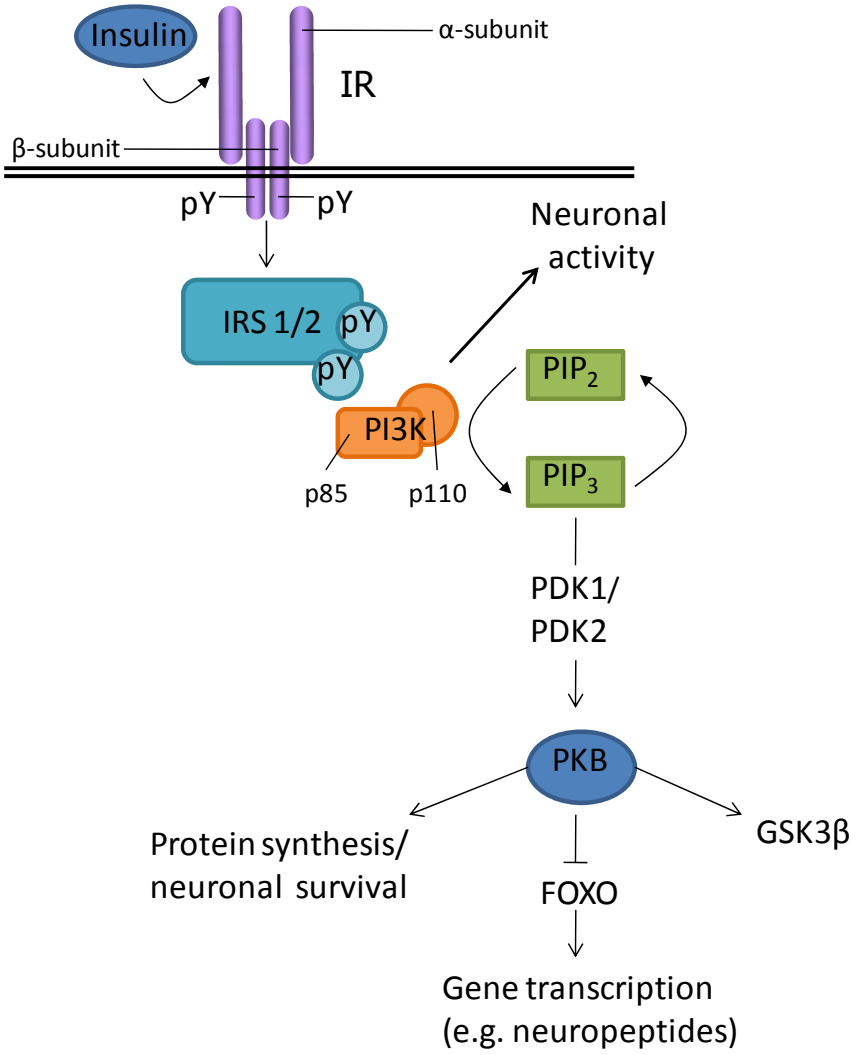


Figure 4. Neuronal Insulin Receptor Signaling Cascade

Binding of insulin to the extracellular α-subunits of the insulin receptor induces a conformational change thereby activating the tyrosine kinase activity of the β-subunits resulting in receptor auto-phosphorylation and subsequent phosphorylation of intracellular IRS proteins. PI3K is recruited to phosphorylated IRS proteins leading to activation of the p110 catalytic subunit of PI3K which mediates the conversion of PIP2 to PIP3 thereby initiating downstream signaling via serine/threonine kinases (i.e. PDK and PKB). These signals result in the diverse biological effects of insulin signaling in the CNS including regulation of gene transcription by FOXO proteins. Like leptin, insulin activation of PI3K in hypothalamic neurons and appears to alter neuronal activity and neuropeptide release. Figure adapted from Plum *et al.*. [141].

elevated levels of insulin and leptin act within the CNS to inhibit anabolic pathways stimulate catabolic pathways. Conversely, in response to decreases in adiposity, reduced levels of insulin and leptin act within the CNS to "activate" anabolic pathways (i.e. remove inhibition) and "inhibit" catabolic pathways (i.e. reduce activation). This coordinated regulation promotes the stability of body fat stores over time.

Integrated Regulation of Energy Homeostasis

The "lipostatic model" of energy homeostasis linking food intake to the amount of energy stored as fat mass in the body as originally articulated by Kennedy [85], postulated that signals proportional to the size of fat stores become integrated with "other" regulators of food intake (i.e. short-term signals). These short-term regulators of food intake can include extrinsic signals, such as social factors and the environment, as well as intrinsic signals including those generated in proportion to fat mass (i.e. the aforementioned adiposity signals) and those generated in response to the consumption of food [204]. The long-term maintenance of body weight and fat mass is determined by the short-term regulation of food intake and energy expenditure on a day to day basis and from meal to meal such that sustained changes in these two factors, over time, will lead to collective long-term effects on body weight and fat mass. Therefore, it is important to understand the relationship between the short- and long-term regulation of food intake and energy expenditure.

Short-term Signals

The ability of short-term signals to impact meal size and number appears to be modulated, either directly or indirectly, by the size of the adipose mass and hence the adiposity signals insulin and leptin [123, 204]. The adiposity signals act as long-term signals to suppress food intake by interacting with the short-term meal-related signals. It

is through this interaction between long-term adiposity signals and short-term meal-related signals that the control of food intake is integrated into the homeostasis of fat stores. Overall, energy homeostasis maintains fat mass stores over time through daily regulation of meal size and meal number [86] in the setting of long-term regulation.

The existence of meal-generated signals, including satiety factors, was first documented in the early 1970s when it was found that administration of the gut peptide cholecystokinin (CCK) to rats prior to food administration caused a dose-dependent decrease in meal size [66]. Since then, satiety factors have been extensively studied. Satiety factors are generated in response to the detection, processing, and absorption of food; accumulate during food consumption; contribute to meal termination; and thereby determine meal size [204]. In addition, there is evidence of an association between meal size and the interval to subsequent meal initiation [96] suggesting that factors determining meal initiation are coupled to those terminating the meal.

Several key features of satiety factors have been identified and are summarized briefly (reviewed in [204]). First, when administered exogenously, satiety factors reduce meal size. Second, blocking the action of endogenous satiety factors with specific antagonists increases meal size. Third, satiety factors can synergistically influence meal size by combining with other satiety factors. Fourth, at doses that elicit modest reductions of meal size, satiety factors do not induce nausea or distress in animals. Fifth, satiety factors signal to the brain via afferent nerves as well as via receptors within the brain. Finally, the repeated administration of satiety factors does not alter body weight despite effects on meal size. For example, while the repeated administration of CCK to rats at the onset of every meal effectively reduces the size of each meal, there is a compensatory increase in the number of meals initiated such that cumulative food intake remains the same and energy balance is maintained [196]. Thus, although satiety

factors can potently affect food intake over the course of individual meals, they have limited influence on long-term adiposity by themselves.

As satiety factors signal meal termination, a complimentary signal exists that can initiate meal consumption. The first peripheral orexigenic hormone, ghrelin, was identified as a twenty-eight-amino acid peptide produced mainly in the stomach [67]. The secretion of ghrelin depends largely on nutritional state with circulating ghrelin levels increasing prior to and decreasing shortly after a meal [91] indicating a potential role for this hormone in meal initiation and satiety. Accordingly, the preprandial increase in ghrelin levels correlate with hunger scores in healthy humans and initiate meals in the absence of time and food-related cues [50]. Ghrelin enhances food intake by increasing the number of meals without altering meal size [83]. The appetite inducing effects of ghrelin are proposed to occur via three different pathways [92]. First, ghrelin released into the bloodstream from the stomach may cross the blood-brain barrier and bind to its receptors in the hypothalamus. Second, ghrelin may reach the brain through vagal afferents to the hindbrain. Third, ghrelin produced locally in the hypothalamus may directly affect the various hypothalamic nuclei. At the level of the hypothalamus, ghrelin stimulates the activity of NPY/AgRP expressing neurons and has an inhibitory effect on POMC neurons [91]. Peripherally, ghrelin stimulates gastrointestinal motility, gastric acid secretion, and pancreatic exocrine secretion [49] and also has an effect on immune cell activation and inflammation [91].

Whole-body Physiology

Energy homeostasis is a complex process involving integrated whole-body physiology. Although studies have shown the arcuate nucleus of the hypothalamus to be an important site in the regulation of energy homeostasis by the adiposity signals insulin and leptin, the integration of homeostatic signals involves multiple brain areas

and peripheral tissues [201]. Additional interoceptive information reaches ARC neurons via abundant intra- and extra- hypothalamic connections. Neural inputs to the ARC from regions in the forebrain carry information pertaining to sensory perception, reward expectancies, learned associations, and other emotional needs and behaviors [201]. In the brainstem, the nucleus of the solitary tract (NTS), area postrema, and dorsal motor nucleus of the vagus have all also been implicated in the regulation of energy homeostasis [127]. For example, information from satiety factors generated in the gastrointestinal tract is conveyed to the NTS, located in the brainstem, via afferent nerves as well as via receptors within the brain itself [174, 183]. Information then passes anteriorly through the brainstem to the hypothalamus and other forebrain areas where there are extensive reciprocal connections between the hypothalamus and the brainstem. Energy intake is coordinated on the basis of information received by both regions [163] [45, 54].

In addition to its role in regulating food intake, the hypothalamus also regulates energy storage and expenditure in part through its connections to the autonomic nervous system. The parasympathetic nervous system, through the vagus nerve, promotes energy storage, whereas sympathetic nervous system (SNS) activation increases energy expenditure. The parasympathetic system transmits signals via projections from the hypothalamus to the dorsal motor nucleus of the vagus, which in turn innervates the viscera, including the pancreatic β-cell [80]. Vagal modulation of β-cell function promotes a stoichiometrically excessive insulin hypersecretion in response to a fixed glucose load thereby increasing lipogenesis [80]. Conversely, hormonal, nutrient, and environmental changes modify the activity of projections from other key nuclei of the hypothalamus which lead to SNS activation. SNS activation tends to mobilize energy stores by the following mechanisms: (1) increased circulating catecholamines stimulate glucagon secretion, which antagonizes insulin's effects and indirectly inhibits insulin

secretion; (2) thyroid mediated increase in energy expenditure; (3) increased blood flow and oxygen consumption in skeletal muscle; and (4) increased thermogenesis and lipolysis via activation of β_3-adrenergic receptors in adipose tissue [80].

Although functions as complex as feeding behavior and energy metabolism are undoubtedly controlled by many peptide and non-peptide neurotransmitters interacting at different levels, the coordinated regulation of these opposing pathways via the adiposity signals, insulin and leptin, is central to the maintenance of long-term energy homeostasis mediated by primary neurons located in the ARC. Figure 5 illustrates the integration of peripheral signals to the central nervous system, the reciprocal pathways between regions of the hypothalamus and the hindbrain, and the coordinated response back to the periphery to regulate adipose mass and complete the feedback loop.

Obesity: Disrupted Energy Homeostasis

Despite the evidence supporting a role for the hypothalamus in the regulation of energy homeostasis, the prevalence of obesity in the United States is increasing at an alarming rate. While the mechanisms involved in the development of obesity remain to be fully elucidated, the obese state has been well studied and characterized. It is well known that obesity is characterized by peripheral insulin resistance, but what is less well appreciated is that obesity seems to be characterized by hypothalamic resistance to the adiposity signals, insulin and leptin. Obese individuals have markedly increased serum insulin [7] and leptin [46] levels, reflecting an increase in body adipose mass, yet food intake and energy expenditure are not appropriately regulated as would be predicted based on the homeostatic feedback loop described. This suggests that the homeostatic effects of insulin and leptin are impaired at the level of the CNS, indicative of hypothalamic resistance. Generally speaking, obesity represents a state of positive energy balance (weight gain) in which energy intake exceeds energy expenditure and

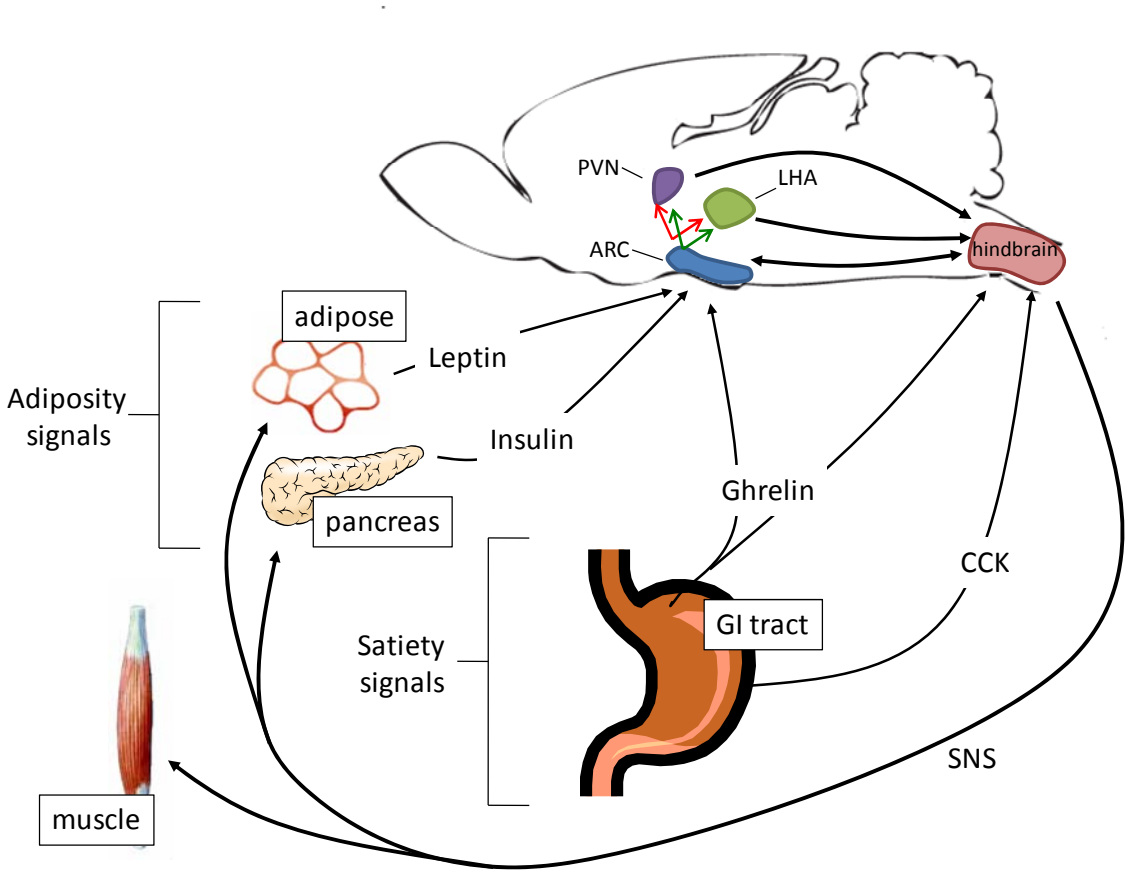


Figure 5. Integration of Central and Peripheral Signals in the Regulation of Energy Homeostasis

Hormonal, nutrient, and environmental cues provide information to the hypothalamus and hindbrain regarding short-term energy metabolism and long-term energy stores. The hypothalamus elicits anorexigenic (green arrows) and orexigenic (red arrows) signals to the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) which leads to efferent outputs from the hindbrain to promote energy storage via activation of the parasympathetic nervous system (vagus nerve) or promote energy expenditure via activation of the sympathetic nervous system. Figure adapted from Isganaitis *et al.*. [80].

the effects of the adiposity signals insulin and leptin fail to appropriately reduce adipose stores via regulation of the hypothalamic circuits controlling food intake and energy expenditure.

Leptin resistance

A number of potential mechanisms have been postulated to underlie central leptin resistance in obesity including defects in leptin transport across the blood-brain barrier, leptin receptor signaling, as well as downstream neurons and signaling molecules that mediate the effects of leptin. Except for leptin-deficient obese mice, most obese mammals have elevated plasma concentrations of leptin [46], yet they exhibit inappropriate levels of food intake and energy expenditure for the given level of leptin [64]. The observation that leptin administered directly into the brain is more potent at curbing appetite in obese mice than peripherally administered leptin has led to speculation that leptin resistance is due to limited availability of the hormone in the CNS [188]. Low CSF leptin levels have been documented in several rodent models of obesity including high-fat feeding and evidence indicates leptin transport is decreased in obesity [10]. However, resistance to the food lowering effects of centrally administered leptin is also observed in genetically obese and HF fed, obese rodents [51, 105], unpublished observations). Thus, inadequate transport does not fully explain the CNS leptin resistance seen in obesity, but appears to be a contributing factor. In addition, the ability of leptin to activate the downstream signaling molecule, STAT3, in hypothalamic neurons is reduced when mice are fed a high-fat diet [57] suggesting a defect in leptin signaling. These data suggest that dietary fat may be involved in multiple sites of hypothalamic leptin resistance from delivery to downstream signaling events by currently unknown mechanisms.

Insulin resistance

Similar mechanisms have been postulated to underlie hypothalamic insulin resistance in obesity. Like leptin, most obese mammals have elevated plasma concentrations of insulin [7, 46] yet exhibit inappropriate levels of food intake and energy expenditure for the level of insulin [64, 204]. Uptake of insulin from the plasma to the brain appears to be reduced in obese Zucker rats [178] and high-fat fed dogs [82], suggesting that insulin transport is a site of resistance. However, previous work from our laboratory has demonstrated resistance to the food lowering effects of insulin administered directly into the brain in rats fed a high-fat diet [146]. Therefore inadequate transport does not fully explain the CNS insulin resistance seen in obesity. Inactivation of insulin signaling via serine phosphorylation of IRS proteins is a common feature of peripheral insulin resistance [211] that is also implicated in the hypothalamus of high-fat fed rats [52]. In addition, studies have shown that activation of PI3K in hypothalamic neurons is required for the ability of centrally administered insulin to reduce food intake and hyperpolarize NPY/AgRP neurons [133, 175]. Thus, impaired signal transduction is another potential mechanism of central insulin resistance in high-fat diet-induced obesity.

Although high-fat diet-induced obesity is characterized by hypothalamic insulin and leptin resistance, the mechanisms involved remain to be fully elucidated. Of note are the similarities between hypothalamic and peripheral insulin signaling as well as the importance of PI3K signaling in both hypothalamic insulin and leptin function. Thus, it seems reasonable that the mechanism of hypothalamic insulin and leptin resistance may be analogous to those involved in peripheral insulin resistance and obesity. Since insulin resistance has been extensively studied in peripheral tissues, findings from these studies may shed light on the mechanisms of resistance present in the CNS. Here, several mechanisms are explored that have been identified in the development of high-

fat diet-induced obesity and peripheral insulin resistance and may also be relevant in the development of hypothalamic insulin and leptin resistance.

Potential Mechanisms Involved in High-Fat Diet-Induced-Obesity

Dietary Fat

Many factors are implicated in the development of obesity including dietary components such as fat and fructose, reduced physical activity, genetic susceptibility, stress, as well as a variety of other factors [30, 195]. Laboratory experiments in animals and clinical studies in humans have repeatedly shown that diet, particularly dietary fat and energy intake are strongly and positively associated with body weight gain [152, 197]. Evidence from cross-sectional and longitudinal epidemiological studies indicates that a high-fat diet is an independent risk factor for increased adiposity and obesity [6] such that there is a direct relationship between dietary fat content and the degree of obesity from which a dose-response curve can be constructed [31, 70]. Furthermore, the type of fatty acids in the diet as well as the efficiency of fatty acid metabolism is reflected in both the plasma and body tissues and has profound effects on physiological and pathophysiological processes in the body [130, 189, 190]. For example, the fatty acid composition of the body is known to affect membrane properties, gene expression, metabolic signaling, as well as energy expenditure [190]. These effects appear to be modulated by fatty acid chain length, degree of fatty acid saturation, and background diet [190]. The obesigenic properties and cellular effects of dietary fatty acids could indicate of a role for these molecules in the development of hypothalamic insulin and leptin resistance.

Essential for a role of dietary fat in the development of hypothalamic insulin and leptin resistance is that fatty acid transport into the brain is an essential process

supplying fatty acids that are not locally synthesized and which are important signaling molecules and components of the phospholipid membrane [73]. In addition, common dietary fatty acids including palmitic acid also rapidly enter the brain and are primarily derived from FA-albumin complexes and to a lesser extent from circulating lipoproteins [176]. A current model of transport proposes that fatty acids cross the blood brain barrier mainly via passive diffusion or potentially with the aid of intracellular fatty acid binding proteins [73]. Acyl CoA synthetases then trap the fatty acids in the cell by forming acyl CoA molecules. The metabolic fate of these molecules depends upon the cellular needs and the specific fatty acid. In the brain, fatty acids function as structural components of the cell, are thought to be oxidized for energy, and can potentially act as signaling molecules [117].

Toll-Like Receptor 4

One recent target of fatty acid signaling that has been implicated in high-fat diet-induced obesity is toll-like receptor 4. The toll-like receptors (TLRs) are receptors expressed by cells of the innate immune system involved in the expression of proinflammatory cytokines [4, 81]. TLRs are stimulated by structural motifs known as pathogen-associated molecular patterns, or PAMPs, including lipopolysaccharide (LPS) [4]. Studies in mice known to have a defective LPS response later identified TLR4 as the specific “LPS receptor” [145]. LPS consists of three parts: lipid A, a core oligosaccharide, and an O side chain [149]. The lipid A moiety of LPS is noteworthy in that it is acylated with saturated fatty acids. Furthermore, removal of these acylated saturated fatty acids results in complete loss of LPS activity and induces an antagonistic effect on native lipid A [125, 143]. These results suggest that acylated fatty acids play a critical role in ligand recognition and receptor activation of TLR4. Additional studies have since demonstrated that saturated fatty acids, independently of LPS, induce TLR4

activity and downstream inflammatory markers whereas unsaturated fatty acids inhibit activation of TLR4 signaling pathways [98, 99].

The expression of TLR4 has been reported in most tissues of the body, including insulin-sensitive tissues [131] making TLR4 an intriguing target of fatty acid induced insulin resistance. Consequently, the role of TLR4 in high fat diet-induced obesity and insulin resistance has been studied in several models of TLR4 deficiency and in various peripheral tissues. For example, Shi *et al.* [165] showed that high-fat feeding activated TLR4 signaling and inflammatory markers in adipocytes and macrophages, but that this effect was blunted in mice lacking TLR4. Furthermore, TLR4 deficient mice were significantly protected from the ability of systemic lipid infusion to 1) suppress muscle insulin signaling and 2) reduce insulin mediated changes in systemic glucose metabolism [165]. Furthermore, mice with a loss-of-function mutation in TLR4 exhibit improved insulin sensitivity and enhanced insulin-signaling capacity in adipose tissue, muscle, and liver compared to control mice during high-fat feeding [184]. Additional studies in these mice demonstrated reduced liver triglyceride content and reduced expression of lipogenic and fibrotic markers with high-fat feeding compared to wild-type controls, indicative of improved hepatic function [142]. Together, these data support a link between TLR4 and high-fat diet-induced insulin resistance.

Elucidation of the TLR4 signaling pathway has revealed potential molecular mechanisms of fatty acid induced insulin resistance. Briefly, ligand binding induces oligomerization of the receptor and recruits downstream adaptor proteins. There are five adaptor proteins including myeloid differentiation primary response gene 88 (MyD88) [134]. TLR4 signaling has been divided into MyD88-dependent and MyD88-independent pathways. From studies using MyD88-deficient macrophages, the MyD88 dependent pathways were shown to be responsible for pro-inflammatory cytokine expression. Upon stimulation, MyD88 recruits and activates downstream signaling molecules resulting in

activation of the IκB kinase (IKK) complex which phosphorylates inhibitor of kappa B (IκB) proteins. This phosphorylation leads to the degradation of IκB proteins and the subsequent translocation of the transcription factor NFκB, which controls the expression of proinflammatory cytokines [109]. Together, this data suggests TLR4 is a link between fatty acid signaling and inflammatory signaling pathways that have been implicated in peripheral insulin resistance (Inflammation as a mediator of insulin resistance will be discussed in detail below).

Phosphoinositide-3 kinase (PI3K) has been implicated in TLR4 signaling pathways and is an important component of hypothalamic insulin and leptin signaling. LPS, a TLR4 agonist, is known to activate PI3K and phosphorylation of PKB/Akt in cells [121, 156]. PKB/Akt further phosphorylates its downstream signaling molecules and has been shown to induce p65 phosphorylation resulting in enhanced NFκB transactivation [111]. Additional studies have been performed to clearly delineate the relationship between fatty acid-induced TLR4 activity and PI3K signaling. Through a series of experiments utilizing inhibitors and dominant negative mutations of proteins in the TLR4 signaling pathway, Lee *et al.* determined that saturated fatty acid induced NFκB activation and inflammatory gene expression was mediated at least in part by TLR4 signaling involving MyD88 and PI3K pathways [100]. These studies also established that saturated and polyunsaturated fatty acids reciprocally modulate the TLR4 signaling pathways. Saturated fatty acids induced TLR4 activation, phosphorylation of PKB/Akt, and NFκB activation, whereas unsaturated fatty acids inhibited TLR4 activity, phosphorylation of PKB/Akt, and NFκB activation ([100], Figure 6). This data links fatty acids with components of the insulin and leptin signaling pathways (via PI3K) as well as inflammatory signaling pathways.

In addition to TLR4 expression in most peripheral tissues, mRNA expression has also been detected in the brain of humans [131]. Of all the TLR members, TLR4

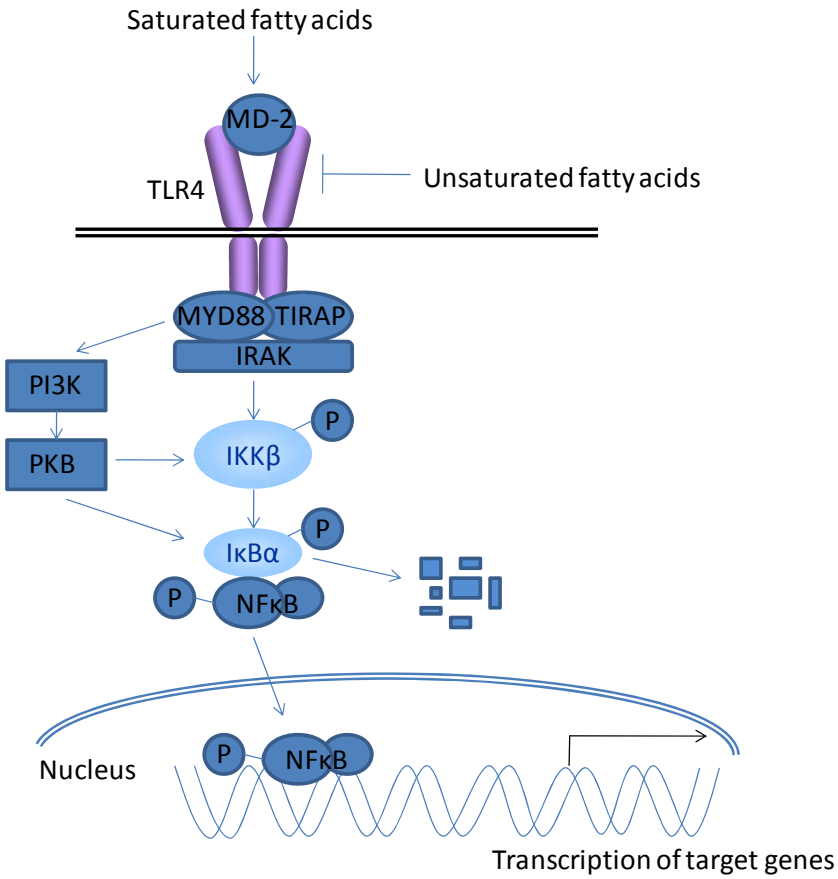


Figure 6. Reciprocal Modulation of Toll-like Receptor 4 Signaling Cascade

Ligand binding induces oligomerization of the receptor and recruits downstream adaptor proteins and signaling cascades divided into MyD88-dependent and MyD88-independent pathways. Shown here is a MyD88-dependent signaling cascade that results in activation of the IKK-IκBα-NFκB pathway. Activation of IKKβ results in phosphorylation and targeted degradation of IκBα, thereby permitting translocation of the transcription factor NFκB to the nucleus where it controls the expression of proinflammatory cytokine target genes. NFκB activation and inflammatory gene expression is also mediated, at least in part, by TLR4 signaling involving PI3K-PKB signaling. In addition, TLR4 signaling activity is reciprocally modulated by saturated and unsaturated fatty acids such that saturated fatty acids induce activation of TLR4, PKB, and NFκB, whereas unsaturated fatty acids inhibit activity of these molecules. Figure adapted from Lee *et al.* [100].

showed the highest expression in brain, liver, and skeletal muscle [131]. In rodents, TLR4 expression has also been documented in the CNS [95] with a low to moderate level of basal expression observed in the median eminence and several adjacent brain regions including the ARC and other hypothalamic nuclei involved in energy homeostasis [34]. Following a single injection of intravenous LPS (40ug/kg), TLR4 gene expression within the ARC of Sprague-Dawley rats was significantly increased at 15min but was quickly restored to basal levels by 30 min and down-regulated by 90 min [34]. Although levels were low under basal conditions, the constitutive expression of TLR4 in these different brain regions as well as the response observed to LPS in the ARC, suggests a potential role of TLR4 signaling in the hypothalamus. Activation of TLR4 by dietary fatty acids could induce hypothalamic insulin (and leptin) resistance as observed in peripheral tissues. Furthermore, hypothalamic insulin (and leptin) resistance could potentially be mediated by the proinflammatory effects of TLR4.

Inflammatory IKKβ Signaling

Compelling evidence linking inflammation to insulin resistance derives from both epidemiological studies and experimental data in humans and animal models. Epidemiological data suggest that subclinical inflammation may represent an additional novel risk factor in the development of obesity and T2DM [55, 159]. As such, obesity is marked by a broad inflammatory response. The first molecular link between obesity and inflammation was discovered by Hotamisligil *et al.* in 1993 [78] in work demonstrating the inflammatory cytokine tumor necrosis factor α (TNFα) is constitutively expressed in adipose tissue and over-expressed in rodent models of obesity. Conversely, body weight reduction in obese individuals is associated with a reduction in both TNFα expression and improved insulin sensitivity [77]. Furthermore, in support of a direct role for inflammation in the development of insulin resistance, in vivo inhibition of TNFα in

obese rats significantly improved insulin sensitivity [78]. Since these initial findings, the IKK/NFκB pathway has been implicated in the development of insulin resistance at the cellular level and can be activated by several proinflammatory receptors [167] including TLR4. These data provide convincing support for inflammation as a contributor to insulin resistance, and provide insights into the underlying molecular pathways.

Inhibition of the IKKβ pathway (the catalytic subunit of the IKK complex) with high doses of salicylates was originally used to lower blood glucose in diabetic patients [13, 151]. Although no longer used to treat diabetes, this effect of IKKβ inhibition to reduce glucose levels led to studies investigating the relationship between IKKβ and insulin sensitivity. Yuan *et al.* [208] reported that activation or over-expression of IKKβ attenuated insulin signaling in cultured cells, whereas inhibition of IKKβ reversed insulin resistance. Furthermore, heterozygous deletion of IKKβ protected mice against the development of high-fat diet-induced insulin resistance [208]. These findings further support the role of inflammation in the pathogenesis of high-fat diet-induced insulin resistance, specifically via IKKβ signaling. The molecular mechanism of IKKβ induced insulin resistance may involve both direct and indirect effects on the insulin signaling pathway to induce resistance. As described previously, IKKβ induces transcriptional activity via activation of the transcription factor NFκB. Several proinflammatory cytokines are direct target genes of NFκB, including TNFα and Interluekin 6 (IL-6), both of which are known inducers of insulin resistance [87]. In addition, evidence indicates a direct effect of IKKβ on the insulin signaling pathways via its function as a serine/threonine kinase. Serine phosphorylation of the insulin receptor and IRS proteins by IKKβ reduces tyrosine phosphorylation and activity of these molecules thereby preventing the association and activation of PI3K and downstream effects [211]. It seems plausible that similar mechanisms may also be involved in the development of hypothalamic insulin and leptin resistance.

Pathogenesis of High-Fat Diet-Induced-Obesity.

High-fat fed, insulin resistant animal models of obesity are characterized by increased activation of IKKβ in peripheral tissues [168]. A significant amount of research has focused on establishing cause versus consequence in this relationship between dietary fat, inflammation, and insulin resistance in peripheral tissues. Studies have demonstrated that infusion of FFAs induces peripheral insulin resistance [40] and that FFA infusion activates PKCθ [71], a known activator or IKKβ [106], whereas inhibition of IKKβ suppresses FFA-induced insulin resistance [88]. Furthermore, studies have shown an even stronger relationship between the accumulation of intracellular fatty acids and insulin resistance [93, 139, 140]. One model to explain this relationship suggests that increases in plasma free fatty acids with high-fat feeding leads to the accumulation of intracellular fatty acid metabolites, such as fatty acyl CoAs, diacylglycerol, and/or ceramides. Accumulation of these molecules can then activate an inflammatory signaling cascade involving IKKβ, which leads to serine phosphorylation of IRS proteins and downregulation of the insulin signaling pathway [169]. In addition, it seems plausible that activation of IKKβ through the TLR4 signaling pathway could also contribute to insulin resistance via a similar mechanism (Figure 7).

Although the model of fatty acid induced insulin resistance was originally described in peripheral tissues, a similar mechanism may also be involved in the development of hypothalamic insulin (and leptin) resistance. In support of this hypothesis, De Souza *et al.* demonstrated that the consumption of a fat-rich diet induces the expression of several pro-inflammatory cytokines and inflammatory responsive proteins in the hypothalamus which was associated with reduced insulin signaling and increased serine phosphorylation of IR and IRS-2 compared to chow controls [52]. In addition, previous work from our laboratory has demonstrated that high-fat fed obese

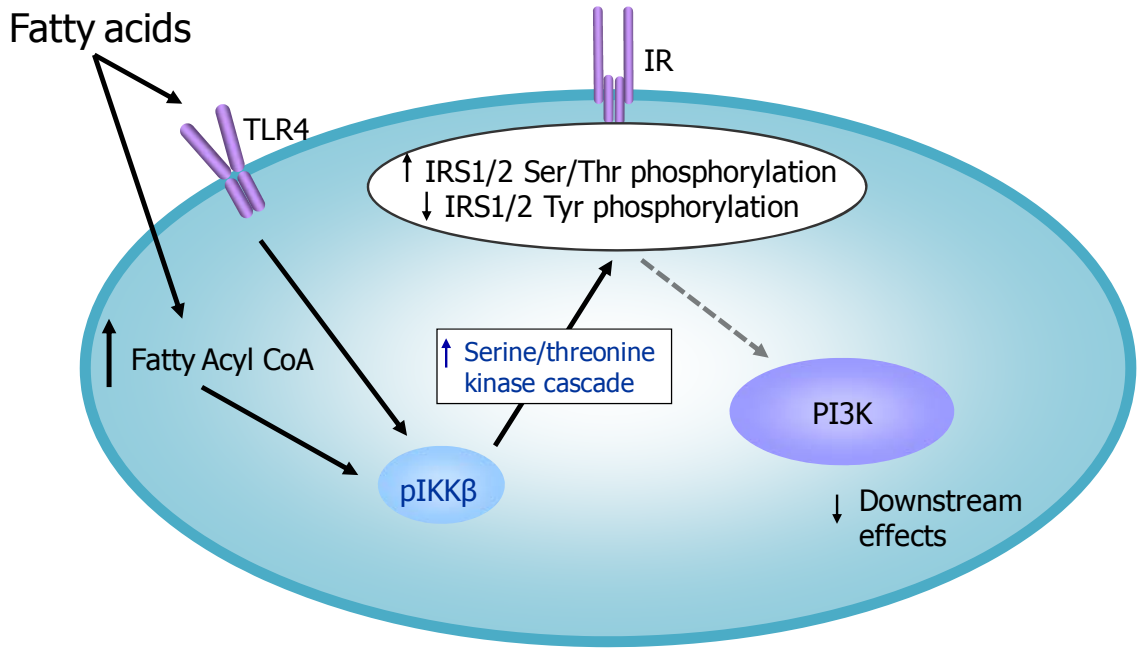


Figure 7. Proposed Cellular Mechanism of Fatty Acid-Induced Insulin Resistance

An increase in delivery of fatty acids or decrease in intracellular metabolism of fatty acids leads to an increase in intracellular fatty acyl CoA levels. The accumulation of fatty acyl CoA molecules induces activation of a serine/threonine cascade potentially mediated by IKKβ resulting in serine/threonine phosphorylation of the IR and IRS molecules and reduced activation of PI3K and other downstream effects of insulin signaling. Alternatively, extracellular saturated fatty acids may contribute to insulin resistance via activation of the TLR4 signaling cascade via a similar mechanism of IKKβ induced serine/threonine phosphorylation of the IR and IRS molecules independent of intracellular fatty acyl CoA accumulation. Figure adapted from Shulman [169].

rats, characterized by hypothalamic insulin and leptin resistance, have increased accumulation of saturated long-chain CoA molecules and increased hypothalamic IKKβ activity compared to low-fat fed controls ([146], Figure 8). These findings establish an important association between consumption of a HF diet, inflammation, and insulin and leptin resistance within key hypothalamic regions involved in the control of energy homeostasis. Based on the current model of central nervous system control of energy homeostasis described, resistance to the effects of insulin and leptin to maintain adipose stores via regulation of hypothalamic circuits regulating food intake and energy expenditure would result in positive energy balance and obesity. Thus, I hypothesize that establishing cause versus consequence of central LC-CoA accumulation and IKKβ activity in the development of hypothalamic insulin and leptin resistance will be crucial in delineating the pathogenesis of high-fat diet-induced obesity.

Overview of Aims

In this body of work, I sought to elucidate the mechanisms involved in the development of high-fat diet-induced hypothalamic insulin and leptin resistance and how this may contribute to the onset of obesity. **My overall hypothesis is that dietary fat per se and not excess caloric intake contributes, either directly or indirectly, to the development of hypothalamic insulin and leptin resistance resulting in impaired regulation of body fat and the development of obesity.**

The degree of fatty acid saturation, location of unsaturated bonds, and fatty acid chain length, have all been shown to influence insulin sensitivity. The ability of fatty acids to modulate these various physiological processes in a chain length and saturation dependent manner suggests that fatty acids possess intrinsic obesogenic properties. Thus, as described in Chapter III, I investigate the potential of dietary fats with varying degrees of saturation to induce obesity and insulin resistance in free-feeding rats.

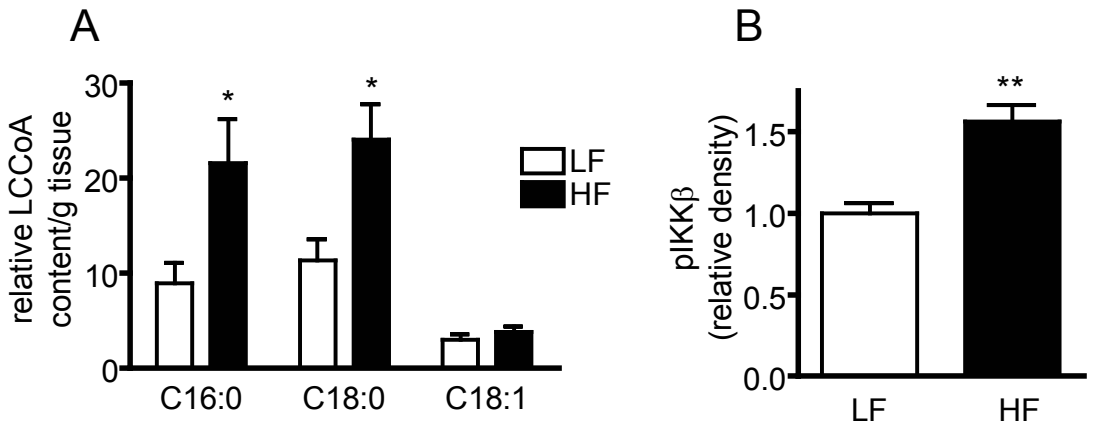


Figure 8. Hypothalamic LC-CoA Accumulation and IKKβ Activity in Obese, HF Fed Rats.

Effect of DIO on hypothalamic long-chain fatty acyl-CoA content and inflammatory signaling. Hypothalamic content of palmitoyl-, stearoyl-, and oleoyl-CoA (A) and IKKβ phosphorylation (B) in low- and high-fat fed rats. Error bars represent the standard error of the mean (SEM). Student's t-test was used to determine significance for comparisons between diet groups. * $p<0.05$, ** $p<0.01$.

However, it is well established that exposure to a high fat diet induces a characteristic hyperphagic response in animal models of diet-induced obesity. This observation raises the question of whether dietary fat simply represents a more calorically dense food source. Therefore I also utilize a pair-feeding paradigm (i.e. caloric restriction of the HF diet to the level of caloric intake of LF free-feeding animals) to delineate the obesogenic effects of dietary fats from the increase in caloric intake observed with high-fat feeding.

In Chapter IV, I directly assess the effects of two structurally distinct fatty acids that are major components of a common dietary fat (lard) on the hypothalamus. I sought to determine whether exposure of the hypothalamus to excess saturated, but not unsaturated fat leads to hypothalamic accumulation of LC-CoAs that trigger inflammatory signaling (elevated IKKβ activity) and blunt insulin signaling in lean chow fed rats given an acute icv infusion of the saturated fat palmitate or the monounsaturated fat oleate.

The effect of the structural characteristics of dietary fatty acids on cellular function, in this case insulin sensitivity, may be explained by a receptor-ligand like interaction between specific fatty acids and "fat sensing" molecules. Although several molecules are known to respond to fatty acids, we have identified Toll-like receptor 4 (TLR4) as a potential target of high-fat diet-induced hypothalamic resistance and impaired body fat mass regulation. TLR4 signaling is activated by saturated fatty acids, whereas unsaturated fatty acids inhibit saturated fatty acid-induced activation of TLR4 signaling [99]. In Chapter V, I investigate the effect of TLR4 deficiency on high-fat diet-induced obesity and hypothalamic insulin resistance.

Current models of energy homeostasis clearly implicate hypothalamic insulin and leptin signaling in the regulation of adipose stores. Accordingly, these signaling pathways are thought to be disrupted in order for obesity to develop. In Chapter VI, I examine the onset of high-fat diet-induced obesity to determine whether the initial onset

of hypothalamic insulin and leptin resistance in high-fat fed rats share a common time-course and mechanism of development. If these changes in hypothalamic insulin and leptin sensitivity are primary to the development of high-fat diet-induced obesity, then they should occur prior to the accumulation of excess body fat. Thus, in these studies I first compared relative insulin sensitivities in central and peripheral tissues of HF fed rats, and second, determined whether the observed HF diet-induced changes in LC-CoA and IKKβ activation occur over a time-course consistent with a role in the onset of hypothalamic insulin and leptin resistance. These changes in insulin and leptin sensitivity would in turn impair energy homeostasis, resulting in pathological weight gain and obesity.

The findings and interpretations from the experiments presented in this dissertation are summarized in Chapter VII. Study limitations and caveats are also addressed. These results raise several unanswered questions and future directions for study in understanding the mechanisms involved in the development of high-fat diet-induced obesity.

CHAPTER II

MATERIALS AND METHODS

Animal Care and Husbandry

All animal studies were performed in accordance with the Vanderbilt Institutional Animal Care and Use Committee guidelines under the supervision of the division of Animal Care.

Rats

Male Long-Evans rats were purchased from Harlan Indianapolis and housed individually when metabolic studies required an accurate measurement of food intake. Diet induced obesity was generated by feeding animals *ad libitum* for 8-10 wks a purified, micronutrient matched diet high in fat content relative to the low fat control diet (45% fat relative to kcal content vs 10%, D12451 vs D12450, Research Diets Inc; New Brunswick, NJ). Nutritional information for all diets and custom diets are shown in Table 1 (Research Diets Inc; New Brunswick, NJ). The established model of high, saturated fat diet-induced obesity creates fasting hyperglycemia, hyperinsulinemia, impaired glucose tolerance, and hypothalamic insulin and leptin resistance in rodents with many characteristics similar to typical human obesity; thus it is an appropriate model for study. Typical metabolic parameters of a diet high in saturated fat from our laboratory are shown in Appendix A. In all diet studies, animals were acclimated to the low fat control diet for 5-7 days and baseline body weight and adiposity were measured. Animals were assigned to diet treatment groups after being randomized to minimize differences in adiposity.

Table 1: Composition of Diets

	LF (D12450)	**HF** (D12451)	**MU** (custom)	**PU** (custom)	**O3** (custom)
%kcal					
Protein	20	20	20	20	20
Carbohydrate	70	35	35	35	35
Fat	10	45	45	45	45
kcal/gm	3.85	4.73	4.73	4.73	4.73
g					
Soybean Oil	25	25	25	25	25
Lard	20	177.5	0	0	0
High Oleic Safflower Oil	0	0	177.5	0	0
Safflower Oil	0	0	0	177.5	0
Menhaden Oil	0	0	0	0	177.5

Table 1: Macronutrient content and fat source are listed for all the diets used in rat and mouse studies. The diets enriched in unsaturated fatty acids (MU, PU, and O3) were custom formulated and matched to the macronutrient content of the HF diet except varying in fat source and fatty acid composition. All diets were purchased from Research Diets Inc (New Brunswick, NJ) and micronutrient matched.

Mice

Male and female wild-type (WT, C57BL/10SnJ) and TLR4 deficient (T4, C57BL/10ScNJ) mice were purchased from Jackson laboratories for in house breeding. The TLR4 deficient mice are homozygous for a null mutation that corresponds to deletion of a 74-kb genomic fragment encompassing the TLR4 gene locus resulting in absence of both mRNA and protein [145]. Lines were maintained separately to eliminate potential differences caused by competition for nutrients, since T4 mice weigh significantly less than their WT counterparts at weaning and throughout diet administration. To confirm loss of TLR4 expression and function, a TLR4 receptor ligand, lipopolysaccharide (LPS, 12.5 μg), was administered by intraperitoneal (ip) injection to a cohort of WT and T4 male mice. Trunk blood, liver, and hypothalami were collected 1 hour post-injection to measure plasma and tissue interleukin-6 (IL-6) levels, a cytokine generated in response to LPS stimulation of the TLR4 receptor. Additionally, a direct measure of TLR4 gene expression was made in WT and T4 mice by quantifying TLR4 mRNA levels. For diet induced obesity studies, four-week old male and female WT and T4 mice were fed a HF (60% kcal fat, see Table 1) or LF diet *ad libitum* for 10 wks. Food intake, body weight, and body composition were measured twice a week. After 10 wks of diet administration, animals were sacrificed and tissues collected for analyses. Trunk blood was collected for plasma metabolite measurements. Indirect calorimetry studies were performed in a separate cohort of male WT and T4 mice in the Mouse Metabolic Phenotyping Center at Vanderbilt University.

Intracerebroventricular (icv) Cannulation

Cannulation of the third ventricle in the brain allows perfusion of structures lying adjacent to third ventricle, i.e. the hypothalamus. Placement of third ventricle cannula into Long Evans rats was performed using proper sterile technique and under general

anesthesia induced and maintained by inhalation of isoflurane. Animals were secured in a stereotaxic apparatus and the surgical area shaved and thoroughly cleaned with Betadine. A small anterior to posterior incision was made along the midline of the head and cleaned with sterile swabs to expose the skull. The skull was leveled and properly aligned using lambda and bregma as reference points at the cranial plate junctions. After removing a small section of the skull at the insertion site, the cannula was targeted to the third ventricle by placing it 2.2 mm caudal to bregma and 7.5 mm ventral to the midsagittal sinus. Dental cement (methyl methacrylate) was used to secure the cannula in place and it was affixed to the skull by prior placement of three small screws that served as anchor points. Antibiotic (ceftriaxone, 0.1 g/kg body weight, ip) was given on the day of surgery and 2 days post-operatively. Animals were allowed to recover for 5-7 days after surgery during which body weight was monitored. Surgical recovery was defined by steady weight gain and final body weight not less than 10% below pre-surgery body weight. Correct placement of cannula was verified by an angiotensin II drinking test. If the cannula is placed correctly, angiotensin II activates the thirst center of the hypothalamus and direct administration to the third ventricle stimulates a measurable thirst response (>5 mls per hour for a 350-400 g rat). For this test, 1 µl of a 10 ng/µl angiotensin II solution was injected via ICV cannula and water consumption was measured over 30 min.

Body Composition Analysis

Body composition was determined by nuclear magnetic resonance (NMR) spectroscopy to assess the percentage of body weight composed of adipose and lean mass. These measurements were performed using the Echo MRI 700 (Echo Medical Systems, Houston, TX) for rats and the Minspec mq7.5 (Bruker Instruments, city, state) in the Mouse Metabolic Phenotyping Center at Vanderbilt University for mice. Animals

were weighed immediately prior to collecting body composition measurements and measurements were performed at the same time of day throughout each study to minimize any fluctuations due to feeding status of the animals. Measurement frequency was from once a day to once a week, which was dependent upon the study protocol. Animals were handled frequently and acclimated to the procedure prior to the start of each study to minimize any stress or novelty responses.

Intraperitoneal Glucose Tolerance Test (IPGTT)

Glucose tolerance tests were performed in 4 hour fasted animals. Blood glucose levels were measured prior to and after a bolus of ip glucose injection (50% dextrose solution in saline) using blood collected from the tip of the tail (Freestyle Flash glucometer, Abbott Laboratories, IL). In rats, blood glucose measurements were collected at -20, 0, 20, 40, 60, 80, 100, and 120 min after a 3 g/kg lean mass dose of glucose administered at time zero. In mice, blood glucose measurements were collected at -30, 0, 5, 15, 30, 60, 90, and 120 min with a 1 g/kg lean mass dose of glucose administered at the 0 minute time-point. The dose and time-points measured were chosen based on previously reported data and reflect species differences in glucose metabolism between rats and mice. As an index of glucose tolerance, the area under the curve was calculated from the blood glucose profiles using the 0 minute time-point value as the baseline.

Pair-Feeding Study

Male Long-Evans rats were divided into three treatment groups; low-fat, high-fat, and high-fat diet that was pair-fed (PF). The low-fat and high-fat groups were given unrestricted access to diet containing 10% or 45% kilocalories fat (Table 1), respectively. The pair-fed group was given the same 45% high-fat diet, but access was limited to

match the caloric intake of the low-fat group and two-thirds of total daily calories were given to pair-fed rats at “lights off” with the remaining one-third given at “lights on”. An IPGTT was performed after 3 wks of diet administration. After 4 wks of diet administration, 4 hour fasted blood glucose levels were measured and animals given an intrapteritoneal injection of either saline or glucose (3 g/kg lean mass, 50% dextrose) to stimulate insulin secretion. Blood glucose was measured again 15 min following intrapteritoneal injection, animals were euthanized, and tissues collected for analysis of p(S473)PKB, p(S177/181)IKKβ, and total IκBα levels. Trunk blood, a mixture of arterial and venous blood collected via decapitation, was used for plasma insulin measurements.

Fatty Acid Infusion

Palmitic (C 16:0) and oleic (C 18:1) fatty acids were obtained from Alltech Associates, Inc (Deerfield, IL) and 100 mM stock solutions of each were prepared as described by Cousin et al. [47]. Basically, fatty acids were dissolved in 0.1 M NaOH at 70 °C in a shaking water bath and then complexed with bovine serum albumin (BSA, fatty acid-free) at 55 °C for 10 min to a final concentration of 1 mM fatty acid/10% BSA solution. After cooling to room temperature, fatty acid solutions were sterile filtered (0.45 μm pore size membrane filter) and stored at -20 °C. On the day of a study, the fatty acid solution was heated to 55 °C for 15 min and cooled to room temperature immediately prior to use. Following a 6 hour fast, previously cannulated male Long Evans rats maintained on standard chow diet (Lab Diet #5001) were subjected to icv infusion using a micropump (Harvard Apparatus 11 Plus Syringe Pump, Holliston MA) of fatty acid or vehicle at a rate of 0.5 μl/min for 4 hours, which delivers 0.5 nmoles fatty acid per minute for a total dose of 120 nmoles. Animals were either euthanized and hypothalami collected for biochemical analyses, e.g. measurement of long chain acyl-CoA (LC-CoA)

content, or non euthanized animals were further assessed, i.e. insulin sensitivity. To test insulin sensitivity, following icv fatty acid infusion, an icv bolus injection of either insulin or vehicle was administered. Animals were euthanized and hypothalami collected for biochemical analyses.

Long Chain Acyl-CoA Measurements

For quantitative analysis of hypothalamic acyl-CoA content, samples were prepared and CoA species purified on solid phase oligonucleotide purification columns, according to Deutsch et al. [53]. Samples were derivatized with n-butylamine for gas chromatography-electron ionization-mass spectrometry (GC-EI-MS) analysis, using a 15-m DB-1 column (J&W Scientific; Folsom, CA) and selected ion monitoring using a Hewlett-Packard (HP) 6890 gas chromatograph coupled to an HP 5973 mass detector operated in the positive EI mode. C16:0 (palmitoyl CoA), C18:0 (stearoyl CoA), and C18:1 (oleoyl-CoA) were quantified by calculating their respective peak areas. A synthetic standard (C17:0, heptadecanoyl CoA) was used as an internal control.

IKK Inhibitor Study

A pharmacological inhibitor of IKK (Inhibitor of kappa B kinase), PS-1145, was purchased from Sigma (St. Louis, MO) and its efficacy was determined by western blot analysis of its downstream target molecule, Inhibitor of kappaB alpha (IκBα), which is degraded after IKK phosphorylation. Efficacy was determined from protein extracts prepared from the hypothalami of rats given an icv dose of PS-1145 (3 µg in saline) into the third ventricle (Appendix B). For food intake studies, icv cannulated animals were fed either a low-fat or high-fat diet *ad libitum* for 8 wks and then received an icv dose of either vehicle or PS-1145 (10 µg) following a 4 hour fast. Food intake was measured over a 4 and 24 hour period after treatment. During food intake studies, animals were

also provided with kaolin pellets, a clay-like substance consumed in response to nausea or 'visceral illness'. Kaolin consumption was measured to monitor any indications of visceral illness (a non-specific reduction in food intake) due to icv treatments [118] (Appendix C). For insulin sensitivity measurements, animals were pretreated with PS-1145 (3 µg) via icv injection for 6 hours prior to insulin sensitivity measurements (described below) to determine the effect of IKK inhibition on insulin signaling.

Cholecystokinin Study

Rats were habituated to regular handling and injections (ip saline) for 1 week prior to any study. Immediately prior to onset of the dark cycle and after a 4 hour fast, either sulfated cholecystokinin (CCK) octapeptide (Bachem Inc; Torrance, CA) at a dose of 0.5 µg or saline vehicle was injected via ip in a final volume of 2.0 ml. Food consumption was measured for 30 min post ip injections (i.e., the first 30 min of the dark cycle). The protocol was adapted from Morton et al. [123]. The selection of the CCK dose was based on a dose-response study in which a 30-minute feeding response was measured relative to vehicle (saline) after an ip injection of CCK doses at 0, 0.5, 1, and 5 µg just prior to onset of the dark cycle (Appendix D).

Insulin and Leptin Sensitivity Meaurements

Hypothalamic insulin and leptin sensitivity was assessed by measuring activation of downstream signaling molecules in response to a direct insulin and leptin stimulation, respectively. Cannulated animals received an icv injection of insulin (10 mU in 2 µl saline), leptin (3 µg in 2 µl PBS, phosphate buffered saline) or vehicle (2 µl). Animals were euthanized 60 min post-injection, and mediobasal hypothalami dissected, snap frozen, and stored at -80°C for analyses. Insulin sensitivity was determined by assessing insulin-stimulated activation (phosphorylation) of PKB/Akt, assessed by

western blotting for p(S473)Akt. The icv insulin dose that was used induces a similar increase in hypothalamic pPKB as compared to the physiological insulin response induced by a fasting-refeeding cycle (Appendix E). Leptin sensitivity was determined by leptin-stimulated activation via phosphorylation of Stat3, assessed by western blotting for p(Y705)Stat3. The icv leptin dose used in these studies is similar to those reported in the literature to assess leptin action in the hypothalamus.

Protein Extraction and Quantification

Total protein was extracted from hypothalamus, liver, muscle, and plasma samples. Tissues were sonicated in a solution of T-Per Tissue Protein Extraction Buffer (Pierce/Thermo Scientific; Rockford, IL) containing 1:100 (v:v) of protease inhibitor cocktail and 1:100 (v:v) of phosphatase inhibitor cocktail (Sigma; St. Louis, MO). For muscle samples, prior to sonication, tissues were pulverized with a tissue pulverizer. Following sonication, all samples were clarified by centrifugation at 10,000 x *g* for 20 min at 4 °C. The protein extract (supernatant) was saved and its protein concentration determined. Protein concentration was assessed in duplicate using a bicinchonic acid colorimetric assay (Pierce/Thermo Scientific; Rockford, IL) performed according to the manufacturer's instructions with BSA as a protein standard. Samples were diluted with T-Per buffer to the desired concentration for further analyses.

SDS PAGE and Western Immunoblotting

Protein samples were mixed with 4x XT Sample Buffer and 20x XT Reducing Agent (Bio-Rad; Hercules, CA), heated to 85 °C for 10 min, and immediately loaded (20 µg total protein per lane) along with a Kaleidoscope Precision Plus Protein Standard (Bio-Rad; Hercules, CA). Protein samples and standard were subjected to denaturing electrophoresis on 10% Bis Tris gel with MOPS Running Buffer or 7% Tris Acetate gels

with XT Tricine Running Buffer using the Criterion XT Electrophoresis System according to the manufacturer's instructions (Bio-Rad; Hercules, CA). For immunoblotting, protein was transferred from the gel to a 0.2 μm nitrocellulose membrane using the Criterion Blotter module, according to the manufacturer's instructions (Bio-Rad; Hercules, CA). Membranes were then blocked in StartingBlock T20 blocking buffer (Pierce/ThermoScientific; Rockford, IL) for 1 hour at room temperature and incubated with primary antibody in blocking buffer overnight at 4 °C with gentle rocking, washed three times in TBS (Tris Buffered Saline; 150mM NaCl, 20mM Tris pH 7.5) with 0.1% (v/v) Tween 20 (Sigma) for 10 min at room temperature, and incubated with species-specific peroxidase-conjugated secondary antibody in 50/50 blocking buffer and TBS-T for 1 hour at room temperature. Wash steps repeated, and antibody detection was performed using ECL Western Blotting Detection Reagents (Amersham Biosciences; Piscataway, NJ) and BioMax XAR scientific imaging film (Kodak; Rochester, NY). The luminescent image corresponding to each protein of interest was analyzed by densitometry using ImageJ software (National Institutes of Health, NIH).

Primary antibodies used for immunoblotting included: rabbit anti-p(S473)Akt (1:1,000; #9271, Cell Signaling), rabbit anti-p(S177/181)IKKβ (1:1000; #2687, Cell Signaling), rabbit anti-p(Y705)Stat3 (1:1000; #2687, Cell Signaling), total IκBα (1:1000; #9242, Cell Signaling), and goat anti-actin (1:5000; sc-1616, Santa Cruz). Secondary antibodies used for immunoblotting included: horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000; W401B, Promega) and bovine anti-goat IgG (1:7500; sc-2350 Santa Cruz).

Enzyme Linked Immunosorbant Assay (ELISA)

IL- 6 levels, a measure of Toll-like Receptor4 (TLR4) stimulation by LPS, were determined from plasma and tissue (liver and hypothalami) in wild-type and T4 mice

using a solid phase sandwich ELISA (Invitrogen; Carlsbad, CA). Briefly, a monoclonal antibody specific for IL-6 is coated on the wells of a microtiter plate. Samples (500ug plasma and 50ug tissue protein extracts) and standards are incubated in the wells and IL-6 binds to the immobilized (capture) antibody. After washing, a biotinylated detection antibody specific for IL-6 is added. During this second incubation, the detection antibody binds to the immobilized IL-6 protein captured during the first incubation. After removal of excess detection antibody, streptavidin-peroxidase (enzyme) is added. This binds to the biotinylated detection antibody to complete the four-member sandwich (capture antibody, protein, detection antibody, enzyme). After removal of all unbound enzyme, a substrate solution is added which is acted upon by the bound enzyme to produce color. The intensity of the colored product is directly proportional to the concentration of the IL-6 in the sample. The absorbance of the color produced is measured spectrophotometrically at 450 nm and the concentration of IL-6 in each plasma and tissue sample is calculated from a standard curve generated from the absorbance values of the standards. Sample values were normalized to the average value of those obtained from low fat vehicle mice. All standards and samples were measured in duplicate.

RNA Isolation and Quantitative Real-Time RT-PCR

Total RNA was extracted from liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to instructions from the manufacturer. Optical density measurements of the reconstituted RNA were taken at A_{260} and A_{280} to determine the concentration and the $A_{260/280}$ ratio, to assess purity of the sample, using the Smart Spec Plus spectrophotometer (Bio-Rad; Hercules, CA). RNA (1.0 µg) was reverse transcribed (Multiscribe RT Reverse Transcriptase, Applied Biosystems) and amplified in a one-step qRT-PCR reaction performed using the MyiQ Real-Time PCR Detection System with iQ

Real-Time SYBR Green PCR Supermix (Bio-Rad; Hercules, CA) and the following mouse-specific primer sets: *TLR4* (receptor) forward 5'-GTTCTTCTCCTGCCTGACAC-3' and reverse 5'-AGGGACTTTGCTGAGTTTCTG-3', *SCD-1* (metabolic enzyme) forward 5'-CGTGGGTTGGCTGCTTGTG -3' and reverse 5'-GCTTCTCGGCTTTCAGGTCAG-3', and *mRPL13a* (internal control) forward 5'-AGATGCACTATCGGAAGAAGAAG-3' and reverse 5'-AGTCTTTATTGGGTTCACACCAG -3'. The starting quantity (SQ) of each sample was calculated using a standard curve derived from a 2-fold serial dilution (0-50ng) of Mouse Reference RNA (SABiosciences, Frederick, MD). The SQ mean was calculated for each sample and normalized to the SQ mean of mRPL13a obtained from the same RNA sample. These normalized values were used for subsequent comparison of the relative abundance of each mRNA of interest between different mice and/or experimental manipulation.

Plasma Hormone and Metabolite Measurements

For all studies, trunk blood was collected in EDTA tubes and placed on ice. Samples were centrifuged at 500 x *g* for 10 min at 4 °C, the plasma supernatants were transferred to a new tube and stored at -80°C. Plasma insulin and leptin concentrations were assayed via radioimmunoassay (RIA) using a double antibody procedure by the Hormone Assay and Analytical Services Core at Vanderbilt University with the respective Rat Insulin and Leptin RIA Kits or Mouse Insulin and Leptin RIA Kits (Linco/Millipore; St. Charles, MO). Plasma free fatty acids (FFA) levels were analyzed using the Wako NEFA C Kit (Wako Chemicals Inc; Richmond, VA), an enzymatic colorimetric assay conducted in 96-well plate format and absorbance measured spectrophotometrically at 550 nm. A standard curve was constructed from known concentrations of oleic acid (0.25-1.0mM) for sample calculations. All samples and

standards were run in duplicate. Plasma triglycerides were measured using an enzymatic colorimetric assay (Riachem; San Diego, CA) conducted in a 96-well plate and absorbance was measured spectrophotometrically at 520 nm. A standard curve was constructed from known concentrations of triolein (0.5-10 mg/dl) for sample calculations. All samples and standards were run in duplicate.

Statistical Analysis

Data were analyzed using GraphPad Prism v4.03. Briefly, mean +/- standard error of the mean (SEM) is reported. Student's T-test analysis was used for two-group comparisons and one-way ANOVA was used for comparison of three or more groups followed by the appropriate post hoc analysis to determine significance between the groups. Repeated-measures two-way ANOVA with Bonferroni's post-test was used to determine points of significance in measurements over time between groups. Correlation analyses using Pearson's correlation was used to determine significance of data relationships. $p<0.05$ was considered significant. Specific analyses performed and sample sizes determined from power calculations are listed in figure and table legends where appropriate.

CHAPTER III

OBESOGENIC PROPERTIES OF HIGH FAT DIETS ENRICHED WITH LONG CHAIN FATTY ACIDS OF VARING SATURATION

Introduction

While many factors are implicated in the development of obesity, studies in humans and animals have repeatedly shown that dietary fat and total energy intake are strongly and positively associated with body weight gain and insulin resistance [197]. The fatty acid composition of the body, which is a reflection of dietary fat consumption and fatty acid metabolism [130, 189], is known to affect several physiological processes such as membrane properties, gene expression, metabolic signaling, and energy expenditure [190]. These effects are dependent upon such structural properties of fatty acids as chain length, degree of saturation, and location of unsaturated bonds [177, 190]. Therefore, I investigated the potential of specific long-chain fatty acid moieties to induce obesity and insulin resistance in free-feeding rats.

It is well known that in animal models of diet-induced obesity, diets high in fat content promote increased caloric intake. This raises the question of whether dietary fat possesses inherent obesogenic properties or simply represents a more calorically dense energy source compared to other macronutrients (~9 kcal/g of fat versus ~4 kcal/g of carbohydrate or protein). To delineate the obesogenic effects of dietary fats from the increase in caloric intake observed with high-fat feeding, I utilized a pair-feeding paradigm. Pair feeding is restricting the consumption of a high-fat diet to the levels of caloric intake observed in free feeding low-fat fed animals. I was able to assess the contribution of intrinsic properties of dietary fat in the absence of increased caloric intake on the development of obesity and insulin resistance. Together, the following studies

address the intrinsic properties of dietary fatty acids on whole body energy homeostasis and the development of central and peripheral insulin resistance.

Results

Saturation Dependent Effects of Dietary Fat in Long Evans Rats

Effects on body weight, adiposity, and plasma hormones

Weight and adiposity matched male Long Evans rats were divided into five dietary groups to investigate the saturation dependent effects of long-chain fatty acids on whole body metabolism. High-fat (HF, 45% of total kcal from fat) and low-fat (LF, 10% of total kcal from fat) groups were fed a lard-based diet which consisted of a mixture of saturated, mono-unsaturated, and poly-unsaturated fatty acids with a composition of 40:45:10 parts, respectively. To determine the effect of specific fatty acid moieties in high-fat diet induced obesity, the experimental groups were fed high-fat diets (also 45% of total kcal from fat) enriched in specific long-chain fatty acids of varying degrees and sites of unsaturation; namely mono-unsaturated (MU), omega-6 poly-unsaturated (PU), and omega-3 polyunsatured (O3) fatty acids. Importantly, these fat type specific diets contained the same number of calories per gram as the lard-based HF diet and the lard-based HF diet is the only diet containing a significant amount of long-chain saturated fat. Rats were housed individually and fed their respective diets ad libitum for 10 wks. The duration of high-fat feeding was chosen based on previous data, which indicated that 8-10 wks was sufficient to induce high-fat diet-induced obesity in Long Evans rats (Appendix A). All data measurements and end-point analyses are reported in Table 2 (p 70).

Body weight and adiposity were measured twice a week throughout the experiment (curves are shown in Figure 9A,B). After 10 wks of diet, the HF group weighed significantly more than the LF group (Figure 9C; HF vs LF $p<0.05$). No other

high-fat diet treatment resulted in a significant gain in body weight compared to the LF group (Figure 9C), which suggests that the saturated fatty acid component of the HF diet was responsible for the weight gain associated with high-fat feeding. This supports human evidence implicating saturated fatty acids in the development of diet-induced obesity [41]. Both the HF and MU diet significantly increased adipose mass compared to the LF diet (Figure 9D; HF vs LF $p<0.01$, MU vs LF $p<0.05$). However, there appeared to be a graded effect of the various high-fat diets on adiposity such that as the degree of unsaturation increased, adiposity decreased. The O3 group gaining the least amount of fat mass, whereas the HF group gained the most amount of fat mass among the high-fat diets thereby implicating the saturated fatty acid component of the HF diet in the increased adiposity associated with HF feeding.

Fasting plasma leptin levels were also measured after 10 wks of diet and were elevated in the HF group compared to the LF group (Figure 9E; $p<0.01$). Even when normalized to body adiposity, significant hyperleptinemia was present in the HF group compared to the LF group (Figure 9F; $p<0.05$) indicative of leptin resistance. However, the other high-fat diets did not induce a significant increase in fasting leptin levels compared to the LF group.

Effects on food intake and energy expenditure

High-fat diets are known to induce significant caloric intake compared to low-fat diets ([198] and our observations). The excess caloric intake is thought to occur, in part, from the caloric density of fat compared to carbohydrates and protein (9 kcal/g of fat vs. 4 kcal/g of carbohydrate or protein), as well as to the palatability of high-fat foods [198]. In this experiment, I determined whether the degree of fatty acid saturation altered the intake of excess energy in response to high-fat feeding. Although all the high-fat diets were similar in percent dietary fat content, food intake was not experimentally controlled,

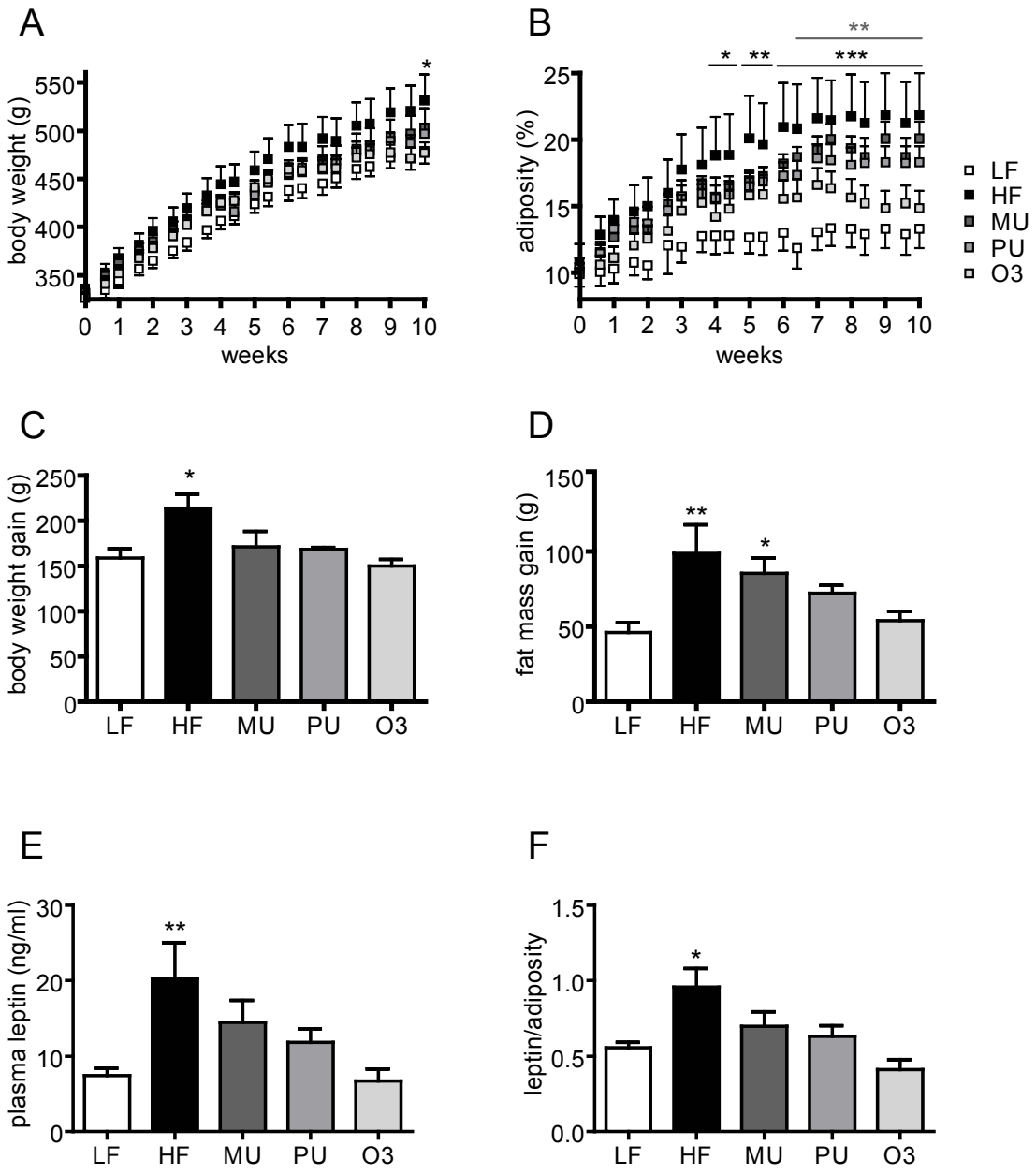


Figure 9: Saturation Dependent Effects of Dietary Fats on Obesity

Male Long Evans rats were fed either a 10%LF diet (LF), or 45% HF diets enriched in saturated (HF), mono-unsaturated (MU), omega-6 poly-unsaturated (PU), and omega-3 polyunsatured (O3) long-chain fatty acids for 10wks. A,B. Body weight and adiposity measurements. C,D. Cumulative body weight and fat mass gain over course of diet treatment. E,F. Absolute plasma leptin levels (4hr fasted) and leptin levels normalized to adiposity. Error bars represent the standard error of the mean (SEM). Two-way ANOVA with Bonferroni's post-tests was used to determine significance in panels A,B (all diets compared to LF, black symbols represent HF vs LF comparison, dark grey symbols represent MU vs LF comparison). One-way ANOVA with Dunnett's Multiple Comparison Test was used to determine significance of all groups compared to a control group in panels C-F (all diets compared to LF). * $p<0.05$, ** $p<0.01$, ***$p<0.001$, n=5-6 per group.

which allowed for any differences in energy intake to be observed between the different high-fat diets. All high-fat diet groups consumed significantly more calories over the course of the study than the LF group (Figure 10A; HF vs LF $p<0.01$, MU vs LF $p<0.05$, PU vs LF $p<0.01$, O3 vs LF $p<0.05$). However, the group fed the lard-based HF diet enriched in long-chain saturated fat consumed the most calories of all the diets high in fat content and no major differences were observed in food intake between the unsaturated high-fat diets.

Caloric intake may account for some, but potentially not all, of the differences observed in body weight gain between the unsaturated high-fat diets (MU, PU, and O3) compared to the lard-based HF diet enriched in long-chain saturated fat. The ability of these diets to induce body weight gain was calculated as feed efficiency. Feed efficiency indicates how effectively the body stores nutrients and accrues mass (as opposed to utilizing nutrients for energy production), calculated as the amount of weight gained per kilocalorie consumed (wt gain/kcal). Weight gain is a function of both food intake and total energy expenditure such that changes in body weight are determined by the relative balance between food intake and energy expenditure. Since body weight gain and food intake are known, relative energy expenditure can be inferred from feed efficiency calculations. For example, a high feed efficiency value (i.e. increased body weight gain per calorie consumed) indicates that energy expenditure is relatively low. Conversely, a low feed efficiency value (i.e. reduced body weight gain per calorie consumed) indicates that energy expenditure is relatively high. No significant difference was observed in feed efficiency between the LF and HF group (Student's T-test, LF vs HF $p=0.2$) indicating that energy expenditure is not appropriately increased with HF feeding to maintain body weight in the setting of increased food intake. However, feed efficiency values were significantly lower in all unsaturated high-fat diet groups compared to the HF group (Figure 10B; MU vs HF $p<0.01$, PU vs HF $p<0.05$, O3 vs HF

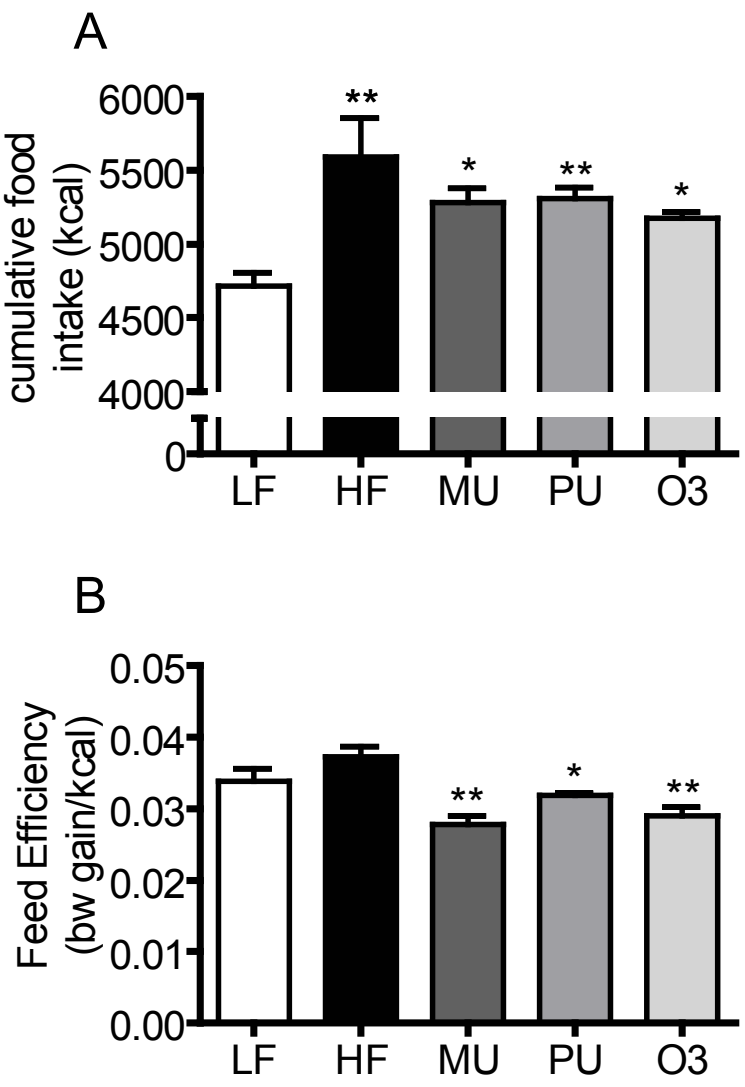


Figure 10: Saturation Dependent Effects of Dietary Fat on Food Intake and Feed Efficiency.

Cumulative food intake measurements (A) and feed efficiency calculations as an index of relative energy expenditure (B) following 10wks of diet treatment in rats. Error bars represent the SEM. One-way ANOVA with One-way ANOVA with Dunnett's Multiple Comparison Test was used to determine significance of all groups compared to a control group (panel A vs. LF, panel B vs HF group). * $p<0.05$, ** $p<0.01$, n=6 per group.

p<0.01). These lower feed efficiency values suggest that per calorie consumed, the animals fed unsaturated fat diets gained less body weight than animals fed the HF diet containing saturated fat. This suggests that consumption of unsaturated fatty acids increase energy expenditure to compensate for the increased caloric intake (observed with all high-fat diets) to maintain energy homeostasis.

Effects of a Restricted High-Fat Diet

Effects on body weight and adiposity

Since animals fed the HF diet enriched in long-chain saturated fats consumed excess energy with respect to their energy needs, the deleterious metabolic effects of HF feeding could arise from increased energy intake, increased dietary fat content, or a combination of both. To determine the contribution of excess caloric intake versus intrinsic obesogenic properties of dietary fat, changes in body weight and adiposity were measured in a separate study that included a group of pair-fed (PF) rats that were fed the HF diet in an amount that was matched to the caloric intake of LF fed controls on a daily basis. All data measurements and end-point analyses are reported in Table 3 for reference (p 70). Total caloric intake of the PF group was precisely matched to the average caloric intake of the LF group over the duration of the study (Figure11A). As previously observed, *ad libitum* HF feeding significantly increased caloric intake (p<0.001) and HF fed animals gained significantly more body weight (p<0.05) and adipose mass (p<0.001) compared to LF fed controls (Figure 11A-E). Caloric restriction in the PF group completely abolished the weight gain observed with high-fat feeding compared to the LF group (Figure 11B,D), which suggests that body weight is a direct corollary of caloric intake in these animals with no apparent effects on energy expenditure. However, the PF group gained approximately 60% more fat mass than the LF group and this equaled approximately 80% of the amount of fat mass accrued by the

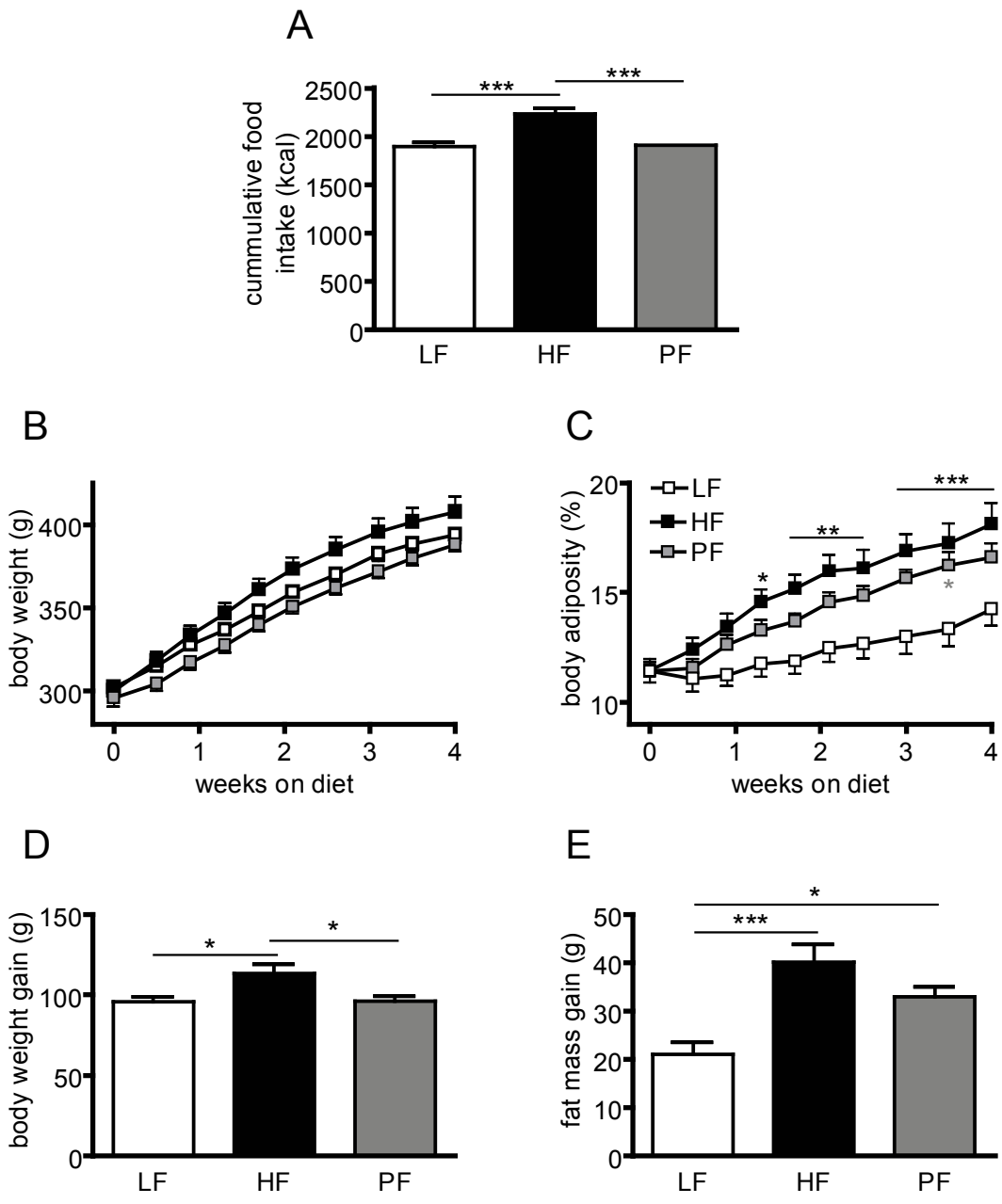


Figure 11: Effects of a Restricted High-Fat Diet on Body Weight and Adiposity.

Food intake of the pair-fed (PF) group was calorically matched to that of the low-fat (LF) diet group fed *ad libitum*. A. Cumulative food intake measurements. The high-fat (HF) group fed *ad libitum* consumed significantly more calories than both the LF and PF groups. B,C. Body weight and adiposity measurements. D,E. Cumulative body weight and fat mass gain over course of diet treatment. Error bars represent the SEM. Two-way ANOVA with Bonferroni's post-tests was used to determine significance in panels B,C (all diets compared to LF group, black symbols represent HF vs LF comparison, grey symbols represent PF vs LF comparison). One-way ANOVA with Tukey's post-hoc analysis was used to determine significance between all groups in panels A,D and E. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=13-14 per group.

HF group (Figure 11C,E; fat mass gain PF vs LF $p<0.05$). Although pair-feeding did not completely recapitulate the level of adiposity observed in the HF group, the amount of long-chain saturated fat consumed by the PF group was 15% less than the HF group. These data indicate that dietary long-chain saturated fats possess intrinsic obesogenic properties which significantly contribute to alterations in body composition wherein adiposity increases at the expense of lean mass.

Effects on glucose tolerance

A glucose tolerance test was performed to determine whether impaired glucose tolerance generally observed with long-term high-fat feeding is mediated by excess caloric intake or the intrinsic obesogenic properties of a diet enriched with long-chain saturated fat. After 3 wks of diet treatment, the glucose excursion curves of the HF and PF group were similarly elevated compared to the LF group (Figure 12A) although area under the curve only reached significance in the PF group (Figure 12B; vs LF $p<0.01$). This is likely attributable to the moderately elevated basal glucose levels observed in the HF group as area under the curve was calculated from baseline glucose values. These data suggest that inherent properties of dietary fat significantly contribute to the impaired glucose tolerance associated with high-fat feeding by increasing adiposity.

High-Fat Feeding, Independent of Increased Caloric Intake is Sufficient to Induce Hypothalamic Insulin Resistance

To determine whether dietary fat enriched in long-chain saturated fat, independent of excess caloric intake, would impair hypothalamic insulin signaling, a glucose bolus was administered via intraperitoneal injection to stimulate endogenous insulin secretion and assess hypothalamic insulin sensitivity in vivo. Hypothalamic tissue samples were collected 15 min post-injection and protein extracts assayed for

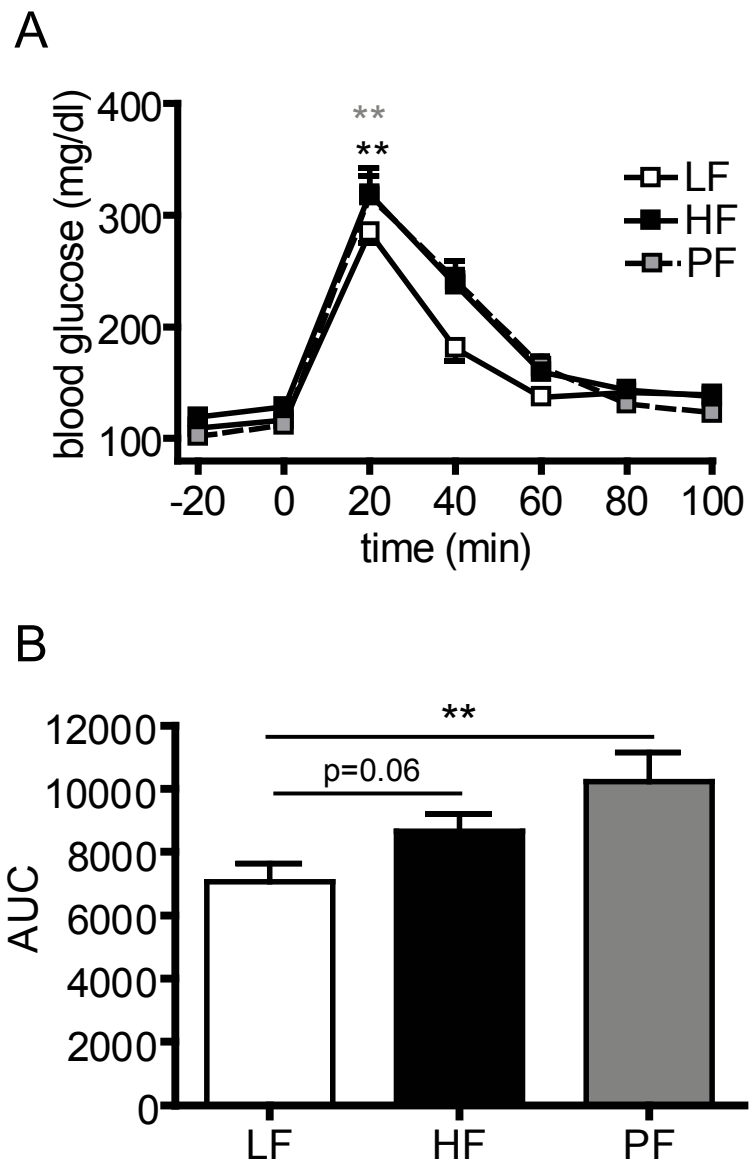


Figure 12: Effect of a Restricted High-Fat Diet on Glucose Tolerance

Dietary fat content, independent of caloric intake, induced a similar blood glucose excursion (A) and elevated AUC in response to intraperitoneal glucose tolerance test (B) compared to LF fed animals. Error bars represent the SEM. Two-way ANOVA with Bonferroni's post-tests was used to determine significance in panel A (black symbols represent HF vs LF comparison, grey symbols represent PF vs LF comparison). One-way ANOVA with Tukey's post-hoc analysis was used to assess significance between groups in panel B. * $p<0.05$, ** $p<0.01$, n=9-11 per group.

activation of the downstream insulin signaling molecule PKB. Mean blood glucose concentrations and plasma insulin levels were similarly elevated in all three groups compared to vehicle controls following glucose injection despite slightly lower baseline glucose and insulin levels in the PF group (likely owing to the longer period of fasting imposed by the pair-feeding protocol; Figure 13A,B). High-fat feeding, in both restricted and free-feeding animals, resulted in slightly higher baseline levels of phosphorylated PKB at serine 473 (pPKB, phosphorylation site leading to activation) compared to LF fed animals (Figure 13C, white bars). Baseline pPKB levels were not further increased in response to glucose-stimulated insulin secretion in the HF or PF group, whereas in the LF group, glucose-stimulated insulin secretion was associated with a significant increase in pPKB (Figure 13C, white vs black bars; LF group $p<0.01$). Moreover, when plotted versus plasma insulin levels, PKB phosphorylation was significantly correlated with plasma insulin concentration in the LF group (Figure 13D; $R=0.73$, $p<0.05$), but not in the HF or PF group.

High-Fat Feeding, Independent of Increased Caloric Intake, is Sufficient to Activate Inflammatory Molecules

Previous observations from our laboratory demonstrated an activation of hypothalamic proinflammatory signaling in high-fat fed obese rats as measured by an increase in phosphorylation of IKKβ. To determine whether activation of IKKβ with high-fat feeding is from excess caloric intake or intrinsic properties of a diet enriched in saturated fat, hypothalamic activation of IKKβ (phosphorylated active form, pIKKβ) and levels of the downstream target molecule, IκBα, were measured. Activation of IKKβ leads to the phosphorylation and degradation of IκBα and subsequent activation of the transcription factor NFκB to propagate proinflammatory signaling thought to contribute to insulin resistance. The inflammatory signaling pathway was activated in the HF group

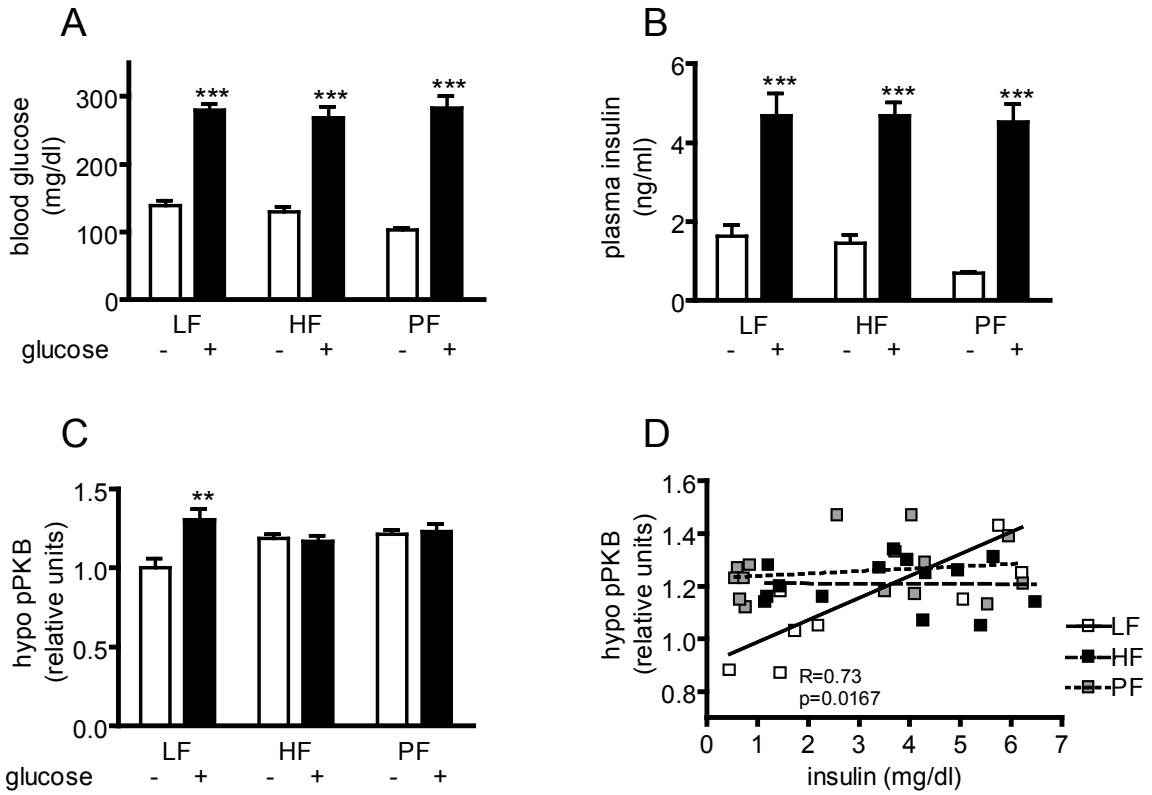


Figure 13: High-Fat Feeding, Independent of Increased Caloric Intake, Induces Hypothalamic Insulin Resistance.

Effect of pair-feeding on glucose-stimulated hypothalamic insulin signaling. A. Effect of dietary fat content on blood glucose, B. plasma insulin, and C. PKB activity following 15-min vehicle (white bars) or glucose (3 g/kg lean mass; black bars) treatment administered intraperitonally. D. Relationship between plasma insulin levels and hypothalamic PKB activity. Error bars represent the SEM. Student's t-test was used to determine significance for comparison within diet treatment groups in panels A-C. Pearson's correlation was used to determine significance in panel D. ** $p<0.01$, *** $p<0.001$, n=6-7 per group.

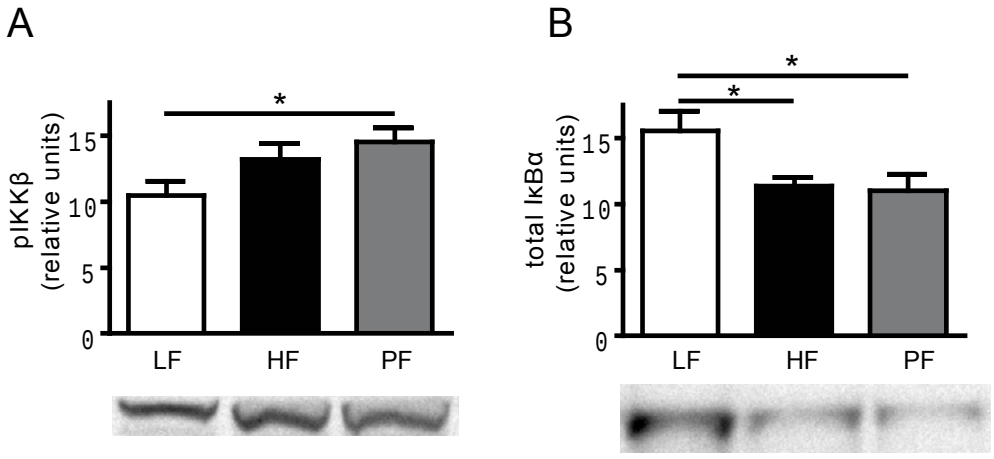


Figure 14: High-Fat Feeding, Independent of Increased Caloric Intake, Activates Hypothalamic Inflammatory Signaling Pathways

Effect of pair-feeding on hypothalamic IKKβ activity. Pair-feeding resulted in increased hypothalamic phosphorylation of IKKβ (A) and decreased total IκBα content (B) following 4wks of diet treatment. Error bars represent the SEM. One-way ANOVA with Tukey's post-hoc analysis was used to assess significance between groups * $p<0.05$, n=13-14 per group.

as indicated by a ~30% increase in pIKKβ and ~30% reduction in total IκBα levels compared to LF fed controls (Figure 14A,B; $p<0.05$ in panel B only). A similar effect was observed in the PF group in which consumption of a high-fat diet, even when limited to the caloric intake of the LF group, was associated with a significant increase in pIKKβ and reduction in total IκBα levels (Figure 14A,B; PF vs LF $p<0.05$).

Discussion

It is well known that diets high in fat induce obesity and insulin resistance in rodents and humans. Evidence suggests that the ability of high-fat feeding to induce these effects is dependent upon both the amount and type of fatty acids in the diet [190]. In these studies the saturation dependent effects of long-chain fatty acids on whole body energy homeostasis were investigated.

Long-Evans rats fed a high-fat diet enriched in long-chain saturated, but not unsaturated, fatty acids were characterized by significantly elevated body weight and body adiposity compared to low-fat fed rats after 10 wks of diet treatment. These findings suggest that, whereas long-chain saturated fatty acids promote weight gain associated with high-fat feeding, long-chain unsaturated fatty acids protect against high-fat diet-induced weight gain. Furthermore, there appeared to be a graded effect of the long-chain unsaturated high-fat diets on adiposity such that as the degree of unsaturation increased, adiposity decreased. Notably, animals fed the omega-3 enriched high-fat diet gained the least amount of fat mass of all the high-fat diet fed groups suggesting that the location of unsaturated bonds also determines the obesogenic potential of specific fatty acids.

A current model of energy homeostasis proposes that body adiposity is regulated by the hormones insulin and leptin [163]. Both hormones function as negative feedback signals to reduce food intake and increase energy expenditure through actions mediated

by key brain regions involved in energy homeostasis [163]. In the setting of positive energy balance (weight gain, energy intake in excess of expenditure), this model predicts that increased circulating insulin and leptin levels elicit neuronal responses that reduce food intake, increase energy expenditure, and protect against increases in fat mass. Indeed, this integrated response is well-described in lean animals [164], but appears to be disrupted in common forms of human obesity and in rodent models of high-fat diet-induced obesity. In these forms of obesity, increased fat mass is accompanied by elevated plasma insulin and leptin levels, yet, food intake remains normal or elevated [102] and energy expenditure is hypothesized to be reduced for the degree of adiposity. This suggests that DIO is characterized by functional hypothalamic resistance to insulin [52] and leptin [126]; which in turn, contributes to pathological weight gain and adiposity.

In these studies, elevated plasma leptin levels were accompanied by significant hyperphagia (excess caloric intake) in the HF group compared to the LF control group indicating functional hypothalamic resistance to leptin. However, the high-fat diets enriched in unsaturated fatty acids did not induce a significant increase in fasting plasma leptin levels compared to the LF group and the hyperphagic response was moderately attenuated compared to the HF group. In addition, feed efficiency values for the high-fat diets enriched in unsaturated fatty acids were significantly lower than the feed efficiency value of the HF diet containing saturated fat. This suggests that energy expenditure is relatively elevated in these groups and that unsaturated fatty acids may promote the oxidation of excess energy. This concept is supported by evidence that the structure of fatty acids appears to affect the degree of oxidation and deposition of fats. Animal and human studies suggest that some fatty acids are prone to oxidation while others lead to fat storage. For instance, evidence in the literature indicates that poly-unsaturated fatty acids have higher oxidation rates than saturated fatty acids [42] and produce a greater

thermogenic effect [179], increase in oxygen consumption [166] and higher sympathetic nervous stimulation [113] compared to saturated fatty acids due to both direct and indirect effects of fatty acids on lipid metabolism and lipid metabolic genes. Furthermore, i*n vivo* and *in vitro* studies have shown that poly-unsaturated, but not mono-unsaturated or saturated fatty acids, suppress the expression of lipogenic genes [206]. This suggests that the consumption of unsaturated fatty acids increases energy expenditure to compensate for the increase in caloric intake associated with high-fat feeding to maintain body weight at levels similar to low-fat fed rats. This counter-regulatory response suggests that hypothalamic insulin and leptin sensitivity remain, at least partially, intact in the setting of an unsaturated fat diet, but not a saturated fat diet. Together, these data suggest that long-chain saturated fatty acids are an important contributing factor in the development of hypothalamic resistance to the adiposity hormones after high-fat feeding.

Since animals placed on a high-fat diet are characteristically hyperphagic (excess energy intake) (our observations and, [101, 205]), they are consequently exposed to both excess calories and excess dietary fat. Although the previous data implicate long-chain saturated fatty acids as the most potent contributing factor in high-fat diet-induced obesity, the deleterious metabolic effects of high-fat feeding could arise from increased energy intake, increased dietary fat content, or a combination of both. To determine the contribution of excess caloric intake versus the intrinsic obesogenic properties of dietary fat to diet-induced obesity, I performed a pair-feeding study. Caloric restriction in the PF group completely abolished the weight gain observed with *ad libitum* high-fat feeding; suggesting that body weight is a direct corollary of caloric intake in these animals with no apparent effects on energy expenditure during this study. However, the PF group gained approximately 60% more fat mass than the LF group, which equaled approximately 80% of the amount of fat mass accrued by the HF group.

In addition, glucose tolerance was impaired with high-fat feeding independent of caloric intake. Taken together, pair-feeding nearly recapitulated the level of adiposity achieved in the free-feeding HF group and completely recapitulated the impaired glucose tolerance associated with high-fat feeding. These data indicate that dietary fats possess intrinsic obesogenic properties that significantly contribute to the deleterious metabolic effects of high-fat feeding independent of caloric intake.

Fatty acids are also known to modulate the production of cytokines and inflammatory responses. Saturated fatty acids are associated with pro-inflammatory effects, whereas unsaturated fatty acids are associated with anti-inflammatory effects [27, 192]. The pro-inflammatory signaling associated with the consumption of saturated fat is known to induce peripheral insulin resistance by promoting inhibitory serine phosphorylation of key elements within the insulin signaling pathway involving signal transduction through the IKKβ pathway (a serine/threonine kinase cascade [76, 168]). However, it has been unclear whether the effects of a high-fat diet enriched in long-chain saturated fat on IKKβ activity and insulin sensitivity were mediated by excess caloric intake or excess dietary fat.

Given the similarities in the insulin signal transduction pathway between central and peripheral tissues, I hypothesized that a similar mechanism of high-fat diet-induced peripheral insulin resistance occurs within the hypothalamus and asked whether it is mediated by excess caloric intake or excess dietary fat. In these studies, I observed insulin resistance in the hypothalamus of both calorically restricted and *ad libitum* high-fat fed animals as evidenced by loss of glucose-stimulated insulin signaling above basal levels. However, basal pPKB levels were moderately elevated in both HF and PF groups compared to LF controls. This basal increase in hypothalamic pPKB levels with HF feeding has been observed by others [185], but does not appear to couple with insulin mediated changes in food intake and energy homeostasis. This basal increase in

pPKB could potentially be due to insulin independent activation of the PI3K-PKB pathway as PI3K is a key regulatory protein involved in a variety of signaling cascades. For example, recall that the inflammatory TLR4 pathway also induces PI3K-PKB signaling associated with activation of the transcription factor NFκB (Chapter I). Nevertheless, insulin failed to stimulate further phosphorylation of PKB in hypothalami of HF and PF animals, and the increase in phosphorylation of PKB in response to insulin stimulation appears to determine insulin action. In addition to the hypothalamic insulin resistance observed with HF feeding, hypothalamic activity of the inflammatory molecule IKKβ, a serine/threonine kinase, was increased in both calorically restricted and *ad libitum* high-fat fed animals compared to LF fed controls. Taken together, HF feeding induced hypothalamic inflammation and insulin resistance irrespective of whether hyperphagia occurred, suggesting that dietary fats possess intrinsic obesogenic properties.

In summary, these studies demonstrated a graded effect of dietary fatty acids on adiposity, food intake, and feed efficiency in a saturation dependent manner and specifically implicate long-chain saturated fatty acids as the most potent mediator of the deleterious effects of high-fat feeding. Furthermore, a diet enriched in long-chain saturated fat, independent of excess caloric intake, is sufficient to induce impaired glucose tolerance, hypothalamic inflammation, and hypothalamic insulin resistance. These findings support the hypothesis that dietary fat possesses intrinsic obesogenic properties in a saturation dependent manner.

Table 2: Saturation Dependent Effects of Dietary Fat

	LF	HF	MU	PU	O3
Body wt (g)	479 ± 13	531 ± 27*	502 ± 20	497 ± 10	476 ± 12
Body wt gain (g)	159 ±10	214 ± 16*	171 ± 17	168 ± 3	151 ± 7
Adiposity (%)	13.3 ± 1.4	20.4 ± 2.8*	20.0 ± 1.3*	18.2 ± 1.2	14.8 ± 1.3
Fat mass gain (g)	46 ± 6	99 ± 19**	86 ± 10*	72 ± 5	54 ± 6
Leptin (ng/dl)	7.4 ± 1.0	20.3 ± 4.7**	14.5 ± 2.9	11.8 ± 1.8	6.7 ± 1.6
Leptin/adiposity	0.56 ± 0.03	0.96 ± 0.12*	0.70 ± 0.07	0.63 ± 0.06	0.41 ± 0.06
Total food intake (kcal)	4714 ± 94	5594 ± 263***	5281 ± 98*	5308 ± 72*	5175 ± 45
Feed efficiency (bw gain/kcal)	0.034 ± 0.002	0.037 ± 0.001	0.028 ± 0.001*,##	0.032 ± 0.000	0.029 ± 0.001##

Table 2: Metabolic parameters of rats following 10wks of diet treatment are reported as the group mean ± SEM. One-way ANOVA with Dunnett's Multiple Comparison Test was used to determine significance of all groups compared to a control group. * symbols represent comparison to LF group and # symbols represent comparison to HF group. One symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$, n=5-6 per group.

Table 3: Metabolic Effects of a Restricted High-Fat Diet

	LF	HF	PF
Total food intake (kcal)	1890 ± 44	2235 ± 59***	1906###
Body wt (g)	394 ± 5	408 ± 9	388 ± 4
Body wt gain (g)	96 ± 3	114 ± 5**	96 ± 3##
Adiposity (%)	14.3 ± 0.8	18.1 ± 1.0***	16.6 ± 0.6
Fat mass gain (g)	21.1 ± 2.4	40.2 ± 3.6***	33.0 ± 2.1#
AUC	6872 ± 566	8566 ± 553	10230 ± 920**
Basal glucose (mg/dl)	111 ± 9	120 ± 12	103 ± 10##

Table 3: Metabolic parameters of rats following 4wks of diet treatment are reported as the group mean ± SEM. One-way ANOVA with Tukey's post-hoc analysis was used to assess significance between groups. * symbols represent comparison to LF group and # symbols represent comparison to HF group. One symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$, n=13-14 per group.

CHAPTER IV

SATURATED FAT ACTIVATES INFLAMMATORY PATHWAYS AND IMPAIRS INSULIN SIGNALING DIRECTLY WITHIN THE HYPOTHALAMUS

Introduction

Diet-induced obesity is associated with hypothalamic resistance to the adiposity feedback signals insulin and leptin [52, 126]. A potential mechanism of insulin resistance in peripheral tissues proposes that intracellular accumulation of lipid metabolites such as long-chain fatty acyl-CoA molecules can activate inflammatory serine/threonine kinase signal transduction cascades involving IKKβ [169]. Activation of IKKβ is known to induce peripheral insulin resistance by promoting inhibitory serine phosphorylation of the insulin receptor and IRS proteins, thereby preventing the association and activation of PI3K and other downstream effects [211].

Findings from the previous chapter suggest that the saturated fatty acid component of a HF lard diet is the most potent mediator of the deleterious effects of high-fat feeding and is sufficient to induce hypothalamic inflammation (activation of IKKβ), and insulin resistance independent of excess caloric intake. The two primary fatty acid components of lard are palmitate, a saturated fat, as well as oleate, a monounsaturated fat. Thus, in this chapter I sought to determine whether the deleterious effects of high-fat feeding are specifically attributable to 1) saturated, and not unsaturated fat, and 2) direct action of saturated fat within the CNS on hypothalamic inflammation and insulin resistance. Specifically, whether exposure of the hypothalamus to excess saturated fat leads to hypothalamic accumulation of LC-CoAs that trigger inflammatory signaling (elevated IKKβ activity) and blunt insulin signaling in lean chow fed rats given an acute icv infusion of palmitate or oleate.

Results

ICV Infusion of Fatty Acids Leads to Hypothalamic Accumulation of LC-CoA, Activation of IKKβ, and Impaired Insulin Signaling

To determine whether the effects of high-fat feeding on the accumulation of hypothalamic LC-CoA content can be mimicked by an acute local elevation in the long-chain saturated fatty acid palmitate, palmitic acid was infused via icv into the third ventricle of the brain of rats maintained on a standard chow diet. Following the 4 hour icv infusion of palmitate (0.5 nmole/min, 120 nmoles total), the hypothalamic content of palmitoyl- and stearoyl-CoA (16:0 and 18:0 respectively), two saturated fatty acid species, and oleoyl-CoA (18:1), a mono-unsaturated fatty acid species, were significantly elevated compared to the control icv vehicle infusion group (Figure 15A; white vs black bars: palmitoyl-CoA 14.9±1.3 vs 21.4±1.7, $p<0.05$; stearoyl-CoA 16.3±1.4 vs 23.2±1.5, $p<0.01$; oleoyl-CoA 17.9±1.3 vs 25.7±1.1, $p<0.05$). In addition to accumulation of these hypothalamic LC-CoA fatty acid species; hypothalamic IKKβ activity was significantly elevated with icv palmitate infusion compared to vehicle controls (Figure 15B; ~15% increase in pIKKβ, $p<0.05$). Concomitant with increased LC-CoA content and elevated IKKβ activity with palmitate infusion, the ability of icv insulin to activate the downstream signaling molecule PKB was severely blunted with palmitate infusion compared to vehicle controls in which activation of PKB was readily detected (Figure 15C; veh-veh vs veh-ins 1.0±0.1 vs 1.8±0.2, $p<0.01$). To determine whether hypothalamic LC-CoA accumulation would occur in response to a long-chain mono-unsaturated fatty acid, oleic acid was also infused into the third ventricle of chow fed rats (4 hour infusion, 0.5 nmole/min, 120 nmoles total). Oleic acid infusion failed to significantly increase any of the fatty acid LC-CoA species measured above those in the control vehicle group (Figure 15A, grey bars).

IKKβ Inhibitor Represses Consumption of a High-Fat, but not a Low-Fat, Diet

While the previous data demonstrate a clear association between saturated fat induced IKKβ activity and insulin resistance, I sought to directly test the role of IKKβ in high-fat diet-induced hypothalamic insulin resistance. Recall that insulin acts as a catabolic hormone within the hypothalamus to reduce food intake and increase energy expenditure. However, food intake is not appropriately reduced in response to hyperinsulinemia associated with HF feeding indicative of hypothalamic insulin resistance. Therefore, to test the role of IKKβ activity on hypothalamic insulin action, a pharmacological inhibitor of IKK (PS-1145, 10 μg) was administered into the third ventricle of rats fed either a high-fat (45%) or low-fat diet and 24 hour food intake measured. As shown in Figure 16, this intervention had no effect on food intake in the LF group (animals that are characterized by relatively low hypothalamic IKKβ activity). In contrast, icv infusion of PS-1145 potently reduced 24 hour food intake in high-fat fed rats (~50% reduction vs HF-veh, $p<0.01$) characterized by hyperinsulinemia and increased hypothalamic IKKβ activity [146]. Efficacy of the inhibitor was confirmed in previous studies by measurements of a downstream IKKβ target and signaling molecule, IκBα. Activation of IKKβ leads to the phosphorylation and degradation of IκBα and subsequent activation of the transcription factor NFκB to propagate proinflammatory signaling thought to contribute to insulin resistance. Activation of IKKβ induces phosphorylation and targeted ubiquitination/degradation of IκBα, thereby reducing protein levels of IκBα. Inhibition of IKKβ would be expected to increase total IκBα protein. Accordingly, hypothalamic IκBα content is significantly increased with inhibitor treatment compared to vehicle controls (Appendix B; ~50% increase vs veh, $p<0.05$). In addition, a dose-dependent effect on food intake was observed with inhibitor treatment as a lower dose of PS1145 (3 μg icv) reduces 4 hour food intake, but not 24 hour food intake (Appendix B). Since food intake was not reduced in LF fed rats, the feeding

effects of PS-1145 do not appear to be due to visceral or non-specific illness. In support of this observation, inhibitor treatment did not increase kaolin consumption, a marker of visceral illness in rodents (Appendix C, [118]).

IKKβ Inhibitor Increases Insulin Signaling in High-Fat Fed, Obese Rats

To further assess the role of IKKβ activity on hypothalamic insulin action, I tested whether pretreatment with the IKK inhibitor would improve insulin stimulated hypothalamic insulin signaling in high-fat fed rats. IKKβ activity is proposed to inhibit the insulin signaling pathway; therefore, inhibition of the IKKβ activity would be expected to, at least partially, restore activation of insulin signaling. Pretreatment with the IKK inhibitor PS1145 (3 μg, 6 h prior to 10 mU icv insulin) in high-fat fed rats significantly increased insulin stimulated phosphorylation of PKB in the hypothalamus (Figure 17; 1.8±0.1 vs 1.4±0.1, $p<0.05$). However, pretreatment with the IKK inhibitor alone (minus insulin) had no affect on phosphorylation of PKB compared to high-fat fed rats without inhibitor pretreatment.

Discussion

Abundant evidence in animals and humans implicates both exogenous (lipid infusion) and endogenous (tissue long-chain acyl-CoAs) fatty acids in the pathogenesis of insulin resistance induced by activation of intracellular inflammatory signals. For example, long-chain acyl-CoA content and IKKβ signaling are elevated in skeletal muscle sampled from morbidly obese, insulin-resistant humans [79]. In addition, exposure of cultured cells to palmitate induces insulin resistance via activation of the IKKβ/NF-κB signaling pathway [171]. A current model of peripheral insulin resistance suggests that lipid mediated activation of pro-inflammatory signaling molecules may contribute to impaired insulin signaling in the obese state. Previous observations from

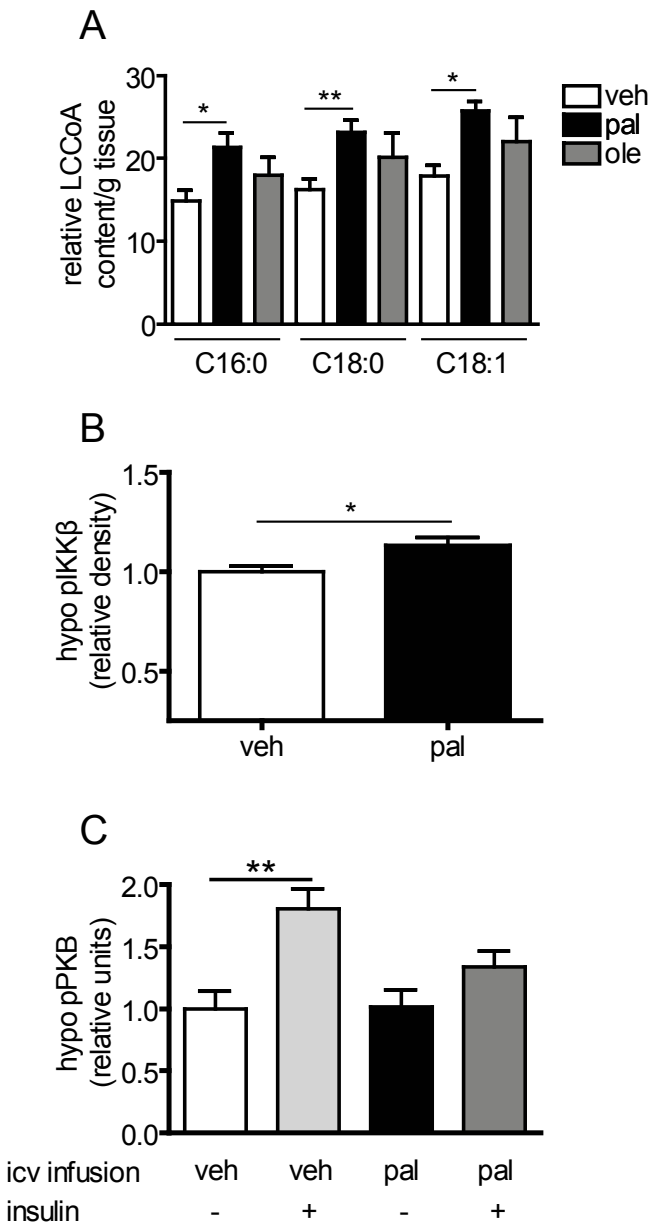


Figure 15: Effect of Intracerebroventricular Fatty Acid Infusion on Hypothalamic Long-Chain Acyl-CoA Content, IKKβ Activity, and Insulin Signaling

A. ICV infusion of palmitic acid (black bars), but not oleic acid (grey bars), leads to increased hypothalamic content of LC-CoA molecules compared to vehicle controls (white bars). B. ICV infusion of palmitic acid increases hypothalamic pIKKβ compared to vehicle controls and C. blunts insulin stimulated pPKB in the hypothalami of chow fed Long Evans rats. Error bars represent the standard error of the mean (SEM). One-way ANOVA with Dunnett's Multiple Comparison Test was used to determine significance of icv infusion groups compared to the vehicle control group for each Acyl-CoA (panel A). Student's t-test was used to determine significance in panels B (vehicle vs palmitate) and C (insulin vs vehicle treatment within each icv infusion group). * $p<0.05$, ** $p<0.01$, n=6-8 per group.

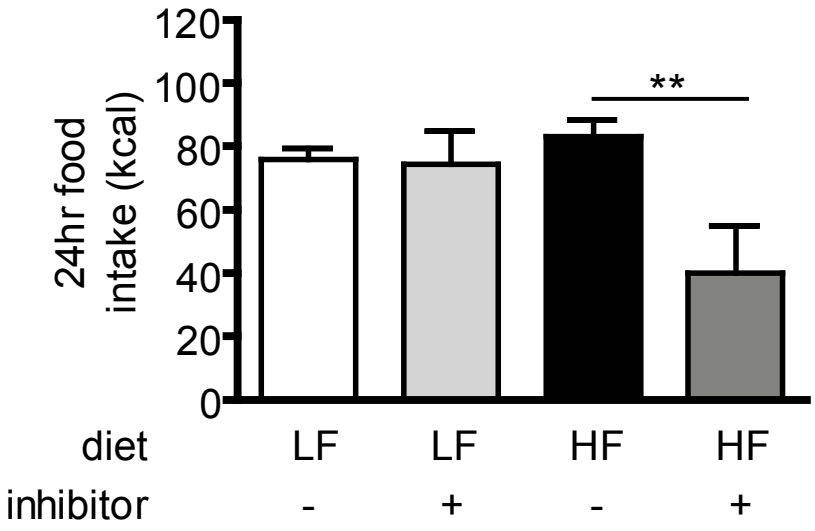


Figure 16: Effect of Pharmacological IKK Inhibitor on Food Intake

Pharmacological inhibition of hypothalamic IKK (icv, 10ug PS-1145) represses food intake in HF, but not LF, fed rats. Error bars represent the standard error of the mean (SEM). Student's t-test was used to determine significance between inhibitor and vehicle treatment on food intake within each diet group. ** $p<0.01$, n=5-8 per group.

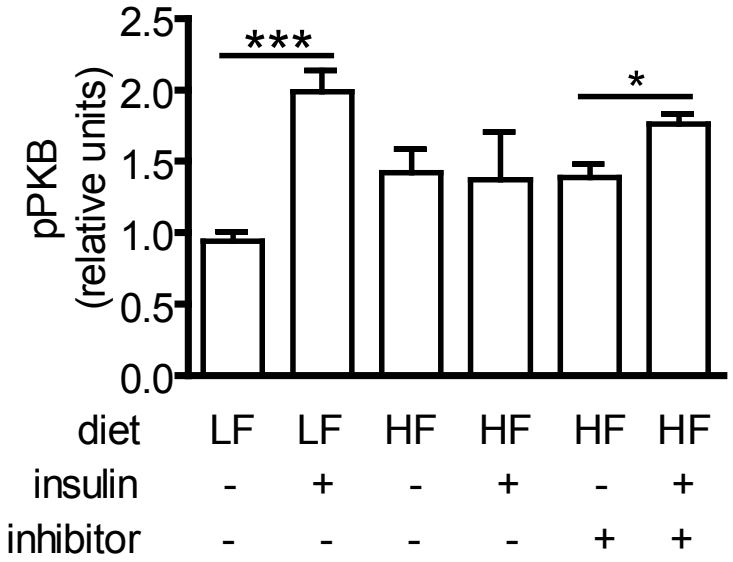


Figure 17: Effect of Pharmacological IKK Inhibitor on Hypothalamic Insulin Signaling in HF Fed, Obese Rats.

Pharmacological inhibition of hypothalamic IKK enhances insulin-stimulated pPKB in HF fed, obese rats. Error bars represent the standard error of the mean (SEM). Two-tail student's t-test was used to assess significance between vehicle and insulin treated groups. * $p<0.05$, *** $p<0.001$, n=4-5 per group.

our laboratory and others [52] suggest that a similar mechanism of high-fat diet-induced insulin resistance may also occur in the hypothalamus of obese animals. Thus, I sought to extend my initial findings in high-fat diet-induced obesity to determine the ability of fatty acids administered directly into the brain of lean, chow fed animals to induce hypothalamic insulin resistance via a similar mechanism.

Long-term high-fat feeding is associated with the accumulation of saturated, but not unsaturated, long chain fatty acyl-CoAs (LC-CoAs) within the hypothalamus of obese rats [146]. To determine if saturated fatty acids mediate the increase in hypothalamic LC-CoA content observed with HF feeding, I investigated the effects of icv palmitate infusion in lean rats maintained on a standard chow diet. Palmitate infusion mimicked the effects of high-fat feeding to increase hypothalamic content of the saturated LC-CoA species, palmitoyl- and stearoyl-CoA, but unlike HF feeding also increased levels of the unsaturated fatty acid, oleoyl-CoA. That oleoyl-CoA was also increased with icv palmitate infusion, but not HF feeding, suggests that the hypothalamic accumulation of LC-CoA under normal conditions (i.e. HF feeding) may also involve indirect effects of dietary fat on hypothalamic lipid metabolism. In keeping with our hypothesis that saturated fatty acids specifically increase hypothalamic LC-CoA content, infusion of the monounsaturated fat, oleate, failed to induce a significant increase in any of the LC-CoA species measured compared to vehicle controls. The accumulation of hypothalamic LC-CoA is hypothesized to lead to activation of inflammatory signaling cascades involving IKKβ.

Previous work from our laboratory has demonstrated that high-fat diet-induced obesity is associated with both elevated LC-CoA content and activation of IKKβ in the hypothalamus [146]. This effect was recapitulated with icv palmitate infusion in lean, chow fed rats suggesting that saturated fatty acids are involved in the inflammatory response induced by high-fat feeding. Activation of IKKβ can induce inhibitory serine

phosphorylation of components of the insulin signaling pathway and impair signal transduction. This mechanism has been implicated in both the peripheral and central insulin resistance associated with HF DIO. Concomitant with increased LC-CoA content and elevated IKKβ activity, icv palmitate infusion in lean rats maintained on a standard chow diet significantly blunted insulin signal transduction in the hypothalamus compared to vehicle controls in which activation of PKB was readily detected. However, in contrast to the effects of long-term HF feeding on phosphorylation of PKB, palmitate infusion did not recapitulate the elevation in basal pPKB. This suggests that chronic exposure to saturated fat is required to increase basal pPKB and impair insulin action via a mechanism similar to long-term depression as discussed in Chapter III, whereas acute exposure to saturated fat directly impairs insulin signal transduction. This raises the possibility that dietary fat impairs hypothalamic insulin action via two distinct mechanisms. Together, these observations support a model in which consumption of a diet high in saturated fat contributes to the development of CNS insulin resistance possibly via a mechanism involving either elevated circulating FFA levels or increased de novo tissue synthesis of saturated fatty acids resulting in accumulation of LC-CoAs in hypothalamic neurons. Furthermore, the finding that hypothalamic IKKβ signaling increases in response to an acute icv infusion of palmitate supports my hypothesis of a direct link between exposure to excess saturated fats and inflammatory signling within the hypothalamus. While these data demonstrate a clear association between fatty acid induced IKKβ activity and insulin resistance in the hypothalamus, they do not establish a direct role of IKKβ in the development of fatty acid induced hypothalamic insulin resistance.

As a direct link between IKKβ activity and insulin resistance has been demonstrated in peripheral tissues [36, 208]; I sought to establish a similar relationship in the hypothalamus of HF fed obese rats compared to LF fed lean controls using a

pharmacological inhibitor of IKK. Since HF feeding significantly increases hypothalamic IKKβ activity and IKKβ activity is proposed to impair hypothalamic insulin action; inhibition of the IKKβ signal would be expected to, at least partially, restore both the behavioral and biochemical effects of insulin action. Recall that insulin acts as a catabolic hormone within the hypothalamus to reduce food intake and increase energy expenditure; effects that are blunted in HF DIO potentially via IKKβ mediated insulin resistance. Accordingly, pharmacological inhibition of hypothalamic IKK potently reduced food intake in HF fed rats characterized by increased IKKβ activity and hypothalamic insulin resistance, but had no effect on food intake in LF fed rats characterized by relatively low IKKβ activity and hypothalamic insulin sensitivity. In fact, IKKβ inhibition reduced food intake in HF fed group to levels below that of LF fed animals. A possible explanation is that HF fed animals are characterized by hyperinsulinemia and hyperleptinemia. If IKKβ activity mediates resistance to these hormones, removal of this signal will permit insulin and leptin signaling and reduce food intake in proportion to the signal (greater than in LF group). In addition, pretreatment with the IKK inhibitor in HF fed rats modestly, but significantly, increased insulin stimulated activation of PKB in the hypothalamus of rats compared to insulin stimulation alone. Together these data support a direct role of IKKβ in HF diet-induce insulin resistance.

In conclusion, these studies demonstrated that icv infusion of a saturated fatty acid (palmitate), but not an unsaturated fatty acid (oleate) recapitulated the effects of the HF lard-based diet to increase hypothalamic LC-CoA content and induce hypothalamic inflammation and insulin resistance. In addition, these studies demonstrate that activation of IKKβ contributes to increased food intake and insulin resistance associated with HF DIO. These results support a model in which cellular exposure to excess nutrients, specifically saturated fat, triggers cellular inflammation and insulin resistance

that in turn contributes to the pathogenesis of obesity. The ability of specific fatty acids to modulate the activation of inflammation may represent a potential mechanism of high-fat diet-induced hypothalamic resistance and obesity. These saturation dependent effects led me to identify potential “fat sensing” target molecules that may be involved in the development of HF diet-induced obesity.

CHAPTER V

DEFICIENCY OF THE PRO-INFLAMMATORY SIGNALING MOLECULE, TLR4, REDUCES BODY WEIGHT AND ADIPOSITY

Introduction

Obesity and Type II Diabetes Mellitus are characterized by central and peripheral insulin resistance that in many cases is accompanied by a low-grade chronic inflammatory state [38, 55, 159, 204]. Current models of high-fat diet-induced obesity suggest that pro-inflammatory signaling molecules, when activated, can negatively impact the insulin signaling cascade to induce insulin resistance in central and peripheral tissues [52, 168, 169]. Although mechanisms linking dietary fat to pro-inflammatory signaling in these tissues is poorly understood, a recent link has been made between fatty acids implicated in high-fat diet-induced obesity and the toll-like receptor 4 (TLR4).

The toll-like receptor family members are expressed by cells as part of the innate immune system and are linked to the promotion of pro-inflammatory cytokines [4, 81]. Lipopolysaccharide (LPS) of gram negative bacteria was identified as the original ligand of TLR4 [145]; specifically, the lipid A component of LPS, which is composed of acylated saturated fatty acids. This saturated acid component is responsible for ligand recognition and receptor activation of TLR4, as removal of this component results in complete loss of LPS activity [125, 143]. Additional studies have demonstrated that long chain saturated fatty acids, independent of LPS, can function as a ligand and induce TLR4 activity along with downstream pro-inflammatory signaling; whereas certain unsaturated fatty acids act as antagonists of TLR4 signaling [98, 99]. Given that most tissues of the body, including the brain and insulin-sensitive tissues, express TLR4 [34,

95, 131], TLR4 is an intriguing target for a mechanism linking dietary derived fatty acids, pro-inflammatory signaling, and insulin resistance.

Elucidation of the TLR4 signaling pathway revealed that activation of IKKβ and the IKK complex promoted the subsequent translocation of the transcription factor NFκB to the nucleus where it regulates expression of several pro-inflammatory cytokines [109]. The IKKβ/NFκB pathway has been implicated in the development of insulin resistance via both direct and indirect effects [167]. IKKβ can directly impair the insulin signaling pathway via inhibitory serine phosphorylation of the insulin receptor and IRS proteins thereby preventing the association and activation of PI3K and other downstream effects as discussed in chapter IV [211]. In addition, activation of NFκB can indirectly impair insulin action via transcription of pro-inflammatory cytokines, such as TNFα and IL-6, both of which are known inducers of insulin resistance [36, 78]. Data from our laboratory and presented here have defined a role of IKKβ centrally in HF DIO, and more specifically a diet enriched in long-chain saturated fat (an endogenous TLR4 agonist). Activation of IKKβ through the TLR4 signaling pathway induced by extracellular saturated fatty acids, may thus contribute to the development of high-fat diet-induced insulin resistance.

The role of TLR4 in high-fat diet-induced obesity and insulin resistance has been studied in several models of TLR4 deficiency and in various peripheral tissues [142, 165, 184]. These studies support a link between TLR4 and high-fat diet-induced peripheral insulin resistance via activation of inflammatory signals. However, lacking from these original studies was discussion of a potential role for TLR4 in central insulin signaling and regulation of energy homeostasis. Since TLR4 is 1) expressed in central tissues associated with the regulation of energy homeostasis, 2) is regulated by dietary fat in a saturation dependent manner, and 3) initiates an inflammatory signaling cascade associated with insulin resistance; it is a likely candidate in the mechanism of high-fat

diet-induced hypothalamic resistance and obesity. It seems plausible that activation of TLR4 by dietary fatty acids may also induce hypothalamic resistance via a similar inflammatory mechanism as diet-induced obesity. Whether dietary fat activates inflammatory molecules via TLR4 signaling in the hypothalamus to induce insulin resistance and impair energy homeostasis is therefore an important unanswered question. I hypothesize that loss of TLR4 will ameliorate high-fat DIO, in part, via centrally mediated mechanisms.

Results

Mouse Model of TLR4 Deficiency

I utilized a mouse model of TLR4 deficiency to study the effects of TLR4 signaling on high-fat diet-induced obesity. Mice originally identified as unresponsive to LPS were determined to lack the gene encoding TLR4 (C57BL/10ScNJ mice, T4). The absence of the TLR4 gene in these mice was confirmed by assessing the inflammatory response to LPS, an agonist of TLR4, and quantitative determination of mRNA levels. LPS treatment failed to induce cytokine expression as measured by IL-6 levels and TLR4 mRNA was undetectable in T4 mice (Figure 18 and 19 respectively). Baseline plasma IL-6 levels were below assay detection (< 7.8 pg/ml) in both wild-type (WT) and T4 male mice (Figure 18A). Following a single intraperitoneal injection of LPS plasma cytokine levels of IL-6 were dramatically elevated in WT mice compared to vehicle controls (3500±585 pg/ml). This effect was almost completely abolished in T4 mice (160±30 pg/mg) and plasma IL-6 levels were significantly reduced compared to LPS treated WT mice ($p<0.001$; Figure 18A). LPS treatment induced a significant increase in liver IL-6 production in WT mice compared to vehicle controls (Figure 18B, # symbols; WT-veh vs WT-LPS 4600±190 vs 3100±360 pg/ml, $p<0.01$), but had no effect in T4 mice (T4-veh vs T4-LPS 1600±180 vs 1900±150 pg/ml, p=ns). Furthermore, the levels of IL-6

in livers of T4 mice were significantly lower than respective WT controls independent of LPS treatment (Figure 18B, * symbols; WT-veh vs T4-veh, $p<0.01$; WT-LPS vs T4-LPS $p<0.001$). Although LPS administered peripherally did not increase local IL-6 production within the hypothalamus in either WT or T4 mice (Figure 18C; veh vs LPS, p=ns), hypothalamic IL-6 levels were significantly lower in T4 mice compared to WT controls independent of LPS treatment (T4 vs WT, vehicle groups, 86±11 vs 160±8 pg/mg, $p<0.01$; LPS groups, 89±9 vs 140±23 pg/mg, $p<0.01$). In addition, TLR4 mRNA levels were measured by real-time RT- PCR in liver samples of male and female mice. TLR4 message was detected in all WT mice, but not in T4 mice, confirming our model of TLR4 deficiency (Figure 19A,B). In addition, diet had no appreciable effect on TLR4 expression.

Effect of TLR4 Deficiency on Body Weight and Composition

As demonstrated in Chapter III, diets enriched in long-chain saturated fat promote obesity. In addition these fats are also associated with chronic inflammation in both rodents and humans [68]. Since saturated fatty acids are known to activate TLR4 and downstream inflammatory signaling pathways associated with insulin resistance and obesity, I sought to determine whether deletion of TLR4 would protect against high-fat diet-induced obesity. Male and female WT and T4 mice were placed on either a low-fat (LF; 10%kcal from fat, Table 1) or high-fat (HF; 45%kcal from fat, Table 1) diet at 4 wks of age and followed for 10 wks. All metabolic data values are listed in Table 4 and 5 (male and female data, respectively; p 105-6). Body weight curves and end-point analyses are shown for both male and female mice (Figure 20A-H). Male and female TLR4 deficient mice weighed significantly less than respective WT controls on the same diet over the course of the 10 week study (Figure 20A,B). However, no overt growth abnormalities were apparent in T4 male or female mice as body weight gain was similar

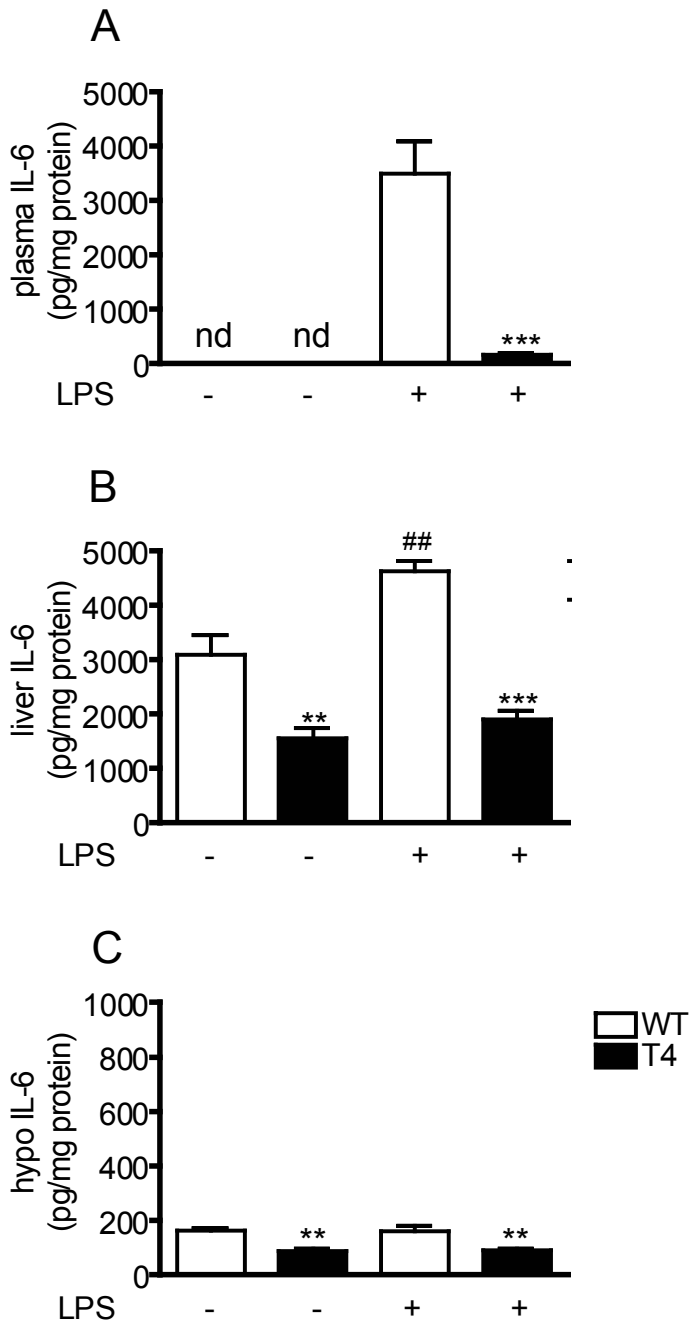


Figure 18: Mouse Model of TLR4 deficiency: LPS Response

Plasma (A), liver (B), and hypothalamic (C) levels of the inflammatory cytokine IL-6 following intraperitoneal injection of vehicle or LPS (12.5µg, 1 hour), a TLR4 agonist, in WT (white bars) and TLR4 deficient male mice (T4, black bars). Error bars represent the standard error of the mean (SEM). One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine significance between selected pairs. * symbols represent WT vs T4 comparisons within treatment groups, and # symbols represent veh vs LPS comparisons within each genotype. Two symbols $p<0.01$, three symbols $p<0.001$, n=4 per group.

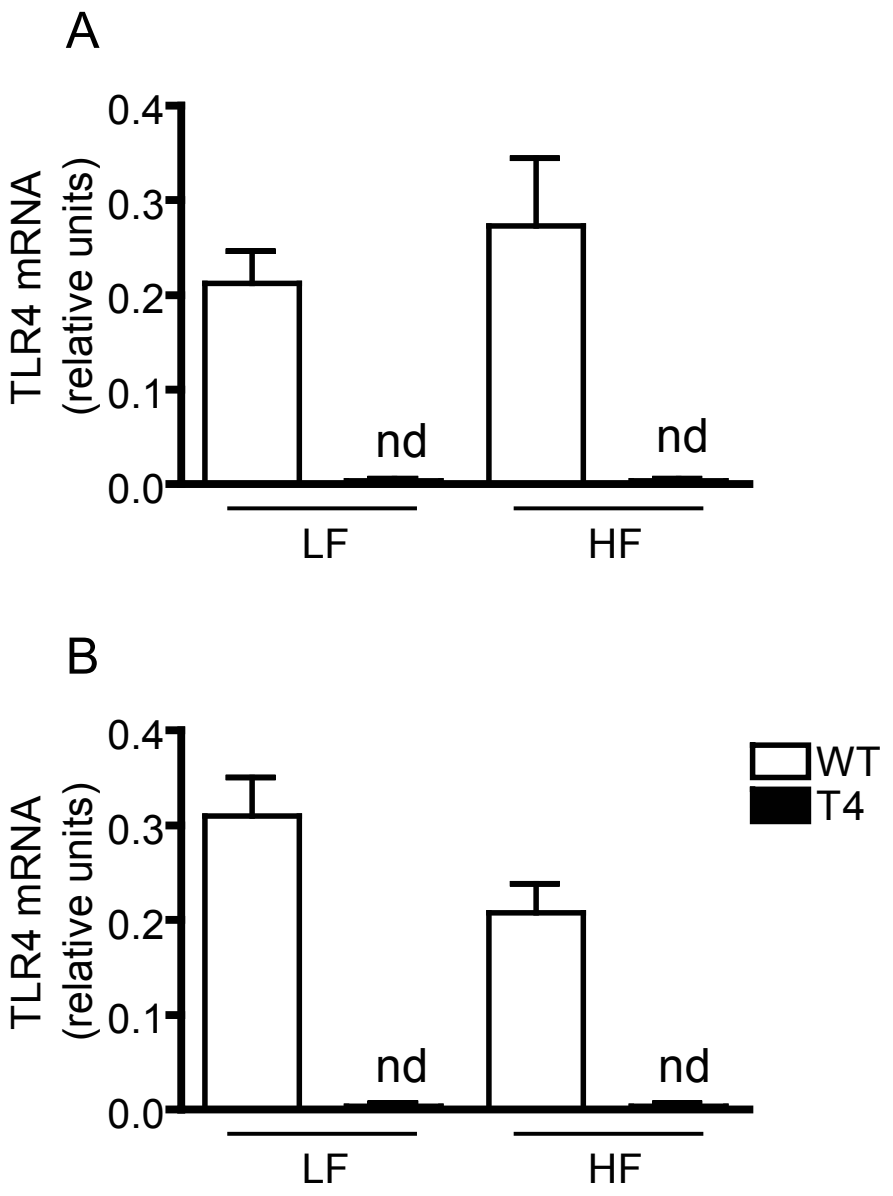


Figure 19: Mouse Model of TLR4 deficiency: TLR4 mRNA

Relative hepatic expression of TLR4 mRNA is shown for both male (A) and female (B) mice. TLR4 mRNA levels were undetectable in all T4 groups (black bars, no statistical analyses performed comparing WT vs T4). In addition, 10 wks of diet treatment had no effect on TLR4 expression in WT mice. Error bars represent the SEM. Student's t-test was used to determine significance comparing LF vs HF diet treatment in WT mice. n=4-5 per group.

to WT controls regardless of diet treatment (Figure 20C,D respectively). Body composition analyses were performed to determine whether TLR4 deficiency altered adiposity or merely resulted in a "smaller" body plan. After 10 wks of diet, T4 mice were leaner and gained less fat mass than WT controls in both male and female mice (Figure 20E-H, T4 vs WT within diet treatments, all comparisons significant except LF female group). High-fat feeding significantly increased adiposity in WT mice compare to LF fed controls (WT-LF vs WT-HF; males $p<0.001$, females $p<0.001$). High-fat feeding induced a similar increase in adiposity of male T4 mice (50% vs 55% in WT mice) although absolute percent adiposity was significantly lower, whereas high-fat feeding had no effect on adiposity in female T4 mice. Female T4 mice were completely protected from HF diet-induced obesity.

Effect of TLR4 Deficiency on Food Intake and Energy Expenditure

Body weight and adiposity are regulated by the relative balance between food intake and energy expenditure through the process of energy homeostasis. To determine whether the differences in body weight and adiposity observed in TLR4 deficient mice could be attributed to a disruption of energy homeostasis, food intake and energy expenditure were assessed in these mice. Food intake data was monitored weekly and reported as cumulative caloric intake (Figure 21). TLR4 deficiency had no effect on total food intake compared to WT controls except in LF fed female mice in which food intake was slightly reduced (T4-LF vs WT-LF, ~10% lower, $p<0.05$). In addition, TLR4 deficiency had no effect on high-fat diet-induced hyperphagia (excess caloric intake). Food intake was similarly elevated in WT and T4 mice compared to LF fed controls (~15% increase in male mice, ~20-25% increase in female mice).

Feed efficiency was calculated as an estimate of energy expenditure and is shown in Figure 22. The rationale for estimating energy expenditure from feed efficiency

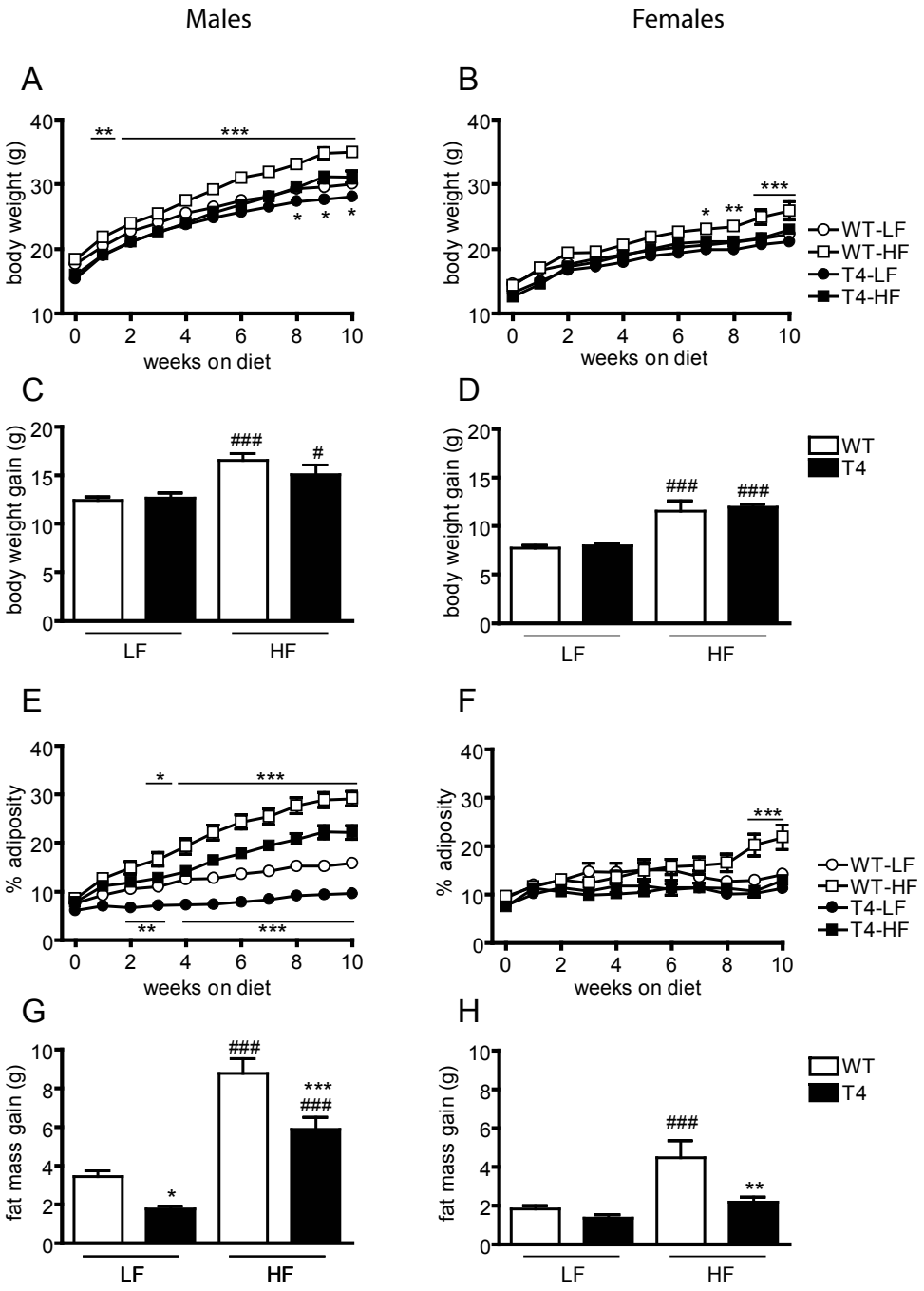


Figure 20: Effect of TLR4 Deficiency on Body Weight and Composition

Body weight and adiposity measurements for male and female WT and TLR4 deficient. Error bars represent the SEM. Two-way ANOVA with Bonferroni post-tests was used to determine significance in panels A-B, E-F. Symbols above the lines represent WT vs T4 on HF diet, symbols below the lines represent WT vs T4 on LF diet. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine significance in panels C-D, G-H. * symbols represent WT vs T4 within diet, and # symbols represent LF vs HF within genotype. One symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$, n=13-20 per group in male mice, n=8-11 per group in female mice.

calculations was discussed in detail in Chapter III. Briefly, feed efficiency is calculated as the amount of weight gained per kilocalorie consumed (wt gain/kcal) and indicates how effectively the body stores nutrients and accrues mass (as opposed to utilizing nutrients for energy production). Since body weight gain and food intake are known, relative energy expenditure can be inferred based on the energy balance equation. No differences were observed in feed efficiency between WT and T4 male mice within the diet groups suggesting that TLR4 deficiency does not affect energy expenditure in male mice. Furthermore, diet did not significantly affect feed efficiency in male mice (HF vs LF, p=ns). In female mice, TLR4 deficiency did not significantly alter feed efficiency (WT vs T4, within diet comparisons p=ns), but HF diet significantly increased feed efficiency in both WT and T4 mice compared to their respective LF fed counterparts (HF vs LF, ~20% increase in WT and T4).

While feed efficiency addresses body weight gain and energy expenditure, a related calculation addresses body composition and energy storage. Calculated as fat mass gain divided by kilocalories consumed this value, termed energy efficiency, indicates how effectively the body stores nutrients in the form of adipose stores (Figure 22C,D). Essentially, a relative increase in energy efficiency means that per calorie consumed, more adipose tissue is accrued. In male mice, high-fat feeding induced a significant increase in energy efficiency in both WT and T4 male mice compared to their LF fed counterparts (Figure 22C; LF vs HF, $p<0.001$ in WT and T4). However, relative to WT controls, energy efficiency was significantly reduced in T4 male mice fed both diets (Figure 22C; T4 vs WT, ~50% decrease in LF $p<0.05$, and ~33% decrease in HF $p<0.001$). In female mice, high-fat feeding induced a significant increase in energy efficiency in WT mice (Figure 22D; HF vs LF, ~50% increase $p<0.01$), but had no effect

in T4 mice. Relative to WT controls, energy efficiency was significantly reduced in HF fed T4 female mice (Figure 22D; T4 vs WT, ~50% decrease $p<0.01$).

To confirm the estimates of energy expenditure derived from the feed efficiency calculations above, indirect calorimetry and activity measurements were collected before and after 10 wks of HF feeding in a cohort of male mice (Figure 23A-C). Indirect calorimetry provides an estimate of basal energy expenditure, calculated as heat production from measurements of oxygen consumption and carbon dioxide production (by-products of energy metabolism). Heat production was normalized to lean mass as lean mass is considered to be metabolically active tissue accounting for the majority of energy expenditure, as well as to account for differences observed in body weight and body composition attributed to genotype and/or diet effects. In accordance with feed efficiency calculations in male mice above, heat production (i.e. energy expenditure) was similar across all groups (Figure 23A). In addition, no significant differences in locomotor activity (# of beam crosses) were observed between WT and T4 mice, or between baseline and 10 wks of HF diet (Figure 23B). Respiratory quotient (RQ) is an index of substrate utilization calculated as the ratio of carbon dioxide production to oxygen consumption. Based on oxidation equations for glucose and fat metabolism, a RQ value of 1 indicates carbohydrate utilization, whereas a RQ value of 0.7 indicates fatty acid utilization. The closer the RQ value to 1, the greater the proportion of carbohydrates metabolized for energy, whereas the closer the RQ value to 0.7, the greater the proportion of fatty acids metabolized for energy. No differences were observed in the RQ values of WT and T4 mice at baseline or after 10 wks of high-fat feeding indicating that TLR4 deficiency does not preferentially alter substrate utilization. However, 10wks of high-fat feeding significantly reduced RQ in WT and T4 mice compared to baseline levels (Figure 23C; HF vs LF, $p<0.001$ for both WT and T4).

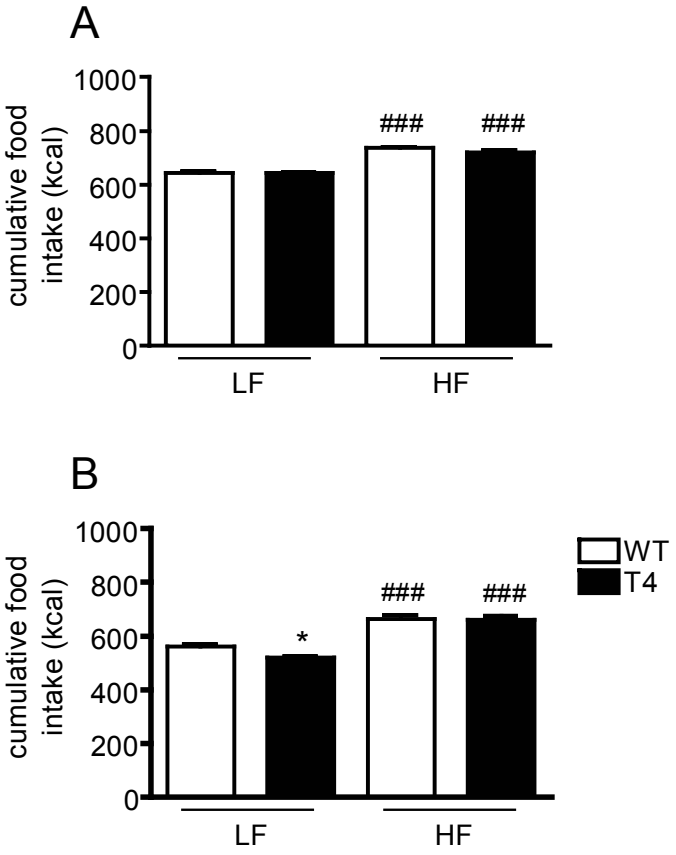


Figure 21: Effect of TLR4 Deficiency on Food Intake

Cumulative food intake in male (A) and female (B) mice. Error bars represent the SEM. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine significance between selected pairs. * symbols represent WT vs T4 within diet, and # symbols represent LF vs HF within genotype. One symbol $p<0.05$, three symbols $p<0.001$, n=13-20 per group in male mice, n=8-11 per group in female mice.

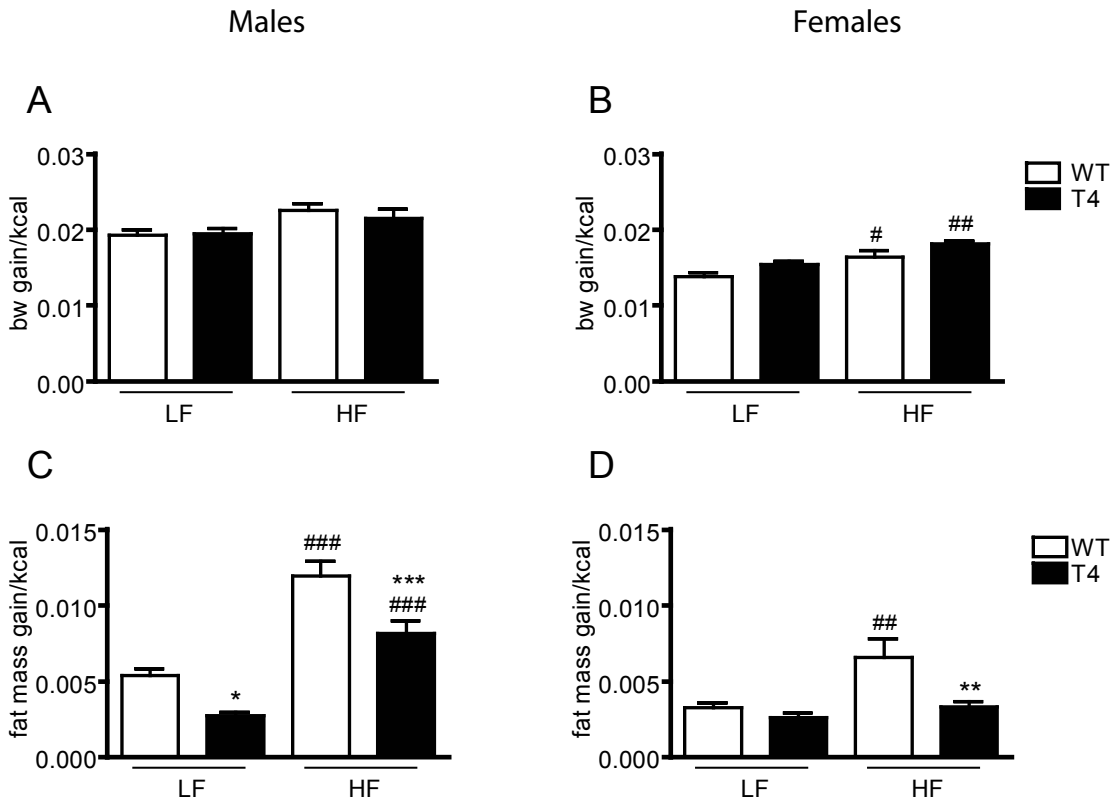


Figure 22. Effect of TLR4 Deficiency on Feed and Energy Efficiency

Feed efficiency was calculated as an index of energy expenditure in male (A) and female (B) mice. A related calculation, energy efficiency, is an index of obesogenic potential in male (C) and female (D) mice. Error bars represent the SEM. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine significance between selected pairs. * symbols represent WT vs T4 within diet, and # symbols represent LF vs HF within genotype. One symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$, n=13-20 per group in male mice, n=8-11 per group in female mice.

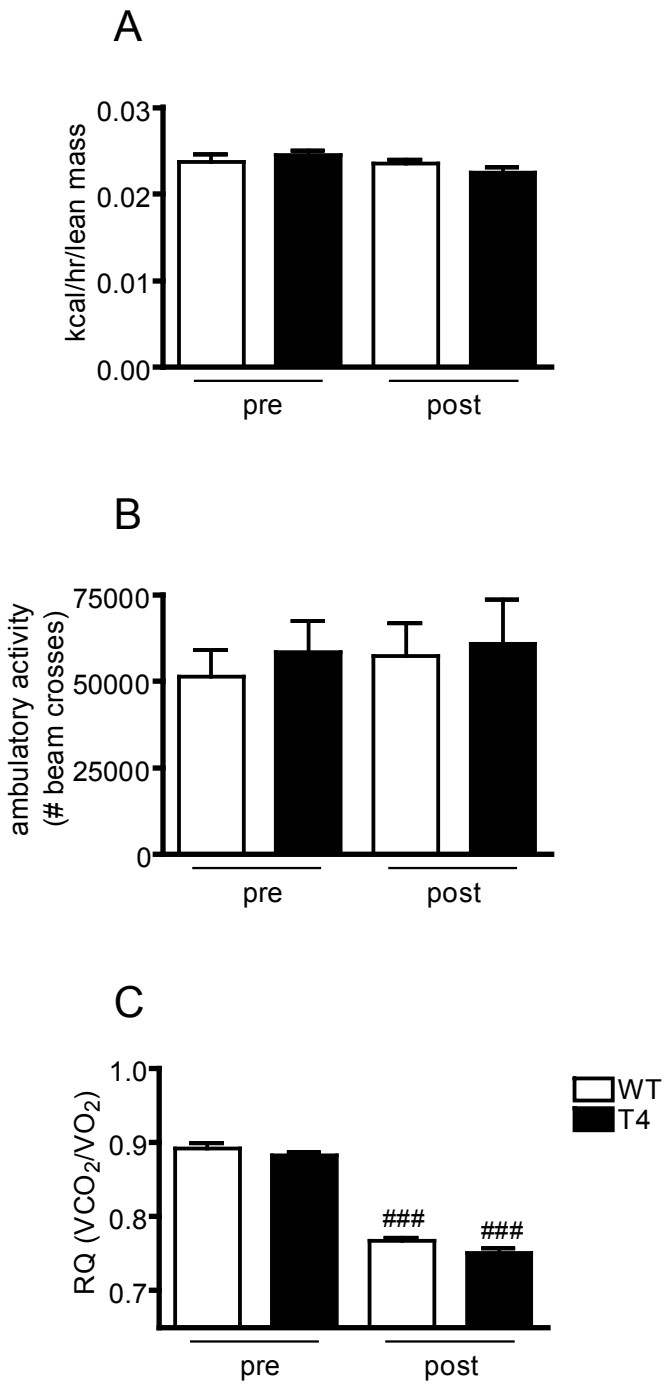


Figure 23: Effect of TLR4 Deficiency on Energy Expenditure

Indirect calorimetry was used to assess energy expenditure in male mice before and after 10 wks of HF feeding. Heat production, normalized to lean mass (A), ambulatory activity (B), and respiratory quotient (C) values were measured in these mice. Error bars represent the SEM. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine significance between selected pairs. # symbols represent LF vs HF comparison within genotype, $p<0.001$, n=6-10 per group.

Effect of TLR4 Deficiency on Glucose Tolerance

In order to determine if TLR4 deficiency had an effect on peripheral glucose metabolism, an intraperitoneal glucose tolerance test (IPGTT, 1g/kg lean mass) was performed following 10 wks of LF and HF feeding. No difference was observed in fasting blood glucose, fasting plasma insulin, and glucose excursion curves quantified as area under the curve (AUC) during an IPGTT between WT and T4 LF fed mice (Figure 24A-F, male and female mice). However, 10 wks of HF feeding induced fasting hyperglycemia, hyperinsulinemia, and impaired glucose tolerance in WT male mice, whereas TLR4 deficient male mice were completely protected from these effects. In female mice, HF feeding did not significantly increase fasting blood glucose or impair glucose tolerance in WT or T4 mice compared to respective LF fed controls and only moderately elevated fasting plasma insulin levels (Figure 24B, D, F). Together, these data suggest that female mice, independent of TLR4 deficiency, are relatively protected from high-fat diet-induced impairments in glucose metabolism; a finding that has been confirmed by others [153]. However, TLR4 deficiency protected male mice against impaired glucose metabolism induced by HF feeding.

Effect of TLR4 Deficiency on Lipid Metabolism

The following parameters were assessed to determine whether the reduced adiposity in TLR4 deficient mice is potentially due to gross alterations in lipid metabolism; fasting plasma free fatty acids (FFA), triglycerides (TG), and leptin levels (Tables 4,5; p 106-7), as well as mRNA levels of stearoyl CoA desaturase-1 (SCD-1; Figure 25), a key mediator of lipid metabolism. No significant differences in plasma FFA or TG levels were observed between WT and T4 male mice fed both diets (except WT vs T4 fed LF diet, ~25% difference, $p<0.05$). In female mice, plasma FFAs and TGs were significantly elevated in LF, but not HF, fed T4 mice compared to WT controls (T4

vs WT, LF diet, ~65% increase in FFA, ~35% increase in TGs). Plasma leptin levels were significantly lower in HF fed T4 mice compared to WT controls (10.2±1.7 vs 43.1±0.9, $p<0.001$). While no differences were observed in leptin levels among female mice, female leptin levels were comparable to levels observed in LF fed WT male mice. SCD-1 is a delta-9 fatty acid desaturase; a lipogenic enzyme catalyzing the synthesis of monounsaturated fatty acids. Along with palmitate, stearate is the major substrate for the enzyme stearoyl-CoA desaturase, which catalyzes the conversion of stearate to oleate [157]. Oleate is the preferred substrate of SCD-1 for the synthesis of TG and other complex lipids [157]. Hepatic mRNA levels of SCD-1 are shown in Figure 25 (HF diet only, WT and T4 male and female mice). SCD-1 mRNA levels were significantly reduced in male T4 mice compared to WT controls (0.09±0.02 vs 0.28±0.06 relative units, $p<0.05$) consistent with reduced liver TG accumulation observed in another mouse model of TLR4 deficiency [142]. Although there was no difference in SCD-1 mRNA levels of WT and T4 female mice, the levels of SCD-1 in female mice were comparable to that of T4 male mice, i.e. significantly lower than WT male mice (WT, 0.14±0.02; T4, 0.09±0.01) suggestive of reduced liver TG accumulation and improved hepatic function.

Effect of TLR4 Deficiency on Inflammatory IKKβ Signaling

The phenotype of decreased body weight and adiposity combined with the changes observed in glucose metabolism of TLR4 deficient mice suggests that TLR4 is involved in energy homeostasis (i.e. central insulin and leptin sensitivity) and peripheral insulin sensitivity. The association between high-fat diet induced obesity and inflammation makes TLR4 a likely candidate as a potential mediator of this relationship because it is activated by fatty acids in a saturation dependent manner and induces an inflammatory signaling cascade known to be involved in the development of insulin resistance. Thus, I investigated whether the metabolic effects of TLR4 are potentially

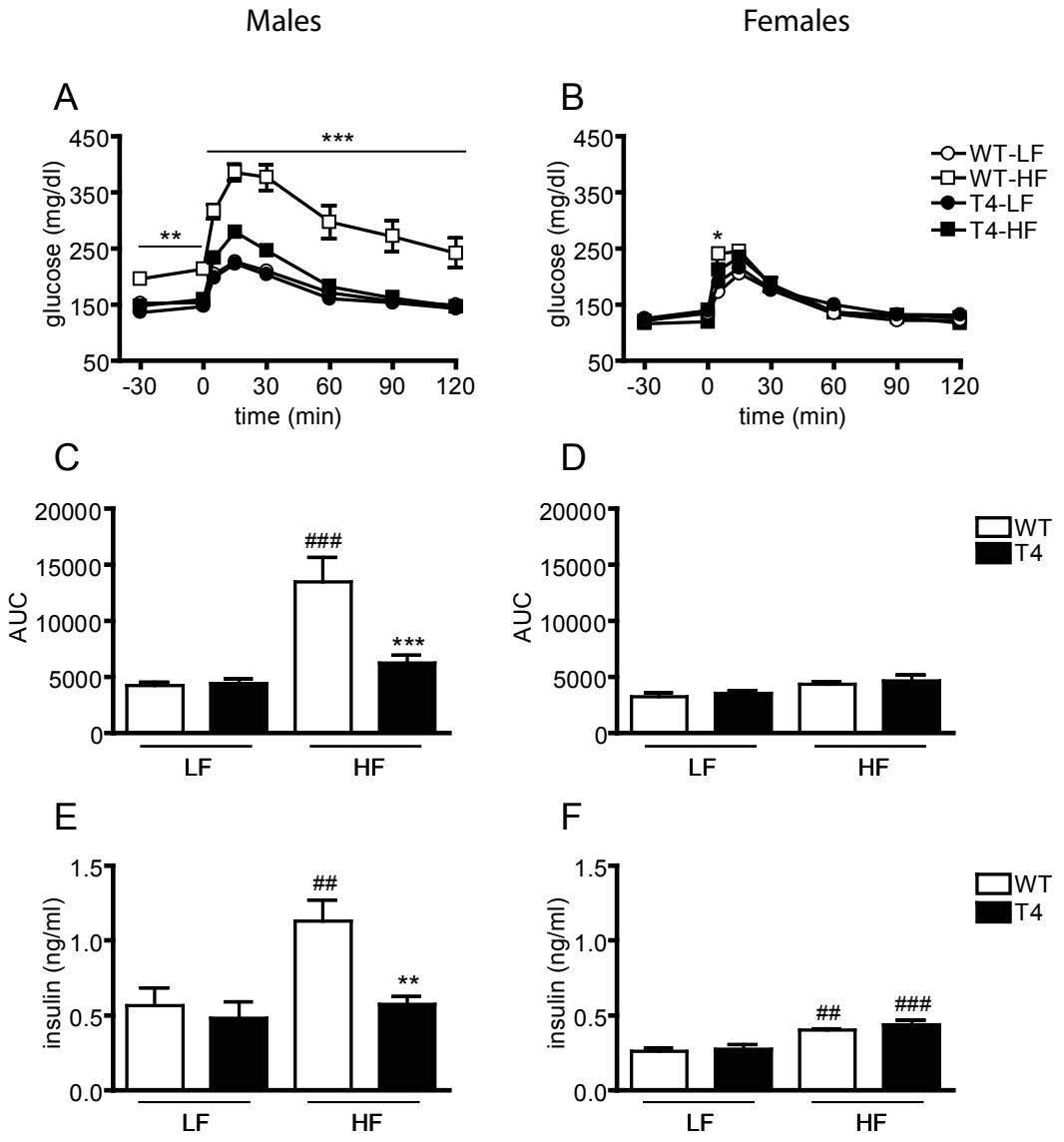


Figure 24: Effect of TLR4 Deficiency on Glucose Tolerance

An intraperitoneal glucose tolerance test was performed after 10 wks of diet treatment in male and female mice (A-B) and area under the curve was calculated from baseline blood glucose values as an index of glucose tolerance (C-D). Four hour fasting plasma insulin levels are shown in panels E,F. Error bars represent the SEM. Two-way ANOVA with Bonferroni post-tests was used to determine significance in panels A-B. Symbols above the lines represent WT vs T4 on HF diet. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine significance between selected pairs. * symbols represent WT vs T4 within diet, and # symbols represent LF vs HF within genotype. One symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$, n=13-20 per group in male mice, n=8-11 per group in female mice.

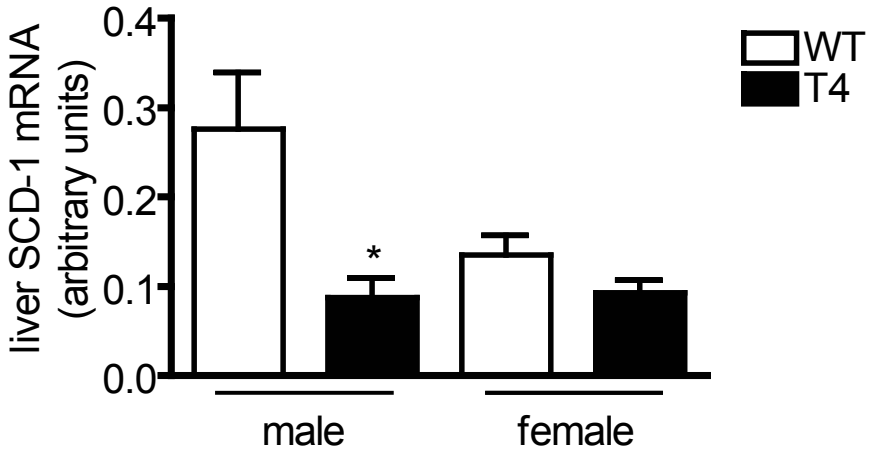


Figure 25: Effect of TLR4 Deficiency on a Major Regulator of Hepatic Lipid Metabolism

Hepatic mRNA levels of stearoyl-CoA desaturase-1 (SCD-1) were determined by quantitative real-time reverse transcriptase PCR in HF fed male and female mice. Error bars represent the SEM. Student's t-test was used to determine significance in male and female mice separately, WT vs T4. * $p<0.05$, n=4-5 per group in male and female mice

mediated via central or peripheral effects, or a combination of both. In this study, hypothalamic pIKKβ levels were elevated following 10 wks of HF feeding in WT and T4 male mice compared to LF fed controls (Figure 26A; LF vs HF, WT 1.00±0.16 vs 1.46±0.20 relative density, $p=0.08$, T4 0.22±0.08 vs 1.02±0.08 relative density, $p<0.01$). However, absolute levels of pIKKβ were lower in TLR4 deficient male mice compared toWT controls (T4 vs WT; LF diet $p<0.01$, HF diet $p=0.09$). No differences were observed in hypothalamic pIKKβ levels among female groups (Figure 26B). In the liver, no differences in pIKKβ were observed between WT and T4 mice (Figure 26C,D).

Discussion

In these studies I utilized a mouse model of TLR4 deficiency to examine the effects of TLR4 signaling in high-fat diet-induced obesity. Dietary fatty acids, specifically saturated fatty acids, activate TLR4 and downstream inflammatory signaling molecules associated with the development of insulin resistance. Previous work investigating TLR4 action in peripheral tissues supports a role for TLR4 in high-fat diet-induced insulin resistance via activation of inflammatory molecules. Since TLR4 is also expressed in regions of the central nervous system associated with the regulation of energy homeostasis, I hypothesized that activation of TLR4 by dietary fatty acids may also induce hypothalamic insulin resistance via a similar inflammatory mediated mechanism in diet-induced obesity. The phenotype of decreased body weight and adiposity combined with the changes observed in glucose metabolism of TLR4 deficient mice indicates that TLR4 may be involved in both energy homeostasis (i.e. hypothalamic insulin and leptin sensitivity) and peripheral insulin sensitivity. Thus, I investigated whether these metabolic effects of TLR4 signaling were mediated via central effects, peripheral effects, or a combination of both.

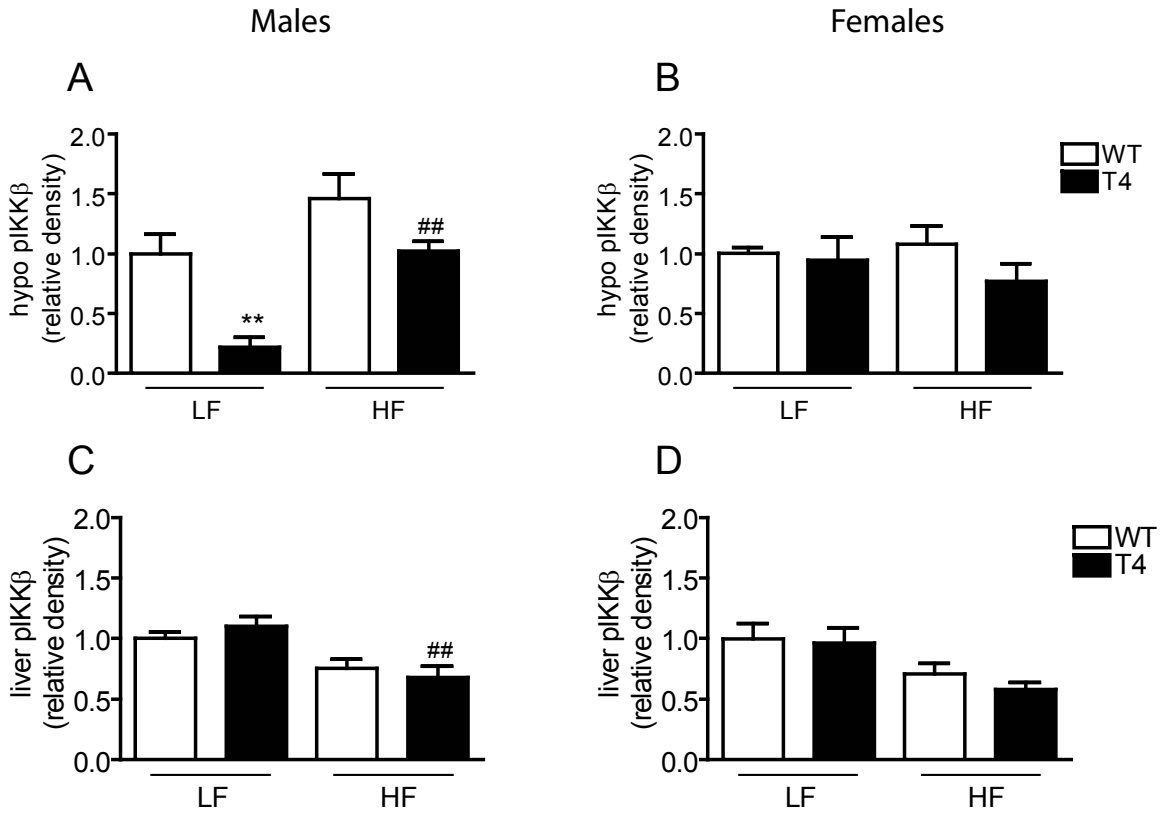


Figure 26: Effect of TLR4 Deficiency on IKKβ Activity

Phosphorylation of IKKβ in hypothalamic (A-B) and liver (C-D) tissues in male and female mice. Error bars represent the SEM. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine significance between selected pairs. * symbols represent WT vs T4 within diet, and # symbols represent LF vs HF within genotype. Two symbols $p<0.01$, n=5-8 per group in male and female mice.

These studies were performed in both male and female mice. Of consequence to these studies are the well appreciated gender specific metabolic differences. For example, data from both clinical and experimental studies have revealed that in addition to its well characterized role in sexual development and reproduction, the sex hormone estrogen exerts beneficial effects on insulin action and glucose homeostasis [107]. In experimental animal models, ovariectomy was shown to impair insulin sensitivity and glucose metabolism, a deleterious metabolic effect reversed by chronic administration of estrogen [94, 191]. Furthermore, evidence indicates that the estrogen receptor ERα is the main receptor involved in energy balance [15] as global deletion of ERα results in obese, insulin resistant mice [75]. Disruption of ERα in the hypothalamus leads to weight gain, increased visceral adiposity, hyperphagia, hyperglycemia and impaired energy expenditure in female mice [128]. However, the mechanisms by which estrogen influence insulin sensitivity and glucose metabolism remains poorly understood. In light of this, the results from male and female mice in these studies are discussed separately.

Since saturated fatty acids are known TLR4 ligands, a diet low in fat would be expected to have a minimal effect on TLR4 activity, whereas a diet high in fat would be expected to increase TLR4 activity in WT mice. Thus, in a setting of low TLR4 activity (LF diet), I predicted there would be no significant differences between WT and TLR4 deficient mice. Conversely, in a setting of elevated TLR4 activity (HF diet), I predicted there would be significant differences between WT and TLR4 deficient mice if TLR4 is a determinant of high-fat diet-induced obesity. Final body weight and adiposity levels were significantly reduced in LF fed female TLR4 deficient mice compared to WT controls. Since an effect was observed with LF feeding, either only a small amount of dietary fat is sufficient to activate TLR4 in WT mice, or TLR4 deficiency exerts additional anti-obesity effects independent of diet. Furthermore, TLR4 deficient female mice were completely protected from increased adiposity associated with HF feeding.

To determine whether the reduced body weight and adiposity observed in TLR4 deficient female mice could be attributed to altered energy homeostasis; food intake and energy expenditure (approximated by feed efficiency calculations) were assessed in these mice. Cumulative food intake was slightly reduced in LF fed T4 female mice compared WT controls whereas TLR4 deficiency per se had no effect on energy expenditure as calculated by feed efficiency. This could potentially explain the lower body weight and adiposity in LF fed T4 mice. However, food intake was similarly increased and energy expenditure similarly decreased with HF feeding in both WT and T4 mice compared to LF fed controls indicating a state of positive energy balance. This may explain the increase in body weight of both WT and T4 female mice compared to LF fed controls. Thus, although T4 mice weighed less than WT controls, TLR4 deficiency failed to protect female mice against HF diet-induced weight gain.

Although TLR4 deficiency did not protect against body weight gain, recall that T4 female mice were completely protected from increased *adiposity* associated with HF feeding. Related to the concept of feed efficiency, energy efficiency calculations indicate how well the body stores nutrients in the form of adipose mass. Results from these calculations indicate that TLR4 deficient female mice store less energy in the form of adipose mass per calorie consumed than WT mice. These data suggest that TLR4 may be involved in the central regulation of adipose stores, potentially via TLR4 mediated activation of IKKβ resulting in impaired hypothalamic insulin sensitivity and disrupted energy homeostasis.

Although there were no clear differences in hypothalamic IKKβ activity in female mice, there was a trend towards reduced pIKKβ levels in HF fed TLR4 deficient female mice compared to HF fed WT controls (~30% reduction, p=ns) in support of my hypothesis that reduced inflammatory signaling in the hypothalamus of TLR4 deficient mice contributes to the reduced adiposity observed in these animals via improved

energy homeostasis. In addition, hepatic IKKβ activity and glucose tolerance were not improved in TLR4 deficient mice suggesting that the reduced adiposity in T4 mice is not due to peripheral effects. However, HF feeding failed to induce a significant inflammatory response, increase plasma insulin and leptin levels, and only modestly impaired glucose tolerance in female mice. The relative protection from high-fat diet induced insulin resistance in female mice may be explained by the beneficial metabolic effects of estrogen.

The same measurements and analyses were performed in male WT and T4 mice. As observed in female mice, final body weight and adiposity levels were significantly lower in male T4 mice compared to WT controls independent of diet. Although TLR4 deficiency did not completely protect male mice from diet-induced obesity, loss of TLR4 activity improved overall energy homeostasis resulting in a lean phenotype.

Energy homeostasis is maintained by the balance of food intake and energy expenditure and these processes are largely regulated by hypothalamic insulin and leptin signaling that become impaired in models of high-fat diet-induced obesity. To determine whether the reduced body weight and adiposity observed in TLR4 deficient male mice could be attributed to improved hypothalamic regulation of energy homeostasis; food intake and energy expenditure were assessed in these mice. However, TLR4 deficiency had no effect on cumulative food intake and energy expenditure compared to WT mice. Although TLR4 deficiency did not significantly alter food intake and energy expenditure, energy efficiency calculations indicate that for the same caloric intake, TLR4 deficient male mice store less energy in the form of adipose mass compared to WT controls independent of diet treatment and may account for the reduced adiposity observed in these mice.

As described above, a reduction in hypothalamic inflammatory signaling may contribute to the reduced adiposity observed in these mice via improved insulin sensitivity and energy homeostasis. In line with this, hypothalamic pIKKβ levels were reduced in T4 mice compared to WT controls independent of diet treatment, whereas no differences were observed in hepatic pIKKβ levels. These data point to a centrally mediated effect of TLR4 deficiency on adiposity. However, TLR4 deficiency also improved high-fat diet-induced hyperglycemia, hyperinsulinemia and impaired glucose tolerance compared to WT controls. These improvements in peripheral glucose homeostasis could account for the reduced adiposity observed in these mice as well. In accordance with a peripheral effect of TLR4, Stearoyl-CoA Desaturase 1 (SCD-1) mRNA levels were reduced in male TLR4 deficient mice compared to WT controls. SCD-1 activity is involved in lipid metabolism and deficiency of SCD-1 reduces triglyceride accumulation, protects against weight gain, and increases insulin sensitivity [119, 150]. Although the mechanism of increased SCD-1 in TLR4 deficient mice is unknown, this could at least partially explain the improved glucose homeostasis and decreased adiposity in male mice.

In conclusion, both male and female TLR4 deficient mice weigh less and are leaner than respective WT controls on both a low-fat and high-fat diet suggesting a potential role for TLR4 in the regulation of adipose stores. I propose that the reduced adiposity observed in both male and female TLR4 deficient mice may occur via a mechanism of decreased hypothalamic inflammatory signaling resulting in improved adiposity hormone signaling and improved energy homeostasis. Although these results suggest that TLR4 mediated inflammatory signaling within the hypothalamus may contribute, at least in part, to modulation of energy homeostatic pathways and adipose accumulation; based on our observations it is not possible to rule out other pathways and mechanisms involved in the development of diet-induced obesity. Whether CNS

resistance occurs early in the course of HF feeding and actively contributes to increased food intake, adiposity and peripheral insulin resistance characteristic of DIO is an additional important question to be addressed.

Table 4: Metabolic Profile of Male Mice

	LF		HF	
	WT	T4	WT	T4
Body wt (g)	30.1 ± 0.3	28.1 ± 0.4*	35.0 ± 0.8###	31.1 ± 0.9***,##
Body wt gain (g)	12.4 ± 0.4	12.7 ± 0.5	16.6 ± 0.7###	15.1 ± 1.0#
Adiposity (%)	15.9 ± 0.8	9.5 ± 0.6***	29.2 ± 1.5###	22.2 ± 1.4***,###
Fat mass gain (g)	3.4 ± 0.3	1.8 ± 0.2*	8.8 ± 0.7###	5.9 ± 0.6***,###
Total food intake (kcal)	645 ± 5	642 ± 4	736 ± 5###	719 ±10###
Feed efficiency (bw gain/kcal)	0.019 ± 0.001	0.019 ± 0.001	0.023 ± 0.001	0.021 ± 0.001
Energy efficiency (fat gain/kcal)	0.005 ± 0.000	0.003 ± 0.000*	0.012 ± 0.001###	0.008 ± 0.001***,###
Heat Production (kcal/hr/g lean mass)	0.024 ± 0.001	0.025 ± 0.001	0.024 ± 0.000	0.023 ± 0.001
Locomotor Activity(# beam crosses)	51600 ± 7580	58500 ± 9070	57300 ± 9630	60900 ± 1280
Respiratory Quotient	0.89 ± 0.006	0.88 ± 0.005	0.77 ± 0.005###	0.75 ± 0.006###
AUC	4220 ± 330	4430 ± 390	13450 ± 2200###	6240 ± 680***
Glucose (mg/dl)	152 ± 7	136 ± 5	196 ± 11###	147 ± 4**
Insulin (ng/dl)	0.56 ± 0.12	0.48 ± 0.11	1.13 ± 0.14##	0.58 ± 0.05**
Leptin (ng/dl)	8.0 ± 0.9	2.8 ± 0.3	43.1 ± 3.9###	10.2 ± 1.7***,#
FFA (mmol/L)	0.41 ± 0.06	0.46 ± 0.05	0.43 ± 0.07	0.47 ± 0.06
TG (mg/dl)	126 ± 5	95 ± 6	112 ± 11	91 ± 6

Table 4: Metabolic parameters of male WT and TLR4 deficient (T4) mice following 10 wks of LF and HF feeding are reported as the group mean ± SEM. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine significance between selected pairs. * symbols represent WT vs T4 within diet, and # symbols represent LF vs HF within genotype. One symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$, n=13-20 per group.

Table 5: Metabolic Profile of Female Mice

	LF		HF	
	WT	T4	WT	T4
Body wt (g)	22.3 ± 0.5	21.2 ± 0.2	25.9 ± 1.4$^{\#\#}$	23.0 ± 0.4*
Body wt gain (g)	7.7 ± 0.3	8.0 ± 0.2	11.6 ± 1.0$^{\#\#\#}$	12.0 ± 0.3$^{\#\#\#}$
Adiposity (%)	14.1 ± 0.7	11.2 ± 0.8	23.2 ± 2.4$^{\#\#\#}$	12.9 ± 1.1***
Fat mass gain (g)	1.8 ± 0.2	1.4 ± 0.2	4.5 ± 0.9$^{\#\#\#}$	2.2 ± 0.3**
Total food intake (kcal)	559 ±11	520 ± 6*	663 ±15$^{\#\#\#}$	662 ±12$^{\#\#\#}$
Feed efficiency (bw gain/kcal)	0.014 ± 0.001	0.015 ± 0.000	0.016 ± 0.001$^{\#}$	0.018 ± 0.000$^{\#\#}$
Energy efficiency (fat mass gain/kcal)	0.003 ± 0.000	0.003 ± 0.000	0.007 ± 0.000$^{\#\#}$	0.003 ± 0.000**
AUC	3240 ± 345	3560 ± 210	4370 ± 220	4660 ± 510
Glucose (mg/dl)	120 ± 5	124 ± 4	120 ± 5	116 ± 2
Insulin (ng/dl)	0.26 ± 0.02	0.27 ± 0.03	0.40 ± 0.01$^{\#\#}$	0.44 ± 0.03$^{\#\#\#}$
Leptin (ng/dl)	5.5 ± 0.7	4.4 ± 0.8	6.5 ± 0.6	3.8 ± 0.9
FFA (mmol/L)	0.28 ± 0.02	0.46 ± 0.06*	0.25 ± 0.03	0.31 ± 0.04
TG (mg/dl)	89 ± 7	122 ± 11*	81 ± 5	75 ± 4$^{\#\#\#}$

Table 5: Metabolic parameters of female WT and TLR4 deficient (T4) mice following 10 weels of LF and HF feeding are reported as the group mean ± SEM. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to determine significance between selected pairs. * symbols represent WT vs T4 within diet, and # symbols represent LF vs HF within genotype. One symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$, n=8-11 per group.

CHAPTER VI

HIGH-FAT DIET-INDUCED HYPOTHALAMIC INSULIN AND LEPTIN RESISTANCE ARE MECHANISTICALLY AND TEMPORALLY DISTINCT AT THE ONSET OF OBESITY.

Introduction

Our laboratory previously demonstrated that high-fat feeding is associated with hypothalamic accumulation of long-chain fatty acyl-CoA (LC-CoA) molecules and activation of the pro-inflammatory molecule IKKβ in diet-induced obese rats. Work from chapter III specifically implicated long-chain saturated fat in DIO independent of increased caloric intake. In support of these findings, work from chapter IV demonstrated that an acute icv infusion of a saturated fatty acid, palmitate, recapitulated the hypothalamic effects observed with chronic HF feeding (i.e. accumulation of LC-CoA and increased IKKβ activity) in lean chow fed rats. In these studies, long-chain saturated fat impaired hypothalamic insulin signaling and this effect is proposed to occur via a mechanism of LC-CoA induced inflammatory signaling similar to the mechanism of insulin resistance described in peripheral tissues.

Current models of energy homeostasis clearly implicate hypothalamic insulin and leptin signaling in the regulation of adipose stores. Accordingly, these signaling pathways are thought to be disrupted in order for obesity to develop. Since insulin and leptin can utilize common intracellular signal transduction pathways [Niswender 2004, Niswender 2001], neuronal resistance to these hormones may involve the same or similar mechanisms. Thus, I hypothesize that dietary long-chain saturated fats induce hypothalamic insulin and leptin resistance via a mechanism of LC-CoA induced inflammatory signaling, ultimately resulting in the development of obesity.

However, it is easy to imagine a scenario in which obesity leads to these changes in LC-CoA accumulation and inflammatory signaling in the hypothalamus, instead of these hypothalamic changes leading to obesity. Obesity is characterized by peripheral insulin resistance, increased lipolysis, and elevated circulating FFA; all of which could potentially lead to the accumulation of lipid within the hypothalamus. In addition, obesity is often associated with low grade inflammation. This could account for the activation of IKKβ within the hypothalamus. Together, these data would suggest that obesity causes hypothalamic insulin and leptin resistance. I propose, however, that high-fat feeding induces obesity via impairments in hypothalamic insulin and leptin sensitivity that result in disrupted energy homeostasis. This is then exacerbated by obesity and peripheral insulin resistance. To address this hypothesis, I first sought to determine whether central resistance precedes peripheral resistance and second, whether the high-fat diet-induced changes in LC-CoA and IKKβ activity occur over a time-course consistent with a role in hypothalamic insulin and leptin resistance, thereby disrupting energy homeostasis and resulting in obesity. If these changes are primary to the development of HF DIO, then they should occur prior to the accumulation of excess body fat. Thus, in the following studies I first compared relative insulin sensitivities in the hypothalamus, liver, and muscle tissue of HF fed rats, and second, I determined whether changes in LC-CoA accumulation and IKKβ activation may contribute to impaired hypothalamic insulin and leptin signaling prior to the onset of obesity in HF fed rats.

Results

Relative Insulin Sensitivity in Central and Peripheral Tissues

To determine whether the hypothalamus is more susceptible to high-fat diet-induced insulin resistance than peripheral tissues, I compared the relative insulin sensitivity of key peripheral tissues to that observed in the hypothalamus of rats from the

pair-feeding study in chapter III. Relative insulin sensitivity was assessed by the ability of a peripheral glucose bolus to induce insulin mediated activation of PKB in the liver, skeletal muscle, and hypothalamus of rats fed a low-fat (LF) or high-fat diet (*ad libitum*, HF, and pair-fed, PF, to the caloric intake of the LF group) for 4 wks (Figure 27A-F). In all tissues of LF fed rats, phosphorylation of PKB was significantly elevated following glucose injection compared to vehicle controls (Figure 27A-C, LF-glu vs LF-veh $p<0.01$). This effect remained intact with high-fat feeding in liver and muscle tissues (Figure 27; panel A, HF $p<0.01$ and PF $p<0.001$; panel B PF $p<0.05$), but was completely blunted in the hypothalamus of high-fat fed rats (Figure 27C). Likewise, when plotted versus plasma insulin levels, PKB phosphorylation was significantly correlated with plasma insulin concentration in all tissues of the LF group indicating insulin sensitivity (Figure 27D-F). Insulin sensitivity appeared intact in liver tissue of HF fed animals (Figure 27; panel D, HF $p<0.001$ and PF $p<0.001$) and in muscle tissue of calorically restricted HF fed animals (Figure 27; panel E, PF $p<0.001$), whereas insulin-stimulated activation of hypothalamic PKB was completely inhibited in HF fed animals (Figure 27F, HF and PF p=ns). These data suggest that the hypothalamus may be more susceptible than peripheral tissues to HF diet-induced insulin resistance.

Early Onset of High-fat Diet-induced Obesity

It is well documented that rats fed a high-fat diet gain significantly more body weight and fat mass compared to low-fat fed controls over time. However, it is remarkable how quickly this change occurs upon exposure to a high-fat diet. I observed a predictable pattern of diet-induced obesity in which body weight significantly diverged by six days (Figure 28A; $p<0.05$), and adiposity significantly diverged by three days(Figure 28B; $p<0.05$) of ad libitum HF feeding compared to LF fed controls. In addition, a predictable pattern of food intake was observed during this period at the

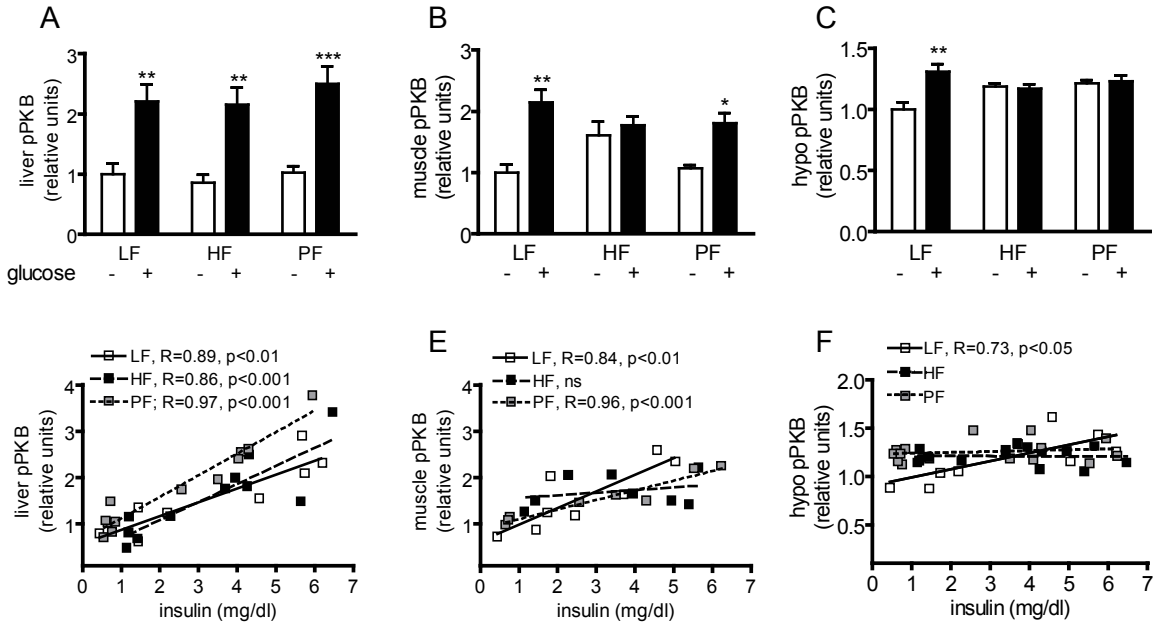


Figure 27: Effect of Dietary Fat Intake on Relative Insulin Sensitivity in Central and Peripheral Tissues

A,B,C. Glucose stimulated pPKB in liver, skeletal muscle, and hypothalamus, respectively. D,E,F. Relationship between plasma insulin levels and phosphorylation of PKB in liver, skeletal muscle, and hypothalamus, respectively. Error bars represent the standard error of the mean (SEM). Student's t-test was used to determine significance of glucose stimulated pPKB compared to vehicle controls for each diet treatment (A-C). Pearson's correlation was used to determine significance of correlation within each diet treatment (D-F). n=4-6 per group for panels A-C, n=8-12 for panels D-F.

onset of obesity. Total gram intake was significantly higher in the HF group at day 2 compared to the LF group (Figure 28C); whereas total caloric intake was significantly higher in the HF group throughout the entire feeding study (Figure 28D).

Based on this time-course of HF DIO, I assessed key molecular mediators thought to be involved in the development of high-fat diet-induced hypothalamic insulin and leptin resistance and obesity. Measures of hypothalamic LC-CoA content, IKKβ activity, and sensitivity to the adiposity signals insulin and leptin were determined at time-points before (2d), concurrent with (3d), and after (7d) adiposity diverged to establish a basis for causality versus consequence of these parameters in the development of HF DIO.

Accumulation of Long Chain CoA and Activation of IKKβ with High-Fat Feeding

Hypothalamic LC-CoA content and pIKKβ levels were measured to determine whether changes in these parameters may contribute to the development of obesity. Although no differences were observed in LC-CoA levels of LF and HF fed rats after two or three days of diet (Figure 29A, C respectively), but all three LC-CoA species measured were significantly elevated in the HF group compared to LF controls after seven days of diet (Figure 29E; 16:0 $p<0.01$, 18:0 $p<0.001$, and 18:1 $p<0.05$). However, a different pattern of IKKβ activity emerged at the onset of HF DIO. High-fat feeding induced a significant increase in IKKβ activity (pIKKβ) after only two days of diet compared to LF controls (Figure 29B; $p<0.05$) and a near significant increase after three days of diet administration (Figure 29D; $p=0.07$). After seven days of HF feeding though, differences in pIKKβ were no longer observed between the groups (Figure 29F). In conjunction with my previously published data illustrating elevated pIKKβ levels with 10 wks of HF feeding [146]; these data indicate that activation of IKKβ is bimodal with elevated pIKKβ levels at early time-points in HF DIO (prior to and concurrent with

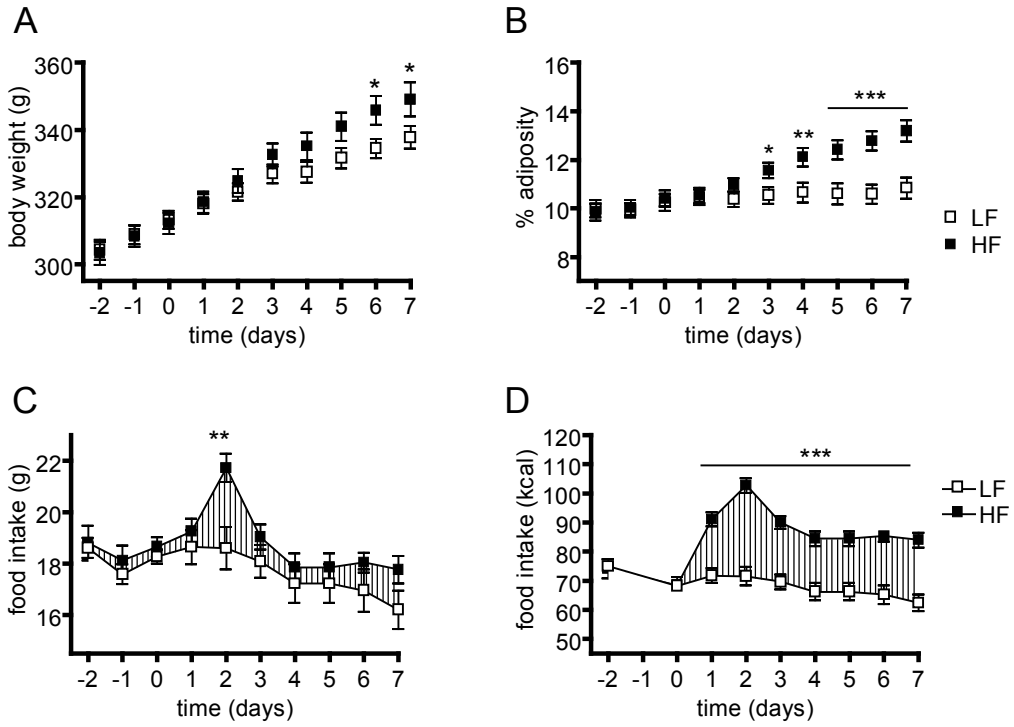


Figure 28: Early Onset of High-Fat Diet-Induced Obesity

A,B. Daily body weight and adiposity measurements. C,D. Daily food intake measurements. Error bars represent the SEM. Two-way ANOVA with Bonferroni's post-tests was used to determine significance. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, n=22-24 per group.

increased adiposity, 2d and 3d respectively), levels restored to basal at an intermediate time-points in HF DIO (after adiposity diverged, 7d), and levels again elevated with long-term HF feeding (10wks).

Progression of Insulin and Leptin Resistance with High-Fat Feeding

To determine whether these changes in hypothalamic LC-CoA content and IKKβ activity with HF feeding altered hypothalamic insulin and leptin sensitivity, activation of their respective downstream signaling molecules PKB and Stat3 were assessed following an icv infusion of insulin or leptin (Figure 30A-F). Insulin-mediated activation of PKB, as determined by a significant increase in phosphorylation compared to vehicle controls, was demonstrated in the LF group at all time-points measured (Figure 30A,C,E). Intact insulin signaling was also observed in the HF group after two and three days of diet (Figure 30A,C). However, insulin-mediated phosphorylation of PKB was completely blunted after seven days of HF diet (Figure 30E). Hypothalamic resistance to insulin signal transduction occurred concurrently with the observation of increased hypothalamic LC-CoA content after seven days of HF feeding. Leptin-mediated activation of Stat3, as determined by a significant increase in phosphorylation compared to vehicle controls, was demonstrated in the LF group at all time-points measured (Figure30B,D,F). However, HF feeding impaired leptin-mediated phosphorylation of Stat3 after two and three days of diet (Figure 30B,D), whereas leptin signaling was intact after seven days of diet (Figure 30F). This pattern of hypothalamic sensitivity to leptin signal transduction is inversely correlated with the pattern of IKKβ activity such that when IKKβ activity is elevated, leptin signaling is impaired (2 and 3d of HF feeding). Conversely, when IKKβ activity is restored to basal levels, leptin signaling is intact (7d of HF feeding).

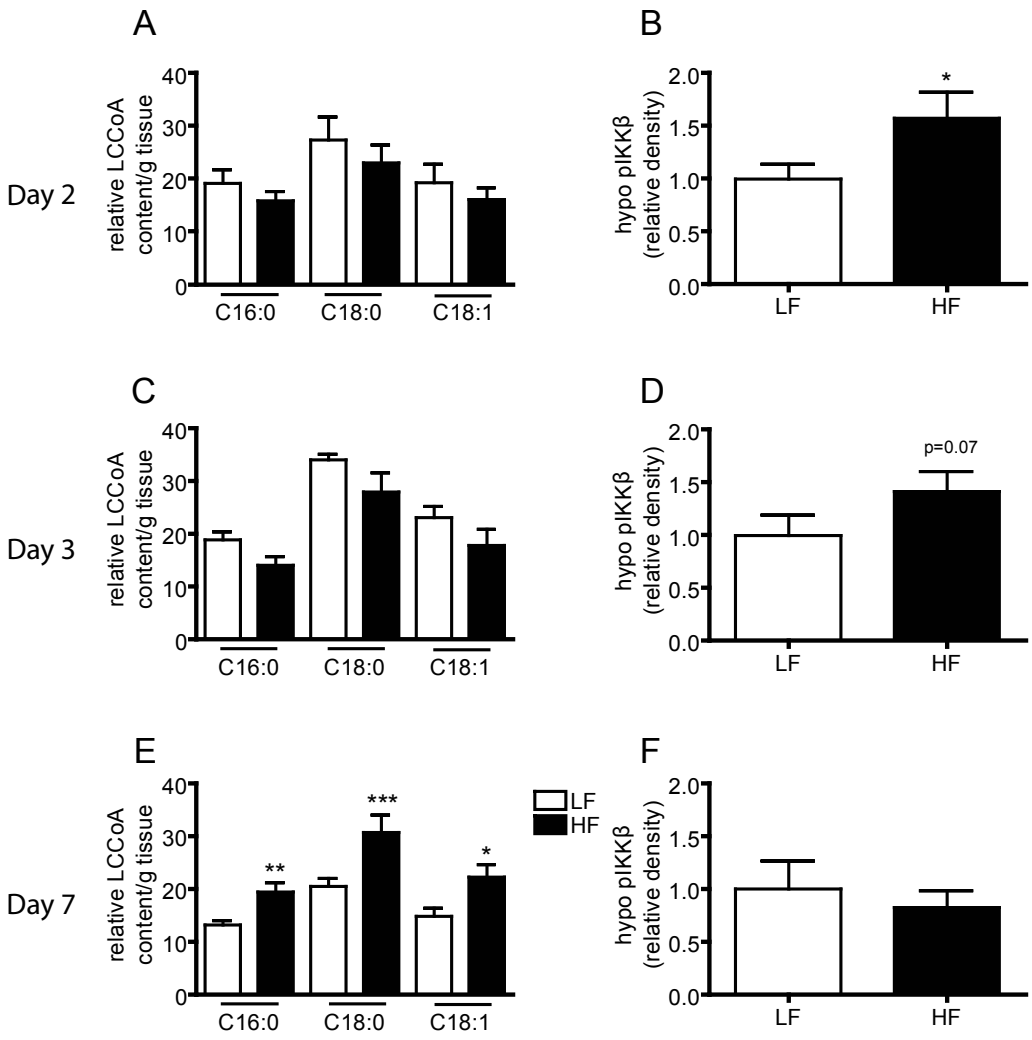


Figure 29: Effect of High-Fat Feeding on Hypothalamic Long Chain CoA Content and IKKβ Activity at the Onset of Diet-Induced Obesity

Hypothalamic content of (A,C,E) palmitoyl-, stearoyl- and oleoyl-CoA and (B,D,F) phosphorylated IKKβ are shown prior to (2days), concurrent with (3days), and after (7days) adiposity diverges in HF fed animals compared to LF fed controls. Error bars represent the SEM. Student's t-test was used to determine significance (points represent HF vs LF for each LC-CoA moiety and pIKKβ). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=6-10 for panels A,C,E and n=5-6 per group for panels B,D,F.

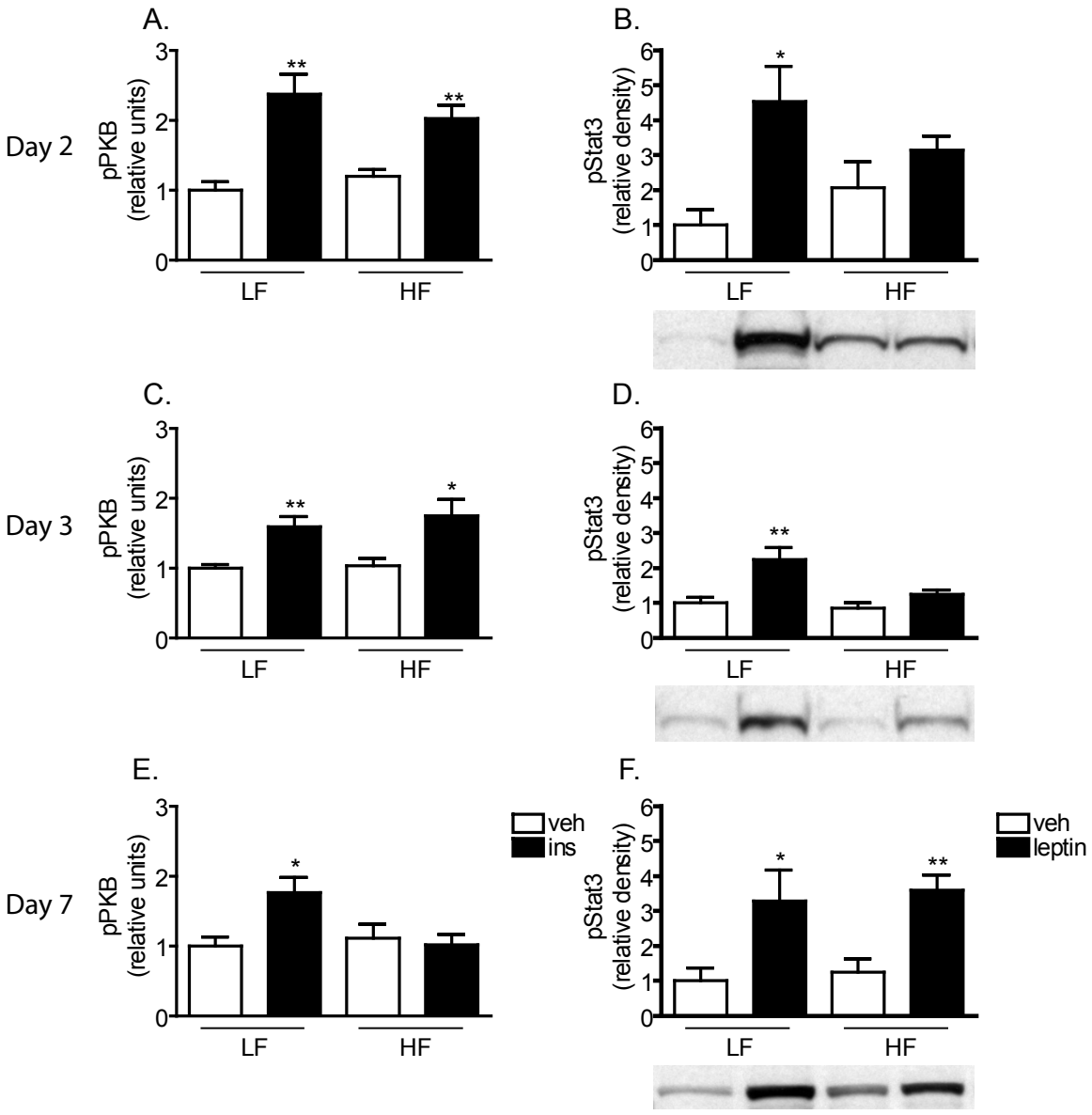


Figure 30: Hypothalamic Insulin and Leptin Sensitivity at the Onset of Diet-Induced Obesity.

Activation of (A,C,E) insulin and (B,D,F) leptin signaling cascades are shown prior to (2days), concurrent with (3days), and after (7days) adiposity diverges in HF fed animals compared to LF fed controls. Error bars represent the SEM. Student's t-test was used to determine significance (points represent treatment vs. vehicle controls in both diet groups). * $p<0.05$, ** $p<0.01$, n=4-6 per group.

Fasting plasma insulin and leptin levels were also measured at these key timepoints. Although plasma insulin levels were slightly elevated in the HF group compared to LF fed controls at each time-point measured, the differences failed to reach statistical significance (Figure 31A). However, plasma leptin levels were significantly higher in the HF group compared to LF fed controls at all time-points measured (Figure 31B; $p<0.05$ at 2, 3, and 7d).

Food Intake Response to Cholecystokinin

In addition to a direct measure of insulin and leptin signaling following icv infusion of these hormones, I utilized an indirect method to probe hypothalamic insulin and leptin sensitivity. Several studies have shown a clear relationship between the long-term energy homeostatic circuits in the hypothalamus and the short-term satiety signals in the hindbrain such that in the setting of hypothalamic resistance, the hindbrain is no longer sensitive to the effects of short-term satiety signals on food intake [123].

I took advantage of this relationship to indirectly test hypothalamic insulin and leptin sensitivity in our model of HF DIO by assessing 30min food intake following an ip injection of cholecystokinin (CCK) in 4 hour fasted rats. Cholecystokinin is a meal-generated satiety factor that signals meal termination and effectively reduces meal size. The results of these studies at time-points of interest are shown in Figure 32. Cholecystokinin effectively reduced food intake in the LF group by 35-60% at 2, 3, and 7 days of diet compared to vehicle controls (panels A, B, and C respectively). In the HF group, CCK failed to reduce food intake at 2 and 3 days of diet compared to vehicle controls (Figure 32A,B). However, the CCK effect was restored in the HF group and reduced food intake by approximately 55% compared to vehicle controls after seven days of diet (Figure 32C). These data suggest hypothalamic resistance occurs with 2 and 3 days of HF feeding; whereas hypothalamic sensitivity is intact after 7 days of HF

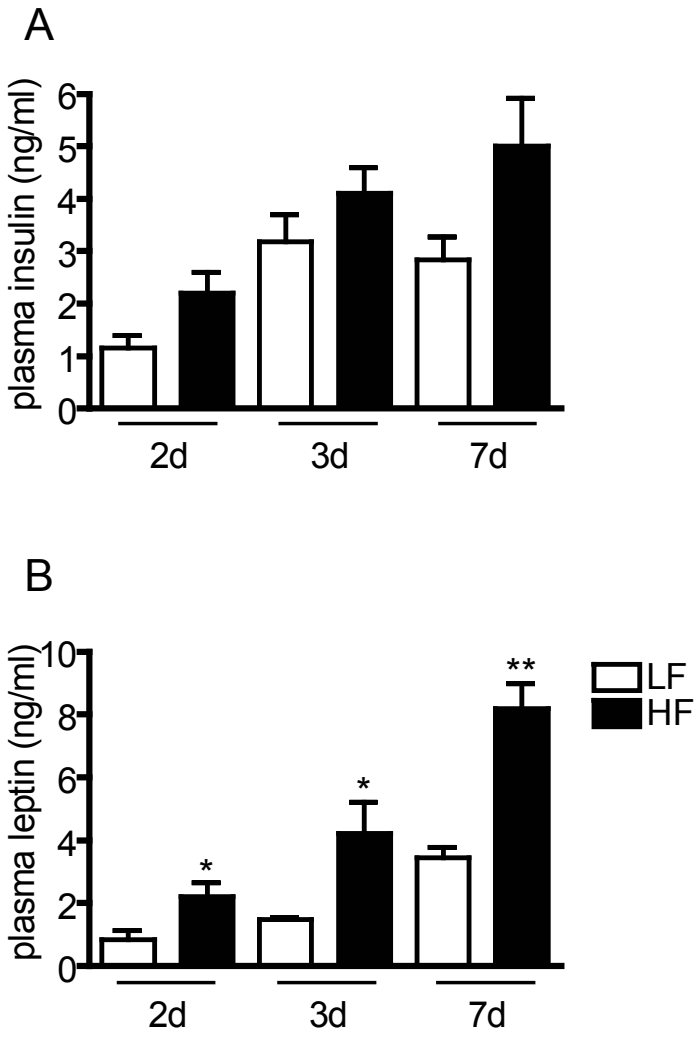


Figure 31: Plasma Insulin and Leptin Profile at the Onset of Diet-Induced Obesity.

Effect of HF feeding on plasma (A) insulin and (B) leptin levels prior to (2days), concurrent with (3days), and after (7days) adiposity diverges. Error bars represent the SEM. Student's t-test was used to determine significance (points represent HF vs LF diet at each time-point). * $p<0.05$, ** $p<0.01$ n=5-6 per group.

feeding. This pattern of hypothalamic sensitivity to CCK mimics the pattern of hypothalamic leptin sensitivity demonstrated by icv injection.

Inhibition of IKKβ Reduces Food Intake in Setting of Elevated pIKKβ

To mechanistically test the role of IKKβ activity on hypothalamic sensitivity and feeding behavior, 24 hour food intake was measured following pharmacological inhibition of IKK via icv injection (same compound used in chapter IV, PS-1145, Figure 33A-C). Inhibition of IKKβ significantly reduced 24 hour food intake in HF fed animals compared to vehicle controls after 2 and 3 days of diet ($p<0.001$ and $p<0.01$, respectively), when IKKβ activity was elevated, but not at 7 days of diet, when IKKβ activity was similar to levels observed in LF fed controls. Inhibition of IKKβ did not affect food intake in LF fed controls at any time-point.

Discussion

In previous chapters, both chronic high-fat feeding a diet enriched in saturated fat (chapter III) and acute intracerebroventricular administration of saturated fat (chapter IV) increased hypothalamic LC-CoA content and IKKβ activity in rats characterized by impaired insulin and leptin signaling. In this chapter, I extended these findings to the onset of diet-induced obesity to determine whether these changes are mechanistically involved in the development of hypothalamic insulin and leptin resistance and obesity. Hypothalamic resistance is proposed to occur via a mechanism similar to peripheral insulin resistance in which accumulation of LC-CoA is thought to activate IKKβ and induce downregulation of both the insulin and leptin signaling pathways within the hypothalamus. Impaired hypothalamic insulin and leptin signaling would result in disrupted energy homeostasis and lead to the development of obesity and peripheral insulin resistance.

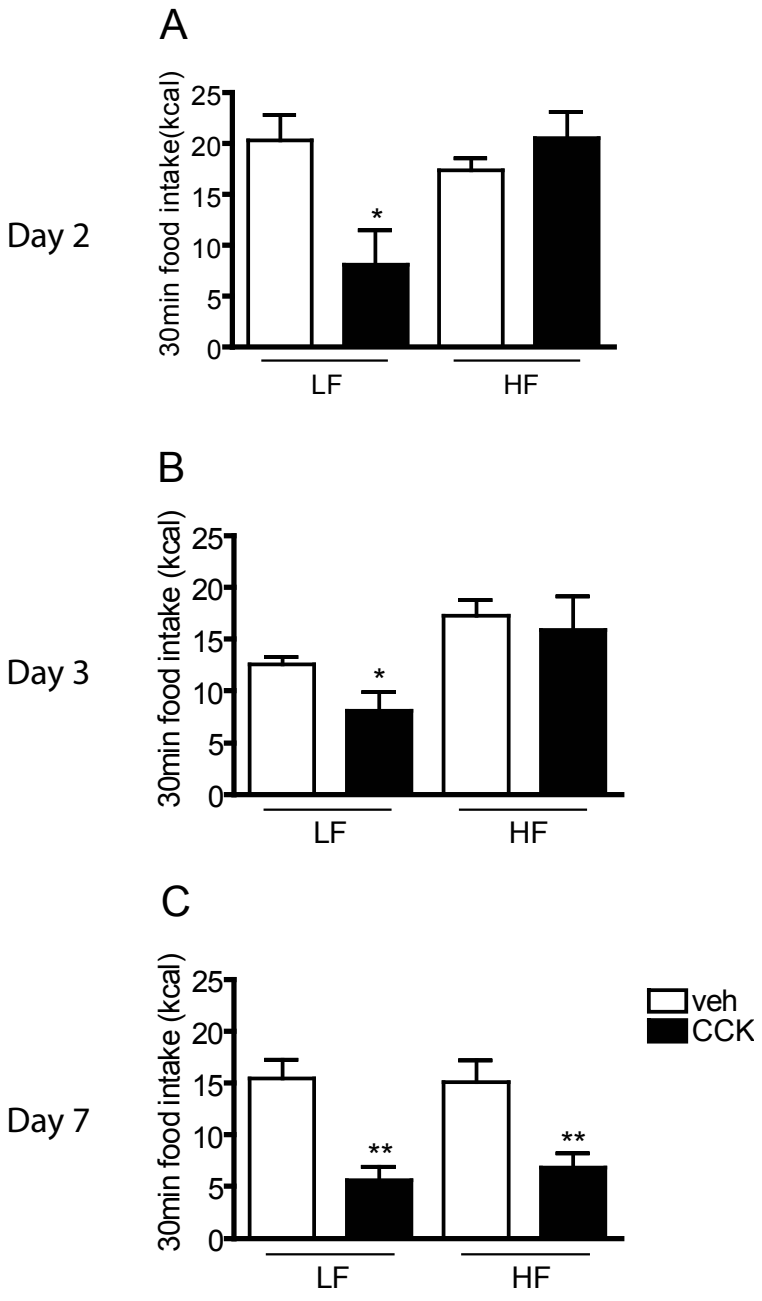


Figure 32: Food Intake Response to Cholecystokinin at the Onset of Diet-Induced Obesity.

Food lowering response to CCK as an indirect measure of hypothalamic sensitivity (A) prior to, (B) concurrent with, and (C) after adiposity diverges in HF fed animals compared to LF fed controls. Error bars represent the SEM. Student's t-test was used to determine significance between groups (points represent CCK vs vehicle treatment within each diet group). * $p<0.05$, ** $p<0.01$, n=6-8 per group.

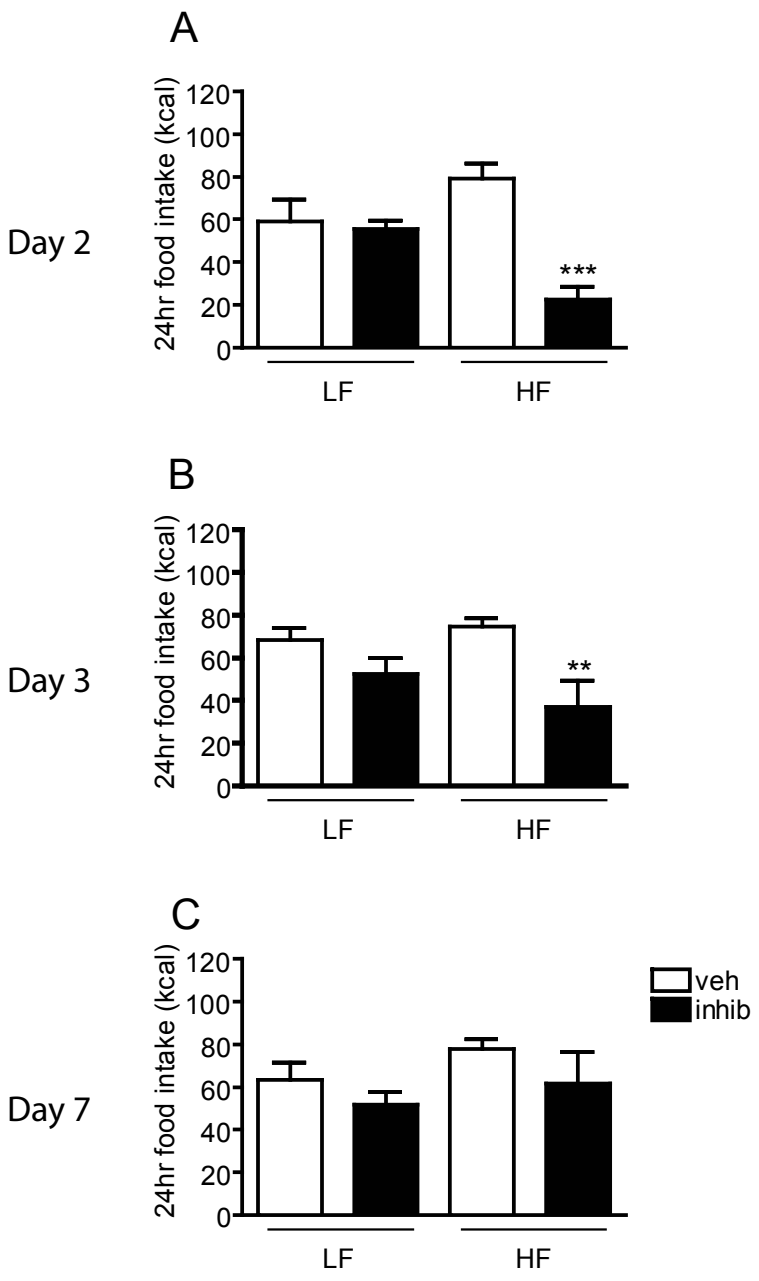


Figure 33: Effect of Pharmacological IKK Inhibitor on Food Intake at the Onset of Diet-Induced Obesity.

Inhibition of IKKβ Reduces Food Intake in Setting of Elevated pIKKβ. Relative IKKβ activity (A) prior to, (B) concurrent with, and (C) after adiposity diverges in HF fed animals compared to LF fed controls. Error bars represent the SEM. Student's t-test was used to determine significance (points represent IKK inhibitor vs vehicle treatment within each diet group). ** $p<0.01$, *** $p<0.001$, n=7-9 per group.

The first premise of my hypothesis is that hypothalamic insulin and leptin resistance cause disruption of energy homeostasis, which in turn leads to the development of obesity and peripheral insulin resistance. Thus, hypothalamic resistance is expected to occur prior to peripheral insulin resistance. Comparison of the relative insulin sensitivity of central and peripheral tissues in calorically restricted HF fed animals to LF fed controls (Figure 27, LF vs PF) indicated that the hypothalamus is more susceptible to high-fat diet-induced insulin resistance than the liver and muscle. This evidence is consistent with my hypothesis that hypothalamic resistance leads to the development of peripheral insulin resistance associated with obesity.

According to my hypothesis, hypothalamic insulin and leptin resistance occurs via a mechanism in which hypothalamic accumulation of LC-CoA molecules activate the IKKβ pathway resulting in impaired insulin and leptin signal transduction. In order to determine whether LC-CoA accumulation and activation of IKKβ occured in a time-frame consistent with a role in the development of hypothalamic insulin and leptin resistance and obesity, a time-course of high-fat diet-induced obesity was established in this rodent model. In this model, a statistically significant increase in adiposity is consistently observed by day 3 of *ad libitum* HF (45%kcal fat) feeding compared to LF fed controls. Based on this time-course of HF DIO, hypothalamic LC-CoA content and IKKβ activity were assessed at time-points before (2d), concurrent with (3d), and after (7d) adiposity diverged to establish a basis for causality versus consequence of these parameters in the development of HF diet-induced hypothalamic insulin and leptin resistance and obesity. Significant hypothalamic accumulation of LC-CoA molecules was observed by day seven of HF feeding, *after* adiposity diverged. These data indicate that hypothalamic accumulation of LC-CoA is not required for the initial development of HF DIO but may be necessary for the persistence of obesity. According to my hypothesis, the accumulation of intracellular LC-CoA molecules induces activation of IKKβ signaling. If this hypothesis

is correct, a similar pattern of IKKβ activity would be expected to occur with HF feeding. However, pIKKβ levels were significantly elevated after two and three days of HF feeding; time-points prior to accumulation of hypothalamic LC-CoAs and prior to increased adiposity. Together these data suggest that 1) hypothalamic LC-CoA accumulation and IKKβ activity are temporally uncoupled with HF feeding at the onset of obesity, 2) hypothalamic activation of IKKβ, but not LC-CoA accumulation, occurs in a time-frame consistent with a potential role in the development of obesity, and 3) hypothalamic activation of IKKβ is bimodal.

To determine whether hypothalamic accumulation of LC-CoA molecules and/or activation of IKKβ could potentially play a role in the development of hypothalamic insulin and leptin resistance, I assessed activation of the respective downstream signaling molecules PKB and Stat3 following icv infusion of insulin or leptin. The adiposity signals insulin and leptin are thought to have largely redundant functions and tightly coupled signaling pathways in the hypothalamus. However, in these studies the sensitivities of these two signals were uncoupled at the onset of HF DIO in this rodent model. Hypothalamic insulin signaling remained intact prior to (2d) and concurrent with (3d) the onset of obesity, but was completely blunted after adiposity diverged (7d). In contrast, hypothalamic leptin signaling was impaired at time-points prior to (2d) and concurrent with (3d) the onset of obesity, but was restored to levels observed in the LF fed controls after seven days of diet. Thus, while insulin and leptin both function as adiposity negative feedback signals within the hypothalamus, the development of HF diet-induced hypothalamic insulin and leptin resistance are temporally and potentially mechanistically distinct. The pattern of hypothalamic insulin resistance corresponded with the pattern of LC-CoA accumulation such that when LC-CoA content was elevated, insulin signaling was impaired. Conversely, the pattern of hypothalamic leptin resistance corresponded with the pattern of IKKβ activity such that when pIKKβ was increased, leptin signaling

was impaired. Together, these results suggest that the accumulation of LC-CoA may be mechanistically involved in the development of HF diet-induced hypothalamic insulin resistance, whereas activation of IKKβ may be mechanistically involved in the development of HF diet-induced hypothalamic leptin resistance. In addition, these results indicate that hypothalamic leptin, but not insulin, resistance occurs in a time-frame consistent with a causative role in the development of HF DIO and may be the primary determinant in HF DIO.

To confirm the results observed in the insulin and leptin signaling studies, I assessed hypothalamic sensitivity indirectly by determining the ability of a short-term satiety signal, CCK, to modulate food intake. Studies have shown that in the setting of hypothalamic insulin and leptin resistance, the hindbrain is no longer sensitive to the effect of CCK to reduce meal size [123]. Peripheral administration of CCK failed to reduce food intake in the HF group at time-points prior to (2d) and concurrent with (3d) increased adiposity. This is consistent with the presence of hypothalamic resistance at these time-points and corresponds with activation of IKKβ and impaired leptin signaling. However, CCK administration effectively reduced food intake in HF fed animals after seven days of diet; when hypothalamic leptin, but not insulin signaling, was intact. This raises the possibility that hypothalamic modulation of the hindbrain response to CCK is dependent upon leptin, but not insulin, signaling. This possibility is supported by evidence in the literature demonstrating a synergistic relationship between leptin and CCK in the regulation of food intake [14, 193].

To determine whether activation of IKKβ, associated with impaired leptin signaling at the onset of HF DIO, directly alters hypothalamic regulation of energy homeostasis, I investigated the effects of IKKβ activity on food intake. Central administration of a pharmacological IKK inhibitor significantly reduced 24 hour food intake in HF fed animals at time-points consistent with elevated IKKβ activity and

impaired leptin signaling, but had no effect on food intake at seven days of HF feeding; when IKKβ activity was at basal levels and leptin signaling was intact. These data support a direct role of IKKβ activity in the development of HF diet-induced hypothalamic leptin resistance and disrupted energy homeostasis.

Together, the results from these studies both support and contradict my original hypothesis that HF DIO occurs via a mechanism in which hypothalamic accumulation of LC-CoA activates IKKβ and impairs hypothalamic insulin and leptin signaling, resulting in disrupted energy homeostasis and the development of obesity. These findings are summarized in the figure below (Figure 34) illustrating the relationship between hypothalamic insulin and leptin sensitivity to hypothalamic LC-CoA accumulation, IKKβ activity, and CCK sensitivity over the course of HF diet-induced obesity. Together these data suggest that 1) LC-CoA accumulation and IKKβ activity are temporally and mechanistically uncoupled in the hypothalamus at the onset of DIO; 2) insulin and leptin resistance are temporally and mechanistically uncoupled in the hypothalamus at the onset of DIO; and 3) suggests leptin, but not insulin, resistance is primary in the development of obesity.

In light of the conclusions drawn from these findings, there are two important points to discuss. First, in these studies HF DIO was defined to occur when adiposity diverged (3d of diet). However, HF diet induced obesity results in both increased adiposity as well as increased body weight in free-feeding rodents. The presence of both increased adiposity and increased body weight was observed between six and seven days of HF feeding; the time-point at which increased LC-CoA content and impaired insulin signaling were observed. Thus, although accumulation of LC-CoA and impaired insulin signaling did not occur in a time-frame consistent with a causative role in the development of HF DIO as defined by increased adiposity, these changes may still be an important determinant in the development of both increased adiposity and body

weight. Second, hypothalamic insulin and leptin signaling were assessed following direct infusion of these hormones into the third ventricle. This route of administration bypasses a potentially critical step in signal transduction, transport across the blood-brain barrier. There is some experimental evidence to suggest that HF diets impair receptor-mediated transport of insulin and leptin across the BBB [11, 82]. Thus, hypothalamic resistance may potentially occur prior to the time-points observed in these studies due to a reduction in transport across the BBB with HF feeding. Specifically, hypothalamic insulin resistance may occur prior to seven days of HF feeding which could potentially indicate a more prominent role of hypothalamic insulin signaling in the development of HF DIO.

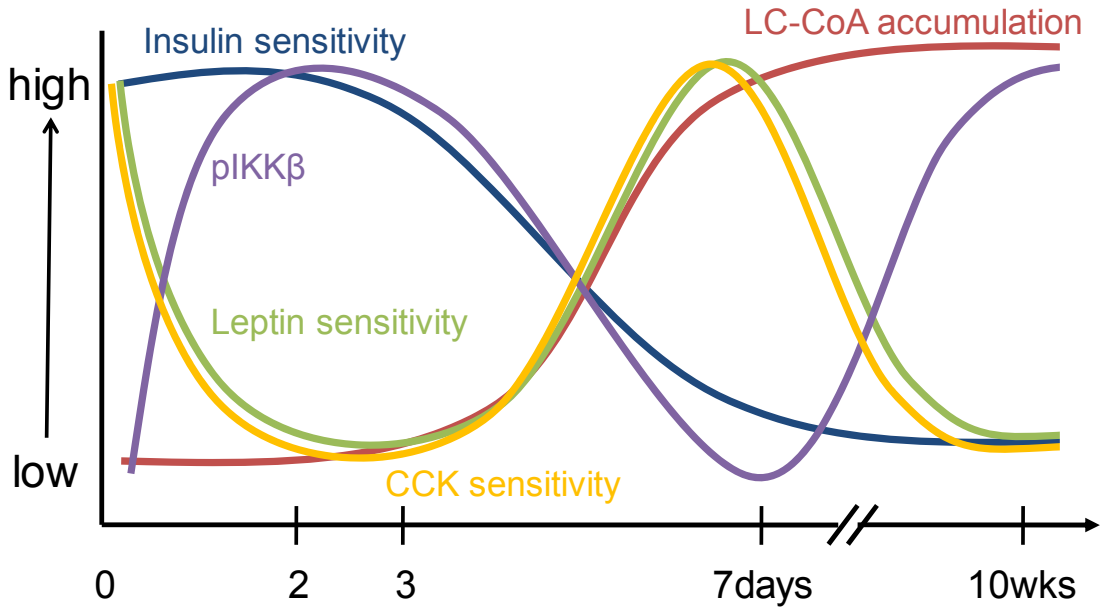


Figure 34: Summary of Relative Changes in Key Molecular Mediators in the Pathogenesis of Diet-Induced Obesity with High-Fat Feeding.

Pattern of high-fat diet induced changes in LC-CoA (red line), pIKKβ (purple line), hypothalamic insulin (blue line) and leptin (green line) sensitivity, and CCK sensitivity (gold line) relative to low-fat fed controls in the development of obesity.

CHAPTER VII

SUMMARY AND FUTURE DIRECTIONS

Obesity has rapidly become a worldwide epidemic with a seemingly uncontrollable rise in prevalence over the last two decades. Evidence suggests that in non-obese individuals, caloric intake is closely matched to energy expenditure to maintain stable body weight and adiposity over time. The regulation of body adiposity can be modeled as a classical endocrine feedback loop which involves the "adiposity signals" insulin and leptin and the CNS. These hormones normally circulate in proportion to body fat mass and directly interact with key neuronal subsets within the CNS, one of these being the arcuate nucleus (ARC) of the mediobasal hypothalamus. Insulin and leptin responsive neurons function coordinately in a series of neural circuits that allow the CNS to co-regulate various aspects of energy homeostasis including food intake and energy expenditure to promote the stability of body fat stores. Conversely, obesity is a state of dysregulated energy homeostasis characterized by hypothalamic resistance to the adiporegulatory effects of insulin and leptin. Understanding the mechanism of hypothalamic resistance is integral in developing strategies to control the obesity epidemic.

While many factors are implicated in the development of obesity, dietary fat remains one of the most potent predictors of obesity [152, 197]. In chapter III, I examined the obesogenic potential of various dietary fats. Results from these studies demonstrated that dietary fats possess intrinsic obesogenic properties in a saturation dependent manner and specifically implicated saturated fatty acids as the most potent mediator of the deleterious effects of HF feeding. Furthermore, a diet enriched in

saturated fat, independent of excess nutrient intake, is sufficient to induce hypothalamic resistance to the negative feedback adiposity signals and increase adiposity.

To determine whether the deleterious effects of high-fat feeding are specifically attributable to direct action of saturated fat within the CNS; in Chapter IV I investigated the direct effects of dietary fat in the brain of lean chow fed animals. More specifically, whether exposure of the hypothalamus to excess saturated fat leads to hypothalamic accumulation of LC-CoAs that trigger inflammatory signaling (elevated IKKβ activity) and blunt insulin signaling in lean chow fed rats given an acute icv infusion of palmitate or oleate. In these studies infusion of a saturated, but not unsaturated fat, mimicked the effects of HF feeding to increase hypothalamic content of LC-CoA molecules, and increase IKKβ activity in lean chow fed rats. Concomitant with increased LC-CoA content and elevated IKKβ activity, icv palmitate infusion (i.e. saturated fatty acid) was sufficient to significantly blunt the ability of insulin to activate the downstream signaling molecule PKB in the hypothalamus. These results demonstrated a clear association between increased LC-CoA content and IKKβ activity with impaired hypothalamic insulin signaling. That pharmacological icv inhibition of IKK potently reduced food intake in obese, HF fed rats characterized by increased IKKβ activity and hypothalamic insulin resistance, but had no effect on food intake in LF fed rats (animals characterized by relatively low hypothalamic IKKβ activity and hypothalamic insulin sensitivity) suggested a direct role of IKKβ on insulin action to reduce food intake. Furthermore, hypothalamic insulin signaling was enhanced in HF fed rats pretreated with the IKK inhibitor. These results support a model similar to that of peripheral insulin resistance in which cellular exposure to excess nutrients, particularly saturated fat, triggers cellular inflammation and insulin resistance that in turn contributes to impaired energy homeostasis and the development of obesity. The ability of specific fatty acids to modulate the activation of inflammation may represent a potential mechanism of high-fat diet-induced hypothalamic

resistance and obesity. These structural effects led me to identify potential “fat sensing” target molecules that may be involved in the development of HF diet-induced obesity.

Since fatty acids are known to function as endogenous ligands for various receptors in a structure dependent manner; in Chapter V I investigated the role of the innate immune receptor, TLR4, in the development of high-fat diet-induced hypothalamic resistance and obesity. TLR4 was identified as a candidate in the mechanism of high-fat diet-induced hypothalamic resistance and obesity since it is 1) expressed in central tissues associated with the regulation of energy homeostasis, 2) is regulated by dietary fat in a saturation dependent manner, and 3) initiates an inflammatory signaling cascade associated with insulin resistance. Body weight and adiposity were significantly reduced inTLR4 deficient mice compared to WT controls; an effect associated with reduced plasma and tissue markers of inflammation in TLR4 deficient mice. In line with my hypothesis, a reduction in inflammatory molecules may lead to improved hypothalamic insulin and leptin signaling and promote energy homeostasis.

The phenotype of decreased body weight and adiposity in TLR4 deficient mice compared to their respective WT controls suggested a potential role for TLR4 in the regulation of adipose stores. Although these results suggested that TLR4 mediated inflammatory signaling within the hypothalamus contributed, at least in part, to modulation of energy homeostatic pathways and adipose accumulation; based on our observations it was not possible to rule out other pathways and mechanisms involved in the development of HF diet-induced hypothalamic resistance and obesity.

Whether hypothalamic resistance occurs early in the course of HF feeding and actively contributes to increased food intake, adiposity, and peripheral insulin resistance characteristic of DIO remains an important unanswered question. Data presented herein have demonstrated that both chronic and acute exposure to saturated fatty acids increase hypothalamic LC-CoA content and IKKβ activity in rats characterized by

impaired insulin and leptin signaling. In chapter VI, I extended these findings to the onset of DIO to determine whether these changes are mechanistically involved in the development of hypothalamic insulin and leptin resistance and obesity. I hypothesized that dietary saturated fat induces hypothalamic insulin and leptin resistance via a mechanism of LC-CoA induced inflammatory signaling, resulting in disrupted energy homeostasis and the development of obesity and peripheral insulin resistance. Results from these studies both supported and contradicted several aspects of this hypothesis. While insulin and leptin both function as adiposity negative feedback signals within the hypothalamus, the development of HF diet-induced hypothalamic insulin and leptin resistance were temporally and potentially mechanistically distinct. Long-Chain CoA accumulation appeared to be mechanistically involved in the development of insulin resistance, whereas activation of IKKβ appeared to be mechanistically involved in the development of leptin resistance. Furthermore, hypothalamic leptin, but not insulin, resistance occurred in a time-frame consistent with a causative role in the development of HF DIO.

While this body of work has focused on the role of long chain saturated fat in the development of diet-induced obesity; several other interesting questions became evident based on our findings that warrant further investigation. For example, when animals were fed the HF diet restricted to the caloric intake of the LF fed animals (i.e. pair-fed), total body weight gain was similar to the LF group yet the proportion of energy stored as adipose tissue was significantly elevated. This difference in adiposity for the same caloric intake may be explained by fuel partitioning, the allocation of metabolic fuels among tissues and metabolic pathways. Under normal circumstances, fuel partitioning serves to maintain a steady supply of energy-yielding substrates to meet the needs of various tissues through the balance between fuel oxidation and storage [65]. However, disturbances in fuel partitioning compromises energy balance such that an imbalance in

favor of fuel storage results in obesity [65]. Given the differences observed in the obesogenic potential of the various fatty acid species we investigated, it is intriguing to ask whether omega-3 or polyunsaturated fatty acids would have a different effect on weight gain and adipose storage under the pair-feeding conditions. Furthermore, it would be exciting to investigate the mechanisms involved in determining the metabolic fate of ingested calories and how the lipid composition alters these mechanisms, either via direct or indirect pathways and either centrally, peripherally, or a combination of both.

One of these potential targets of lipid action is the innate immune receptor TLR4. Although we observed a reduction in whole body inflammation and improved body composition in TLR4 deficient mice compared to WT mice on both diets, it is not possible to attribute these effects entirely to improved central energy homeostatic signaling since these animals were globally deficient in TLR4. Thus, it would be beneficial to determine the contribution of TLR4 signaling specifically in the hypothalamus to HF diet-induced insulin and leptin resistance and obesity via generation of brain specific TLR4 knock-out mice or use of icv pharmacological agonists and antagonists. In fact, a recently published paper investigated the effects of daily *ip* administration of a TLR4-inhibiting antibody in rats fed a HF diet for 8 weeks. Results from these studies demonstrated that inhibition of TLR4 attenuated weight gain and activation of inflammatory cytokines associated with HF feeding [116]. Furthermore, Milanski *et al.* suggests that TLR4 acts as a predominant molecular target for saturated fatty acids in the hypothalamus and is an important mediator of hypothalamic dysfunction during the development of obesity [116].

However, recall that TLR4 deficiency did not completely protect mice against HF diet-induced increases in inflammation, body weight, and adiposity in our studies. This suggests that additional pathways may be involved in the effects of dietary lipids to induce inflammation and obesity. Studies have provided strong evidence for the

contribution of endoplasmic reticulum stress (ER stress) as a mechanism linking the consumption of HF diets and obesity to insulin resistance [129, 138]. ER stress can be induced by metabolic and nutritional factors such as high levels of glucose and lipids and stimulates cytokine production [138]. Cytokine production induced by ER stress could potentially lead to both insulin and leptin resistance in the hypothalamus making this an interesting target for further research. Along these lines, a paper published within the last several months, reported that increased hypothalamic ER stress inhibits leptin receptor signaling and augments HF diet-induced obesity [137].

Another question which remains to be addressed is how dietary lipids ultimately induce a signal within the hypothalamus and alter energy homeostasis within a relatively short period of time at the onset of obesity. Upon ingestion of a HF meal, fatty acids are transported via chylomicrons as trigylcerides to the liver where they are processed and exported to the circulation attached to lipoprotein particles (VLDL). Fatty acids then enter various tissues through a receptor mediated process. Lipoprotein receptors are expressed at the blood-brain-barrier and may be involved in a direct effect of dietary fatty acids in the development of hypothalamic resistance and impaired energy homeostasis. However the contribution of fatty acids from lipoproteins is thought to be a small proportion of total circulating fatty acids. The majority of circulating plasma fatty acids is derived from adipose tissue and closely reflects the composition of lipid stores in the adipose tissue. Adipose stores are relatively stable and changes in dietary lipid composition would take weeks to months to become reflected in these pools. Thus, the short-term effects of HF feeding are not likely due to direct effects of free fatty acids in the hypothalamus but may be mediated by indirect effects of HF feeding. One possibility could be the presence of a gut-derived signal, either a circulating factor or a neurally mediated signal that changes in response to the ingestion of fat and alters hypothalamic insulin and leptin sensitivity. While elucidation of a gut-derived signal is outside the

scope of this project, whether the effect of dietary fatty acids on hypothalamic sensitivity is mediated directly or indirectly is an important question in the mechanism of HF DIO.

Potentially the most interesting finding from this work, however, is the disconnect between the development of insulin and leptin resistance at the onset of obesity. One limitation of these studies though is that the method used to assess insulin and leptin sensitivity (icv infusion and downstream signaling) bypasses a potential site of resistance at the level of hormone transport across the blood-brain barrier. Studies to address transport as well as signaling could potentially reveal a different pattern of insulin and leptin resistance than reported here (i.e. insulin resistance at earlier time-points due to impaired transport) and warrants further investigation. Leptin transport and action in the hypothalamus could possibly be assessed by peripheral administration of a leptin dose and measurement of the food intake response and activation of the signaling cascade. However, the leptin dose required to induce a hypothalamic response is cost prohibitive in rats. Furthermore, this technique would not be suitable to test insulin transport and action in the hypothalamus due to the hypoglycemic effects of a peripheral insulin dose. Instead, insulin transport and action in the hypothalamus could be assessed following a glucose bolus or following a fasting-refeeding paradigm to induce pancreatic insulin secretion. However, the potency of these methods to stimulate insulin secretion can vary across groups and the results not be directly comparable. Clearly these approaches contain inherent flaws as well. However, transport across the blood-brain barrier is still an important component of hypothalamic insulin and leptin action to address at the onset of HF diet-induced obesity as evidence indicates that hormones and circulating metabolites including nutritional status and triglycerides modulate the blood-brain barrier transport system [9, 84].

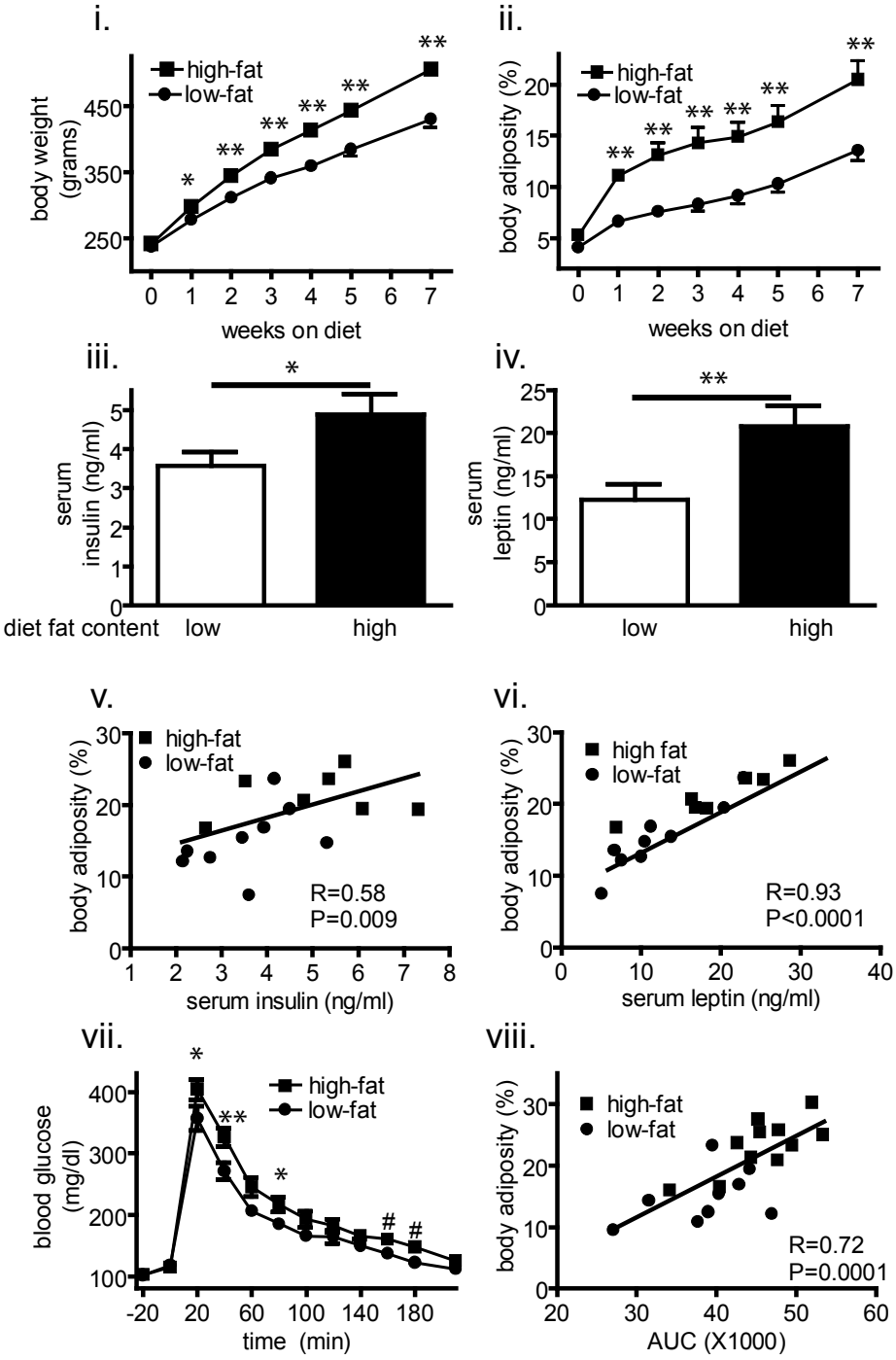
In addition to the disconnect between the development of insulin and leptin resistance is the bimodal response in leptin sensitivity we observed in our studies with

impaired leptin signaling at early and late time-points (day 2,3 and wk10) in the development of obesity and intact signaling at the intermediate time-point (day 7). One potential explanation for the early impairment in leptin signaling with high-fat feeding could be evolutionary in origin. Under conditions of intermittent food supplies, the ability to adapt to starvation is fundamentally important to the survival of the species. Along these lines, evidence suggests that the dominant role of leptin occurs in response to starvation to prevent depletion of energy stores [3]. With the availability of an energy-dense food source, the apparent leptin "resistance" may actually be a permissive response to temporarily allow overconsumption of the high-fat diet as a survival mechanism for future periods of prolonged fasting despite the overabundance of nutrient availability today and may partially explain genetic susceptibility to obesity. Another potential explanation for leptin resistance is the influence of non-homeostatic factors in the control of food intake including palatability and novelty in rodents [22]. It has been hypothesized that cortico-limbic processes can override homeostatic regulatory circuits via neural projections between the accumbens and the hypothalamus [22]. Leptin signaling was restored following the initial exposure to high-fat diet and increase in food intake (day 7), possibly as these initial responses were attenuated, but was again impaired after prolonged exposure to high-fat diet. The leptin resistance following prolonged exposure to high-fat diet may represent a "pathological" resistance in response to dietary fat and occur via a different mechanism. Insight into both the early and late mechanisms of leptin resistance may prove beneficial in the development of pharmacological treatments for obesity.

In conclusion, this body of work has extended previous studies in our laboratory investigating hypothalamic insulin and leptin resistance in HF fed obese rats and describes potential mechanisms involved in the development of HF diet-induced hypothalamic resistance and obesity. However, it is evident that more work is required

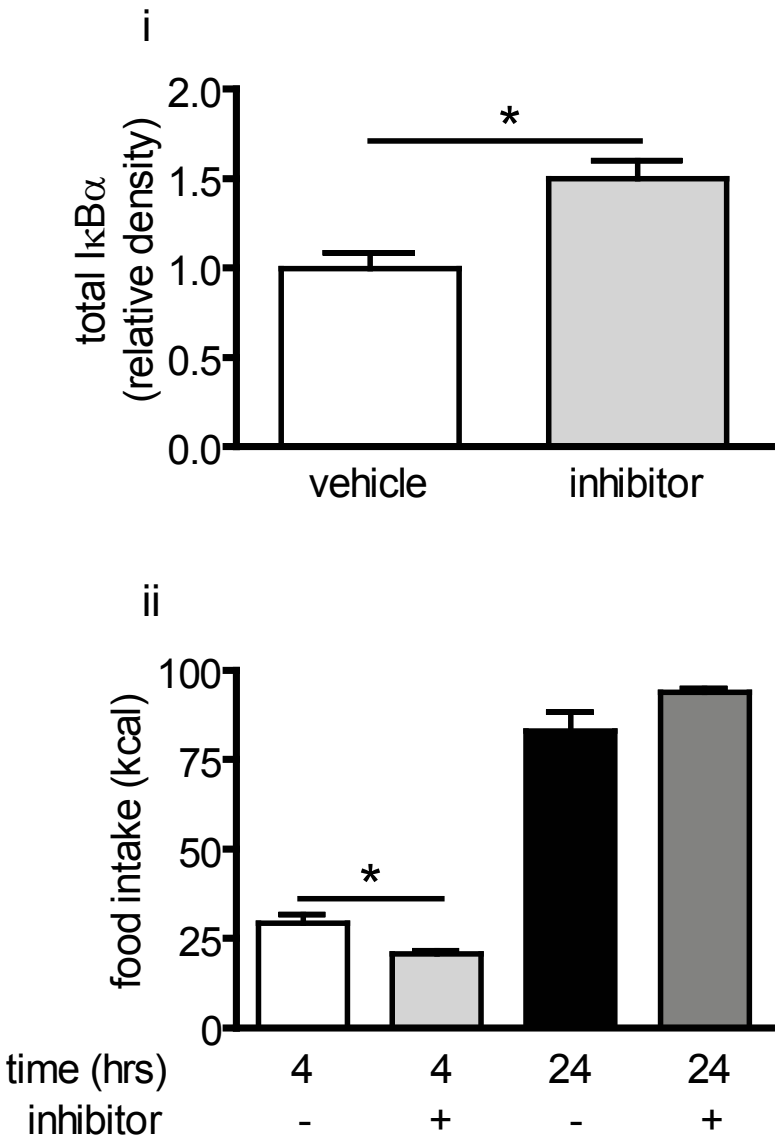
to tease out the exact mechanisms of hypothalamic insulin and leptin resistance as well as the distinct roles of insulin and leptin function as adiposity signals in the hypothalamus. Further elucidation of the mechanisms involved in the development of diet-induced hypothalamic insulin and leptin resistance and distinct functional roles of these adiposity hormones will aid in potential therapeutic treatments to curb the obesity epidemic.

Appendix A. Baseline Metabolic Parameters of High-Fat Diet-Induced Obesity Model in Rats



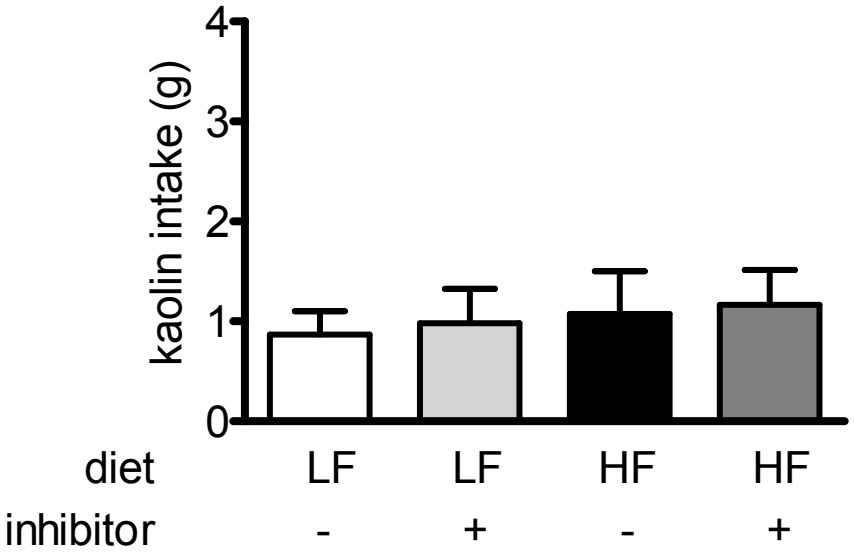
Baseline metabolic parameters in a rat model of HF DIO. Effect of high-fat feeding on body weight (i), body adiposity (ii) and plasma insulin (iii) and leptin (iv) levels compared to low-fat fed controls. Plasma insulin (v) and leptin (vi) levels across groups are significantly correlated with changes in body adiposity, as is area under the curve (AUC, viii) of the glucose excursion curve during an intraperitoneal glucose tolerance test (vii). * p<0.05, **p<0.01.

Appendix B. Efficacy and Dose Response of IKK Inhibition



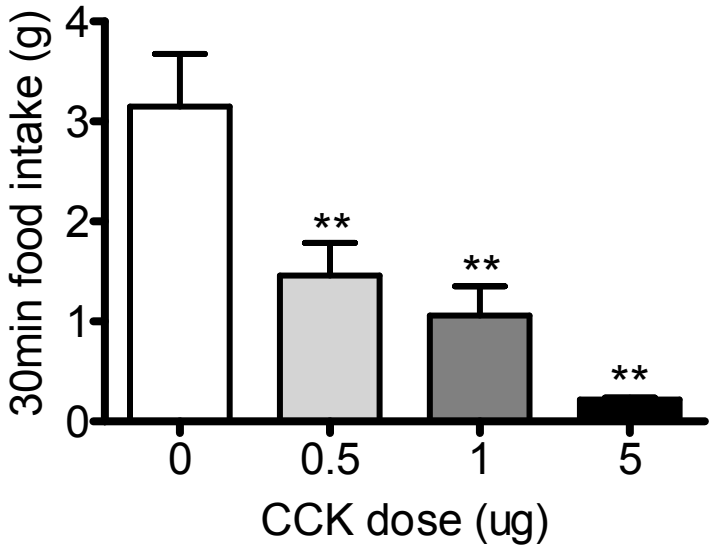
Pharmacological inhibition of IKK was confirmed by measurement of IκBα protein levels following treatment with PS-1145 (3μg). Efficacy of IKK inhibition was dose dependent as a lower dose reduced food intake at 4hr but not 24hr (3μg vs 10μg shown to reduce both 4 and 24hr food intake). * $p<0.05$

Appendix C. Kaolin Intake Test of Visceral Illness with IKK Inhibition



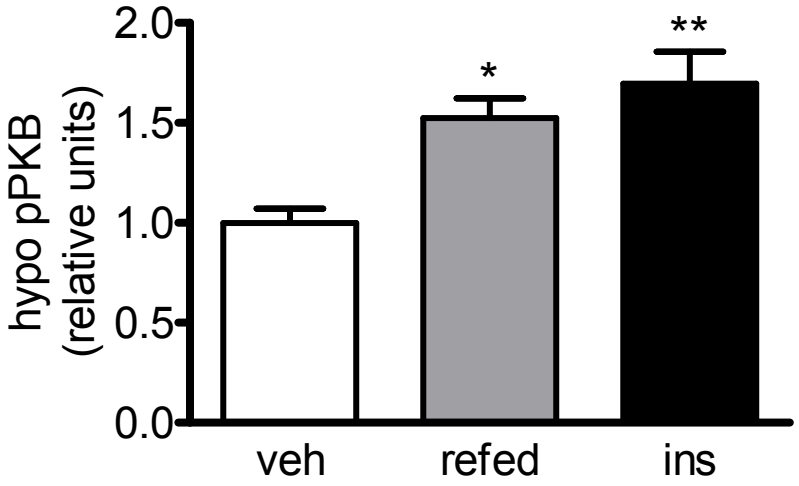
Effect of IKK inhibitor treatment on 24hr kaolin consumption, a marker of “visceral” or non-specific illness.

Appendix D. CCK Dose Response



Effect of intraperitoneal CCK on 30min food intake in 4hr fasted rats. ** $p<0.01$ compared to vehicle control (0ug dose).

Appendix E. Comparison of Pharmacological ICV Insulin Infusion and Physiological Insulin Response to a Fasting-Refeeding Cycle



Hypothalamic phosphorylation of PKB in response to physiological (refed, 4hr access to high-carb diet after fast) and pharmacological (ins, 10mu insulin icv) insulin stimulation in 24hr fasted rats compared to vehicle controls (veh). * $p<0.05$, ** $p<0.01$.

REFERENCES

1. *Chartbook on Trends in the Health of Americans Health*. National Center for Health Statistics 2006, Hyattsville, MD: Public Health Service.

2. *Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults-The Evidence Report*. 1998, Bethesda, MD: National Institutes of Health, National Heart, Lung, and Blood Institute.

3. Ahima, R.S., et al., *Role of leptin in the neuroendocrine response to fasting.* Nature, 1996. **382**(6588): p. 250-2.

4. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.

5. Argetsinger, L.S., et al., *Autophosphorylation of JAK2 on tyrosines 221 and 570 regulates its activity.* Mol Cell Biol, 2004. **24**(11): p. 4955-67.

6. Astrup, A., *Healthy lifestyles in Europe: prevention of obesity and type II diabetes by diet and physical activity.* Public Health Nutr, 2001. **4**(2B): p. 499-515.

7. Bagdade, J.D., E.L. Bierman, and D. Porte, Jr., *The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and nondiabetic subjects.* J Clin Invest, 1967. **46**(10): p. 1549-57.

8. Banks, A.S., et al., *Activation of downstream signals by the long form of the leptin receptor.* J Biol Chem, 2000. **275**(19): p. 14563-72.

9. Banks, W.A., et al., *Triglycerides induce leptin resistance at the blood-brain barrier.* Diabetes, 2004. **53**(5): p. 1253-60.

10. Banks, W.A., C.R. DiPalma, and C.L. Farrell, *Impaired transport of leptin across the blood-brain barrier in obesity.* Peptides, 1999. **20**(11): p. 1341-5.

11. Banks, W.A., S.A. Farr, and J.E. Morley, *The effects of high fat diets on the blood-brain barrier transport of leptin: failure or adaptation?* Physiol Behav, 2006. **88**(3): p. 244-8.

12. Banks, W.A., et al., *Leptin enters the brain by a saturable system independent of insulin.* Peptides, 1996. **17**(2): p. 305-11.

13. Baron, S.H., *Salicylates as hypoglycemic agents.* Diabetes Care, 1982. **5**(1): p. 64-71.

14. Barrachina, M.D., et al., *Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice.* Proc Natl Acad Sci U S A, 1997. **94**(19): p. 10455-60.

15. Barros, R.P., U.F. Machado, and J.A. Gustafsson, *Estrogen receptors: new players in diabetes mellitus.* Trends Mol Med, 2006. **12**(9): p. 425-31.

16. Baskin, D.G., et al., *Insulin and insulin-like growth factors in the CNS.* Trends Neurosci, 1988. **11**(3): p. 107-11.

17. Bates, S.H., et al., *STAT3 signalling is required for leptin regulation of energy balance but not reproduction.* Nature, 2003. **421**(6925): p. 856-9.

18. Baura, G.D., et al., *Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain.* J Clin Invest, 1993. **92**(4): p. 1824-30.

19. Beck, B., et al., *Rapid and localized alterations of neuropeptide Y in discrete hypothalamic nuclei with feeding status.* Brain Res, 1990. **528**(2): p. 245-9.

20. Benoit, S.C., et al., *The catabolic action of insulin in the brain is mediated by melanocortins.* J Neurosci, 2002. **22**(20): p. 9048-52.

21. Bernstein, I.L., et al., *Effect of force-feeding upon basal insulin levels of rats.* Proc Soc Exp Biol Med, 1975. **150**(2): p. 546-8.

22. Berthoud, H.R., *Homeostatic and non-homeostatic pathways involved in the control of food intake and energy balance.* Obesity (Silver Spring), 2006. **14 Suppl 5**: p. 197S-200S.

23. Berthoud, H.R., *Multiple neural systems controlling food intake and body weight.* Neurosci Biobehav Rev, 2002. **26**(4): p. 393-428.

24. Billington, C.J., et al., *Effects of intracerebroventricular injection of neuropeptide Y on energy metabolism.* Am J Physiol, 1991. **260**(2 Pt 2): p. R321-7.

25. Bjorbaek, C. and B.B. Kahn, *Leptin signaling in the central nervous system and the periphery.* Recent Prog Horm Res, 2004. **59**: p. 305-31.

26. Bjorbak, C., et al., *SOCS3 mediates feedback inhibition of the leptin receptor via Tyr985.* J Biol Chem, 2000. **275**(51): p. 40649-57.

27. Blok, W.L., M.B. Katan, and J.W. van der Meer, *Modulation of inflammation and cytokine production by dietary (n-3) fatty acids.* J Nutr, 1996. **126**(6): p. 1515-33.

28. Boden, G., et al., *Effect of fasting on serum leptin in normal human subjects.* J Clin Endocrinol Metab, 1996. **81**(9): p. 3419-23.

29. Brady, L.S., et al., *Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats.* Neuroendocrinology, 1990. **52**(5): p. 441-7.

30. Bray, G.A., *Obesity: the disease.* J Med Chem, 2006. **49**(14): p. 4001-7.

31. Bray, G.A., S. Paeratakul, and B.M. Popkin, *Dietary fat and obesity: a review of animal, clinical and epidemiological studies.* Physiol Behav, 2004. **83**(4): p. 549-55.

32. Broadwell, R.D. and M.W. Brightman, *Entry of peroxidase into neurons of the central and peripheral nervous systems from extracerebral and cerebral blood.* J Comp Neurol, 1976. **166**(3): p. 257-83.

33. Bruning, J.C., et al., *Role of brain insulin receptor in control of body weight and reproduction.* Science, 2000. **289**(5487): p. 2122-5.

34. Bsibsi, M., et al., *Broad expression of Toll-like receptors in the human central nervous system.* J Neuropathol Exp Neurol, 2002. **61**(11): p. 1013-21.

35. Butler, A.A. and R.D. Cone, *The melanocortin receptors: lessons from knockout models.* Neuropeptides, 2002. **36**(2-3): p. 77-84.

36. Cai, D., et al., *Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB.* Nat Med, 2005. **11**(2): p. 183-90.

37. Cheung, C.C., D.K. Clifton, and R.A. Steiner, *Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus.* Endocrinology, 1997. **138**(10): p. 4489-92.

38. Chew, G.T., S.K. Gan, and G.F. Watts, *Revisiting the metabolic syndrome.* Med J Aust, 2006. **185**(8): p. 445-9.

39. Clark, J.T., et al., *Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats.* Endocrinology, 1984. **115**(1): p. 427-9.

40. Clerk, L.H., S. Rattigan, and M.G. Clark, *Lipid infusion impairs physiologic insulin-mediated capillary recruitment and muscle glucose uptake in vivo.* Diabetes, 2002. **51**(4): p. 1138-45.

41. Cnop, M., *Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes.* Biochem Soc Trans, 2008. **36**(Pt 3): p. 348-52.

42. Coiffier, E., R. Paris, and J. Lecerf, *Effects of dietary saturated and polyunsaturated fat on lipoprotein lipase and hepatic triglyceride lipase activity.* Comp Biochem Physiol B, 1987. **88**(1): p. 187-92.

43. Coleman, D.L., *Effects of parabiosis of obese with diabetes and normal mice.* Diabetologia, 1973. **9**(4): p. 294-8.

44. Coleman, D.L., *Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice.* Diabetologia, 1978. **14**(3): p. 141-8.

45. Cone, R.D., *Anatomy and regulation of the central melanocortin system.* Nat Neurosci, 2005. **8**(5): p. 571-8.

46. Considine, R.V., et al., *Serum immunoreactive-leptin concentrations in normal-weight and obese humans.* N Engl J Med, 1996. **334**(5): p. 292-5.

47. Cousin, S.P., et al., *Free fatty acid-induced inhibition of glucose and insulin-like growth factor I-induced deoxyribonucleic acid synthesis in the pancreatic beta-cell line INS-1.* Endocrinology, 2001. **142**(1): p. 229-40.

48. Cowley, M.A., et al., *Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus.* Nature, 2001. **411**(6836): p. 480-4.

49. Cummings, D.E., *Ghrelin and the short- and long-term regulation of appetite and body weight.* Physiol Behav, 2006. **89**(1): p. 71-84.

50. Cummings, D.E., et al., *Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues.* Am J Physiol Endocrinol Metab, 2004. **287**(2): p. E297-304.

51. Cusin, I., et al., *The weight-reducing effect of an intracerebroventricular bolus injection of leptin in genetically obese fa/fa rats. Reduced sensitivity compared with lean animals.* Diabetes, 1996. **45**(10): p. 1446-50.

52. De Souza, C.T., et al., *Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus.* Endocrinology, 2005. **146**(10): p. 4192-9.

53. Deutsch, J., et al., *Isolation and quantitation of long-chain acyl-coenzyme A esters in brain tissue by solid-phase extraction.* Anal Biochem, 1994. **220**(2): p. 321-3.

54. Drucker, D.J., *The biology of incretin hormones.* Cell Metab, 2006. **3**(3): p. 153-65.

55. Duncan, B.B., et al., *Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study.* Diabetes, 2003. **52**(7): p. 1799-805.

56. Egawa, M., H. Yoshimatsu, and G.A. Bray, *Neuropeptide Y suppresses sympathetic activity to interscapular brown adipose tissue in rats.* Am J Physiol, 1991. **260**(2 Pt 2): p. R328-34.

57. El-Haschimi, K., et al., *Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity.* J Clin Invest, 2000. **105**(12): p. 1827-32.

58. Ellacott, K.L. and R.D. Cone, *The central melanocortin system and the integration of short- and long-term regulators of energy homeostasis.* Recent Prog Horm Res, 2004. **59**: p. 395-408.

59. Elmquist, J.K., *Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin.* Physiol Behav, 2001. **74**(4-5): p. 703-8.

60. Elmquist, J.K., et al., *Distributions of leptin receptor mRNA isoforms in the rat brain.* J Comp Neurol, 1998. **395**(4): p. 535-47.

61. Erickson, J.C., et al., *Endocrine function of neuropeptide Y knockout mice.* Regul Pept, 1997. **70**(2-3): p. 199-202.

62. Fei, H., et al., *Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues.* Proc Natl Acad Sci U S A, 1997. **94**(13): p. 7001-5.

63. Finkelstein, E.A., I.C. Fiebelkorn, and G. Wang, *National medical spending attributable to overweight and obesity: how much, and who's paying?* Health Aff (Millwood), 2003. **Suppl Web Exclusives**: p. W3-219-26.

64. Frederich, R.C., et al., *Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action.* Nat Med, 1995. **1**(12): p. 1311-4.

65. Friedman, M.I., *Fuel partitioning and food intake.* Am J Clin Nutr, 1998. **67**(3 Suppl): p. 513S-518S.

66. Gibbs, J., R.C. Young, and G.P. Smith, *Cholecystokinin decreases food intake in rats.* J Comp Physiol Psychol, 1973. **84**(3): p. 488-95.

67. Gil-Campos, M., et al., *Ghrelin: a hormone regulating food intake and energy homeostasis.* Br J Nutr, 2006. **96**(2): p. 201-26.

68. Giugliano, D., A. Ceriello, and K. Esposito, *The effects of diet on inflammation: emphasis on the metabolic syndrome.* J Am Coll Cardiol, 2006. **48**(4): p. 677-85.

69. Glaum, S.R., et al., *Leptin, the obese gene product, rapidly modulates synaptic transmission in the hypothalamus.* Mol Pharmacol, 1996. **50**(2): p. 230-5.

70. Golay, A. and E. Bobbioni, *The role of dietary fat in obesity.* Int J Obes Relat Metab Disord, 1997. **21 Suppl 3**: p. S2-11.

71. Griffin, M.E., et al., *Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade.* Diabetes, 1999. **48**(6): p. 1270-4.

72. Hahn, T.M., et al., *Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons.* Nat Neurosci, 1998. **1**(4): p. 271-2.

73. Hamilton, J.A. and K. Brunaldi, *A model for fatty acid transport into the brain.* J Mol Neurosci, 2007. **33**(1): p. 12-7.

74. Haynes, W.G., et al., *Sympathetic and cardiorenal actions of leptin.* Hypertension, 1997. **30**(3 Pt 2): p. 619-23.

75. Heine, P.A., et al., *Increased adipose tissue in male and female estrogen receptor-alpha knockout mice.* Proc Natl Acad Sci U S A, 2000. **97**(23): p. 12729-34.

76. Hotamisligil, G.S., *Inflammatory pathways and insulin action.* Int J Obes Relat Metab Disord, 2003. **27 Suppl 3**: p. S53-5.

77. Hotamisligil, G.S., et al., *Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance.* J Clin Invest, 1995. **95**(5): p. 2409-15.

78. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.

79. Houmard, J.A., et al., *Effect of weight loss on insulin sensitivity and intramuscular long-chain fatty acyl-CoAs in morbidly obese subjects.* Diabetes, 2002. **51**(10): p. 2959-63.

80. Isganaitis, E. and R.H. Lustig, *Fast food, central nervous system insulin resistance, and obesity.* Arterioscler Thromb Vasc Biol, 2005. **25**(12): p. 2451-62.

81. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.

82. Kaiyala, K.J., et al., *Obesity induced by a high-fat diet is associated with reduced brain insulin transport in dogs.* Diabetes, 2000. **49**(9): p. 1525-33.

83. Karhunen, L.J., et al., *Effect of protein, fat, carbohydrate and fibre on gastrointestinal peptide release in humans.* Regul Pept, 2008. **149**(1-3): p. 70-8.

84. Kastin, A.J. and W. Pan, *Dynamic regulation of leptin entry into brain by the blood-brain barrier.* Regul Pept, 2000. **92**(1-3): p. 37-43.

85. Kennedy, G.C., *The role of depot fat in the hypothalamic control of food intake in the rat.* Proc R Soc Lond B Biol Sci, 1953. **140**(901): p. 578-96.

86. Kersten, A., J.H. Strubbe, and N.J. Spiteri, *Meal patterning of rats with changes in day length and food availability.* Physiol Behav, 1980. **25**(6): p. 953-8.

87. Kim, J.H., R.A. Bachmann, and J. Chen, *Interleukin-6 and insulin resistance.* Vitam Horm, 2009. **80**: p. 613-33.

88. Kim, J.K., et al., *Prevention of fat-induced insulin resistance by salicylate.* J Clin Invest, 2001. **108**(3): p. 437-46.

89. Kitamura, T., et al., *Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake.* Nat Med, 2006. **12**(5): p. 534-40.

90. Kloek, C., et al., *Regulation of Jak kinases by intracellular leptin receptor sequences.* J Biol Chem, 2002. **277**(44): p. 41547-55.

91. Klok, M.D., S. Jakobsdottir, and M.L. Drent, *The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review.* Obes Rev, 2007. **8**(1): p. 21-34.

92. Korbonits, M., et al., *Ghrelin--a hormone with multiple functions.* Front Neuroendocrinol, 2004. **25**(1): p. 27-68.

93. Krssak, M., et al., *Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study.* Diabetologia, 1999. **42**(1): p. 113-6.

94. Kumagai, S., A. Holmang, and P. Bjorntorp, *The effects of oestrogen and progesterone on insulin sensitivity in female rats.* Acta Physiol Scand, 1993. **149**(1): p. 91-7.

95. Laflamme, N. and S. Rivest, *Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components.* Faseb J, 2001. **15**(1): p. 155-163.

96. Le Magnen, J. and S. Tallon, *[The spontaneous periodicity of ad libitum food intake in white rats].* J Physiol (Paris), 1966. **58**(3): p. 323-49.

97. Lee, G.H., et al., *Abnormal splicing of the leptin receptor in diabetic mice.* Nature, 1996. **379**(6566): p. 632-5.

98. Lee, J.Y., et al., *Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids.* J Lipid Res, 2003. **44**(3): p. 479-86.

99. Lee, J.Y., et al., *Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4.* J Biol Chem, 2001. **276**(20): p. 16683-9.

100. Lee, J.Y., et al., *Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids.* J Biol Chem, 2003. **278**(39): p. 37041-51.

101. Levin, B.E. and A.A. Dunn-Meynell, *Defense of body weight depends on dietary composition and palatability in rats with diet-induced obesity.* Am J Physiol Regul Integr Comp Physiol, 2002. **282**(1): p. R46-54.

102. Levin, B.E. and A.A. Dunn-Meynell, *Reduced central leptin sensitivity in rats with diet-induced obesity.* Am J Physiol Regul Integr Comp Physiol, 2002. **283**(4): p. R941-8.

103. Levine, A.S. and J.E. Morley, *Neuropeptide Y: a potent inducer of consummatory behavior in rats.* Peptides, 1984. **5**(6): p. 1025-9.

104. Li, J.Y., et al., *Agouti-related protein-like immunoreactivity: characterization of release from hypothalamic tissue and presence in serum.* Endocrinology, 2000. **141**(6): p. 1942-50.

105. Lin, S., et al., *Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice.* Int J Obes Relat Metab Disord, 2000. **24**(5): p. 639-46.

106. Lin, X., et al., *Protein kinase C-theta participates in NF-kappaB activation induced by CD3-CD28 costimulation through selective activation of IkappaB kinase beta.* Mol Cell Biol, 2000. **20**(8): p. 2933-40.

107. Louet, J.F., C. LeMay, and F. Mauvais-Jarvis, *Antidiabetic actions of estrogen: insight from human and genetic mouse models.* Curr Atheroscler Rep, 2004. **6**(3): p. 180-5.

108. Lu, D., et al., *Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor.* Nature, 1994. **371**(6500): p. 799-802.

109. Lu, Y.C., W.C. Yeh, and P.S. Ohashi, *LPS/TLR4 signal transduction pathway.* Cytokine, 2008. **42**(2): p. 145-51.

110. MacDougald, O.A., et al., *Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes.* Proc Natl Acad Sci U S A, 1995. **92**(20): p. 9034-7.

111. Madrid, L.V., et al., *Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38.* J Biol Chem, 2001. **276**(22): p. 18934-40.

112. Marsh, D.J., et al., *Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides.* Nat Genet, 1999. **21**(1): p. 119-22.

113. Matsuo, T., et al., *Sympathetic activity is lower in rats fed a beef tallow diet than in rats fed a safflower oil diet.* Metabolism, 1995. **44**(7): p. 934-9.

114. Mercer, J.G., et al., *Coexpression of leptin receptor and preproneuropeptide Y mRNA in arcuate nucleus of mouse hypothalamus.* J Neuroendocrinol, 1996. **8**(10): p. 733-5.

115. Mercer, J.G., et al., *Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization.* FEBS Lett, 1996. **387**(2-3): p. 113-6.

116. Milanski, M., et al., *Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity.* J Neurosci, 2009. **29**(2): p. 359-70.

117. Miller, J.C., J.M. Gnaedinger, and S.I. Rapoport, *Utilization of plasma fatty acid in rat brain: distribution of [14C]palmitate between oxidative and synthetic pathways.* J Neurochem, 1987. **49**(5): p. 1507-14.

118. Mitchell, D., et al., *Poison induced pica in rats.* Physiol Behav, 1976. **17**(4): p. 691-7.

119. Miyazaki, M., Y.C. Kim, and J.M. Ntambi, *A lipogenic diet in mice with a disruption of the stearoyl-CoA desaturase 1 gene reveals a stringent requirement of endogenous monounsaturated fatty acids for triglyceride synthesis.* J Lipid Res, 2001. **42**(7): p. 1018-24.

120. Mokdad, A.H., et al., *The continuing epidemics of obesity and diabetes in the United States.* Jama, 2001. **286**(10): p. 1195-200.

121. Monick, M.M., et al., *Lipopolysaccharide activates Akt in human alveolar macrophages resulting in nuclear accumulation and transcriptional activity of beta-catenin.* J Immunol, 2001. **166**(7): p. 4713-20.

122. Morris, B.J., *Neuronal localisation of neuropeptide Y gene expression in rat brain.* J Comp Neurol, 1989. **290**(3): p. 358-68.

123. Morton, G.J., et al., *Leptin action in the forebrain regulates the hindbrain response to satiety signals.* J Clin Invest, 2005. **115**(3): p. 703-10.

124. Morton, G.J. and M.W. Schwartz, *The NPY/AgRP neuron and energy homeostasis.* Int J Obes Relat Metab Disord, 2001. **25 Suppl 5**: p. S56-62.

125. Munford, R.S. and C.L. Hall, *Detoxification of bacterial lipopolysaccharides (endotoxins) by a human neutrophil enzyme.* Science, 1986. **234**(4773): p. 203-5.

126. Munzberg, H., J.S. Flier, and C. Bjorbaek, *Region-specific leptin resistance within the hypothalamus of diet-induced obese mice.* Endocrinology, 2004. **145**(11): p. 4880-9.

127. Murphy, K.G. and S.R. Bloom, *Gut hormones and the regulation of energy homeostasis.* Nature, 2006. **444**(7121): p. 854-9.

128. Musatov, S., et al., *Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome.* Proc Natl Acad Sci U S A, 2007. **104**(7): p. 2501-6.

129. Nakatani, Y., et al., *Involvement of endoplasmic reticulum stress in insulin resistance and diabetes.* J Biol Chem, 2005. **280**(1): p. 847-51.

130. Nikkari, T., et al., *Fatty acid composition of serum lipid fractions in relation to gender and quality of dietary fat.* Ann Med, 1995. **27**(4): p. 491-8.

131. Nishimura, M. and S. Naito, *Tissue-specific mRNA expression profiles of human toll-like receptors and related genes.* Biol Pharm Bull, 2005. **28**(5): p. 886-92.

132. Niswender, K.D., D.G. Baskin, and M.W. Schwartz, *Insulin and its evolving partnership with leptin in the hypothalamic control of energy homeostasis.* Trends Endocrinol Metab, 2004. **15**(8): p. 362-9.

133. Niswender, K.D., et al., *Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia.* Diabetes, 2003. **52**(2): p. 227-31.

134. O'Neill, L.A. and A.G. Bowie, *The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling.* Nat Rev Immunol, 2007. **7**(5): p. 353-64.

135. Ollmann, M.M., et al., *Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein.* Science, 1997. **278**(5335): p. 135-8.

136. Olshansky, S.J., et al., *A potential decline in life expectancy in the United States in the 21st century.* N Engl J Med, 2005. **352**(11): p. 1138-45.

137. Ozcan, L., et al., *Endoplasmic reticulum stress plays a central role in development of leptin resistance.* Cell Metab, 2009. **9**(1): p. 35-51.

138. Ozcan, U., et al., *Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes.* Science, 2004. **306**(5695): p. 457-61.

139. Pan, D.A., et al., *Skeletal muscle triglyceride levels are inversely related to insulin action.* Diabetes, 1997. **46**(6): p. 983-8.

140. Perseghin, G., et al., *Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents.* Diabetes, 1999. **48**(8): p. 1600-6.

141. Plum, L., M. Schubert, and J.C. Bruning, *The role of insulin receptor signaling in the brain.* Trends Endocrinol Metab, 2005. **16**(2): p. 59-65.

142. Poggi, M., et al., *C3H/HeJ mice carrying a toll-like receptor 4 mutation are protected against the development of insulin resistance in white adipose tissue in response to a high-fat diet.* Diabetologia, 2007. **50**(6): p. 1267-76.

143. Pohlman, T.H., R.S. Munford, and J.M. Harlan, *Deacylated lipopolysaccharide inhibits neutrophil adherence to endothelium induced by lipopolysaccharide in vitro.* J Exp Med, 1987. **165**(5): p. 1393-402.

144. Polonsky, K.S., et al., *Quantitative study of insulin secretion and clearance in normal and obese subjects.* J Clin Invest, 1988. **81**(2): p. 435-41.

145. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.

146. Posey, K., et al., *Hypothalamic proinflammatory lipid accumulation, inflammation, and insulin resistance in rats fed a high-fat diet.* Am J Physiol Endocrinol Metab, 2008.

147. Powis, J.E., J.S. Bains, and A.V. Ferguson, *Leptin depolarizes rat hypothalamic paraventricular nucleus neurons.* Am J Physiol, 1998. **274**(5 Pt 2): p. R1468-72.

148. Pritchard, L.E., A.V. Turnbull, and A. White, *Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity.* J Endocrinol, 2002. **172**(3): p. 411-21.

149. Raetz, C.R. and C. Whitfield, *Lipopolysaccharide endotoxins.* Annu Rev Biochem, 2002. **71**: p. 635-700.

150. Rahman, S.M., et al., *Stearoyl-CoA desaturase 1 deficiency elevates insulin-signaling components and down-regulates protein-tyrosine phosphatase 1B in muscle.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 11110-5.

151. Reid, J., A.I. Macdougall, and M.M. Andrews, *Aspirin and diabetes mellitus.* Br Med J, 1957. **2**(5053): p. 1071-4.

152. Riccardi, G., R. Giacco, and A.A. Rivellese, *Dietary fat, insulin sensitivity and the metabolic syndrome.* Clin Nutr, 2004. **23**(4): p. 447-56.

153. Ropero, A.B., et al., *The role of estrogen receptors in the control of energy and glucose homeostasis.* Steroids, 2008. **73**(9-10): p. 874-9.

154. Rosenbaum, M., et al., *Effects of gender, body composition, and menopause on plasma concentrations of leptin.* J Clin Endocrinol Metab, 1996. **81**(9): p. 3424-7.

155. Sahu, A., P.S. Kalra, and S.P. Kalra, *Food deprivation and ingestion induce reciprocal changes in neuropeptide Y concentrations in the paraventricular nucleus.* Peptides, 1988. **9**(1): p. 83-6.

156. Salh, B., et al., *Activation of phosphatidylinositol 3-kinase, protein kinase B, and p70 S6 kinases in lipopolysaccharide-stimulated Raw 264.7 cells: differential effects of rapamycin, Ly294002, and wortmannin on nitric oxide production.* J Immunol, 1998. **161**(12): p. 6947-54.

157. Sampath, H. and J.M. Ntambi, *The fate and intermediary metabolism of stearic acid.* Lipids, 2005. **40**(12): p. 1187-91.

158. Schindler, C. and J.E. Darnell, Jr., *Transcriptional responses to polypeptide ligands: the JAK-STAT pathway.* Annu Rev Biochem, 1995. **64**: p. 621-51.

159. Schmidt, M.I., et al., *Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study.* Lancet, 1999. **353**(9165): p. 1649-52.

160. Schwartz, M.W., et al., *Central insulin administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food-deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats.* Endocrinology, 1991. **128**(5): p. 2645-7.

161. Schwartz, M.W., et al., *Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans.* Nat Med, 1996. **2**(5): p. 589-93.

162. Schwartz, M.W., et al., *Kinetics and specificity of insulin uptake from plasma into cerebrospinal fluid.* Am J Physiol, 1990. **259**(3 Pt 1): p. E378-83.

163. Schwartz, M.W., et al., *Central nervous system control of food intake.* Nature, 2000. **404**(6778): p. 661-71.

164. Seeley, R.J., et al., *Behavioral, endocrine, and hypothalamic responses to involuntary overfeeding.* Am J Physiol, 1996. **271**(3 Pt 2): p. R819-23.

165. Shi, H., et al., *TLR4 links innate immunity and fatty acid-induced insulin resistance.* J Clin Invest, 2006. **116**(11): p. 3015-25.

166. Shimomura, Y., T. Tamura, and M. Suzuki, *Less body fat accumulation in rats fed a safflower oil diet than in rats fed a beef tallow diet.* J Nutr, 1990. **120**(11): p. 1291-6.

167. Shoelson, S.E., J. Lee, and A.B. Goldfine, *Inflammation and insulin resistance.* J Clin Invest, 2006. **116**(7): p. 1793-801.

168. Shoelson, S.E., J. Lee, and M. Yuan, *Inflammation and the IKK beta/I kappa B/NF-kappa B axis in obesity- and diet-induced insulin resistance.* Int J Obes Relat Metab Disord, 2003. **27 Suppl 3**: p. S49-52.

169. Shulman, G.I., *Cellular mechanisms of insulin resistance.* J Clin Invest, 2000. **106**(2): p. 171-6.

170. Sims, E.A., et al., *Experimental obesity in man.* Trans Assoc Am Physicians, 1968. **81**: p. 153-70.

171. Sinha, S., et al., *Fatty acid-induced insulin resistance in L6 myotubes is prevented by inhibition of activation and nuclear localization of nuclear factor kappa B.* J Biol Chem, 2004. **279**(40): p. 41294-301.

172. Sipols, A.J., D.G. Baskin, and M.W. Schwartz, *Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression.* Diabetes, 1995. **44**(2): p. 147-51.

173. Small, C.J., et al., *Effects of chronic central nervous system administration of agouti-related protein in pair-fed animals.* Diabetes, 2001. **50**(2): p. 248-54.

174. Smith, G.P., C. Jerome, and R. Norgren, *Afferent axons in abdominal vagus mediate satiety effect of cholecystokinin in rats.* Am J Physiol, 1985. **249**(5 Pt 2): p. R638-41.

175. Spanswick, D., et al., *Insulin activates ATP-sensitive K+ channels in hypothalamic neurons of lean, but not obese rats.* Nat Neurosci, 2000. **3**(8): p. 757-8.

176. Spector, A.A., *Plasma free fatty acid and lipoproteins as sources of polyunsaturated fatty acid for the brain.* J Mol Neurosci, 2001. **16**(2-3): p. 159-65; discussion 215-21.

177. Stein, D.T., et al., *The insulinotropic potency of fatty acids is influenced profoundly by their chain length and degree of saturation.* J Clin Invest, 1997. **100**(2): p. 398-403.

178. Stein, L.J., et al., *Reduced effect of experimental peripheral hyperinsulinemia to elevate cerebrospinal fluid insulin concentrations of obese Zucker rats.* Endocrinology, 1987. **121**(5): p. 1611-5.

179. Takeuchi, H., et al., *Diet-induced thermogenesis is lower in rats fed a lard diet than in those fed a high oleic acid safflower oil diet, a safflower oil diet or a linseed oil diet.* J Nutr, 1995. **125**(4): p. 920-5.

180. Tartaglia, L.A., et al., *Identification and expression cloning of a leptin receptor, OB-R.* Cell, 1995. **83**(7): p. 1263-71.

181. Tatemoto, K., M. Carlquist, and V. Mutt, *Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide.* Nature, 1982. **296**(5858): p. 659-60.

182. Thornton, J.E., et al., *Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice.* Endocrinology, 1997. **138**(11): p. 5063-6.

183. Travers, J.B., S.P. Travers, and R. Norgren, *Gustatory neural processing in the hindbrain.* Annu Rev Neurosci, 1987. **10**: p. 595-632.

184. Tsukumo, D.M., et al., *Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance.* Diabetes, 2007. **56**(8): p. 1986-98.

185. Tsuneki, H., et al., *Age-related insulin resistance in hypothalamus and peripheral tissues of orexin knockout mice.* Diabetologia, 2008. **51**(4): p. 657-67.

186. Uotani, S., et al., *Functional properties of leptin receptor isoforms: internalization and degradation of leptin and ligand-induced receptor downregulation.* Diabetes, 1999. **48**(2): p. 279-86.

187. Vaisse, C., et al., *Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice.* Nat Genet, 1996. **14**(1): p. 95-7.

188. Van Heek, M., et al., *Diet-induced obese mice develop peripheral, but not central, resistance to leptin.* J Clin Invest, 1997. **99**(3): p. 385-90.

189. van Staveren, W.A., et al., *Validity of the fatty acid composition of subcutaneous fat tissue microbiopsies as an estimate of the long-term average fatty acid composition of the diet of separate individuals.* Am J Epidemiol, 1986. **123**(3): p. 455-63.

190. Vessby, B., et al., *Desaturation and elongation of Fatty acids and insulin action.* Ann N Y Acad Sci, 2002. **967**: p. 183-95.

191. Wagner, J.D., et al., *Insulin sensitivity and cardiovascular risk factors in ovariectomized monkeys with estradiol alone or combined with nomegestrol acetate.* J Clin Endocrinol Metab, 1998. **83**(3): p. 896-901.

192. Wallace, F.A., E.A. Miles, and P.C. Calder, *Activation state alters the effect of dietary fatty acids on pro-inflammatory mediator production by murine macrophages.* Cytokine, 2000. **12**(9): p. 1374-9.

193. Wang, L., et al., *Synergistic interaction between CCK and leptin to regulate food intake.* Regul Pept, 2000. **92**(1-3): p. 79-85.

194. Weigle, D.S., et al., *Recombinant ob protein reduces feeding and body weight in the ob/ob mouse.* J Clin Invest, 1995. **96**(4): p. 2065-70.

195. Wells, J.C., *The evolution of human fatness and susceptibility to obesity: an ethological approach.* Biol Rev Camb Philos Soc, 2006. **81**(2): p. 183-205.

196. West, D.B., D. Fey, and S.C. Woods, *Cholecystokinin persistently suppresses meal size but not food intake in free-feeding rats.* Am J Physiol, 1984. **246**(5 Pt 2): p. R776-87.

197. West, D.B. and B. York, *Dietary fat, genetic predisposition, and obesity: lessons from animal models.* Am J Clin Nutr, 1998. **67**(3 Suppl): p. 505S-512S.

198. Westerterp, K.R., *Perception, passive overfeeding and energy metabolism.* Physiol Behav, 2006. **89**(1): p. 62-5.

199. White, D.W., et al., *Leptin receptor (OB-R) signaling. Cytoplasmic domain mutational analysis and evidence for receptor homo-oligomerization.* J Biol Chem, 1997. **272**(7): p. 4065-71.

200. White, M.F., *Insulin signaling in health and disease.* Science, 2003. **302**(5651): p. 1710-1.

201. Williams, G., et al., *The hypothalamus and the control of energy homeostasis: different circuits, different purposes.* Physiol Behav, 2001. **74**(4-5): p. 683-701.

202. Woods, S.C., et al., *Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons.* Nature, 1979. **282**(5738): p. 503-5.

203. Woods, S.C. and D. Porte, Jr., *The central nervous system, pancreatic hormones, feeding, and obesity.* Adv Metab Disord, 1978. **9**: p. 283-312.

204. Woods, S.C., et al., *Signals that regulate food intake and energy homeostasis.* Science, 1998. **280**(5368): p. 1378-83.

205. Woods, S.C., et al., *A controlled high-fat diet induces an obese syndrome in rats.* J Nutr, 2003. **133**(4): p. 1081-7.

206. Worgall, T.S., et al., *Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein.* J Biol Chem, 1998. **273**(40): p. 25537-40.

207. Yeo, G.S., et al., *The role of melanocortin signalling in the control of body weight: evidence from human and murine genetic models.* Qjm, 2000. **93**(1): p. 7-14.

208. Yuan, M., et al., *Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta.* Science, 2001. **293**(5535): p. 1673-7.

209. Zarjevski, N., et al., *Chronic intracerebroventricular neuropeptide-Y administration to normal rats mimics hormonal and metabolic changes of obesity.* Endocrinology, 1993. **133**(4): p. 1753-8.

210. Zhang, Y., et al., *Positional cloning of the mouse obese gene and its human homologue.* Nature, 1994. **372**(6505): p. 425-32.

211. Zick, Y., *Insulin resistance: a phosphorylation-based uncoupling of insulin signaling.* Trends Cell Biol, 2001. **11**(11): p. 437-41.