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Fatty Acid Induced Insulin Resistance in the Brain

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FATTY ACID INDUCED INSULIN RESISTANCE IN THE BRAIN

by

Hyoung Il Oh

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biology

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UTAH STATE UNIVERSITY
Logan, Utah

2012
ABSTRACT

Fatty Acid Induced Insulin Resistance in the Brain

by

Hyoung Il Oh, Doctor of Philosophy

Utah State University, 2012

Major Professor: Dr. David A. York
Department: Biology

The prevalence of obesity, which is considered as a disease, has been increasing uncontrollably over the last two decades. Obesity is a state of disregulated energy homeostasis characterized by hypothalamic resistance to adiposity signals (insulin and leptin). While many factors are involved in the development of obesity, excess dietary fat has been proposed as one of the main causal factors. This causes disrupted energy homeostasis by inducing both leptin and insulin resistance in the central nervous system. Although brain tissue was considered to be insulin independent for a long time, insulin is now recognized to have important functions in the brain in the regulation of feeding behavior, energy expenditure and peripheral metabolism to maintain energy homeostasis. Recently, our lab discovered that insulin has an anorectic effect when it is applied into the central nucleus of the amygdala (CeA), a response that is similar to its effect when it is intracerebroventricularly (icv) administered into the hypothalamus. Our lab also demonstrated that rats fed a high fat diet lost the anorectic response to CeA insulin and became insulin resistant. These data suggested that insulin signaling in the amygdala had
an important role in controlling food intake and energy expenditure in similar ways to the hypothalamus. It also suggests that a high fat diet inhibits amygdala insulin signaling in the CeA. Both in vitro cell culture and in vivo animal studies have been used to investigate the effects of dietary fats on insulin signaling in neuronal cells and in the amygdala. Using both hypothalamic GT1-7 cells and primary amygdala cells in culture, the saturated fatty acid palmitic acid was shown to inhibit insulin signaling (Akt phosphorylation). This response appears to be related to the activation of PKCθ since the inhibitory effect of palmitic acid on Akt phosphorylation was greater in GT1-7 cells transfected with PKCθ compared to wild type cells and was abolished in GT1-7 cells transfected with PKCθ siRNA. Further investigations in vivo confirmed that insulin stimulated Akt and mTOR signaling in the CeA of rats and that the insulin stimulation of Akt phosphorylation, but not mTOR phosphorylation, was inhibited in rats fed a high fat diet for 3 days or by infusing palmitic acid into the CeA for 3 days. These experiments also identified that fatty acid and insulin signaling in the CeA differentially affected Akt and mTOR signaling in the hypothalamus and suggest that these neural connections might be important components of the neural pathways through which insulin in the amygdala affects food intake and peripheral metabolism. This research has provided novel insight into the effects of dietary fats on insulin signaling in an area of the brain, the CeA, that is now recognized to have effects on energy balance and peripheral metabolism.
The prevalence of obesity is rapidly increasing; it is now one of the most serious public health problems worldwide. Obesity is thought to reflect the interaction between genetics and modern lifestyle. In particular, high fat diets (HFD) are considered as a major contributing factor to the development of obesity and type 2 diabetes as well as other metabolic disorders such as cardiovascular disease, Alzheimer’s disease and some types of cancer. Recently, it has been suggested that insulin actions in the brain are important in the regulation of energy homeostasis and peripheral metabolism.

With the support of USTAR (The Utah Science Technology and Research program), Hyoungil Oh, a Ph.D student in Dr. York’s research group in the Department of Biology at Utah State University, studied the effect of HFD and elevated saturated fatty acids on brain insulin signaling pathways. The hypothesis investigated was that HFD induce insulin resistance in specific regions of the brain to impair energy homeostasis. The experimental model used was Sprague-Dawley rats fed on either High or Low fat diets in their cages for 3 days. In addition, cultures of brain neuronal cells were used to study the mechanism through which fatty acids inhibited insulin signaling.

The results of these studies confirmed the hypothesis that dietary fat, specifically increased levels of saturated fatty acids inhibited brain insulin signaling in neuronal cells in two parts of the brain, the hypothalamus and amygdala, of rats. It further suggested that a specific enzyme, protein kinase Cθ mediated this fatty acid inhibition of insulin signaling. This work contributes to our understanding of why dietary fat can lead to increased body weight and altered peripheral metabolism by inhibiting insulin actions in specific regions of the brain and may be helpful in the development of new treatment approaches for metabolic disorders such as obesity and Type 2 Diabetes.
ACKNOWLEDGMENTS

This dissertation is dedicated to all the people who continually supported and guided me. I would like to acknowledge my major professor, Dr. York. He has been leading me throughout my doctorate program and supporting me in every way to become a successful student. In particular, his logical way to analyze data was a great lesson to me and assisted with my dissertation. I really respect his analytical skills. I also want to take this opportunity to express my gratitude to Dr. Park. She offered limitless guidance for me in the laboratory. When I first became a member of York’s lab, I did not have any lab techniques. She taught me all the necessary lab techniques and molecular aspects of my studies. One of the important aspects I have learned from her is to perform experiments very meticulously. This helped to obtain clear and consistent data that is easier to analyze. I also want to thank Dr. Boghossian for being a great mentor to me in the field of animal experiments. He taught me all the skills to handle rats and mice and made it possible for me to perform animal experiments. I would like to thank Yu Ho Kim, graduate student, who was my colleague and a good friend. I would also like to express my appreciation to all the committee members. Dr. Tim Gilbertson provided me a great opportunity to learn neurobiology and made excellent suggestions to improve the discussion for my dissertation. Dr. Daryll DeWald helped me to learn cell biology and basic biological knowledge. He also offered good comments to improve my dissertation. Dr. Ilka Nemere provided a chance for me to learn about the field of endocrinology and suggested various ways to analyze my data. In addition, I also
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CHAPTER 1
INTRODUCTION

1. Rationale for the proposed studies

Obesity and its comorbidities are becoming a major health problem worldwide and high fat diets are considered as one of the major contributing factors to the development of obesity. In type 2 diabetes, association of obesity with peripheral insulin resistance is well recognized. Also, the molecular mechanisms through which insulin regulates metabolism in adipocyte, muscle and liver cells have been well investigated. However, there has been little investigation of insulin’s effects in the brain because it was considered that the brain was insulin independent for a long time. However, it is now known that insulin has many effects in the brain including the very important roles in regulation of peripheral metabolism, feeding behavior, memory and maintenance of neural functions [1-3]. Insulin receptors are widely expressed in the brain [4]. Neuron specific insulin receptor knockout mice that lack insulin receptors in the CNS become sensitive to diet-induced obesity with increased body fat and become moderately insulin resistant and hyperinsulinemic [5]. These results illustrated the link between central insulin actions and the regulation of body weight and energy homeostasis.

Like insulin sensitive peripheral cells, altered or defective insulin action in the brain may cause or promote metabolic diseases such as obesity, diabetes or Alzheimer’s disease suggesting that central insulin resistance might be not only a consequence but also a starting point for the development of obesity and possibly of type 2 diabetes [2]. However, current approaches used for prevention and treatment of insulin resistance and type 2 diabetes are only partially effective. Therefore, to develop new and more efficient
therapeutic strategies for these metabolic disorders, an increased understanding of the complexity of insulin signaling in the brain and the interactions between central and peripheral effects of insulin is desirable [2].

It appears that dietary fat, especially saturated fat, may have a critical role in the development of central insulin resistance. According to recent data from our laboratory, insulin has an anorectic response in the rat’s amygdala and a high fat diet induces complete amygdala insulin resistance before the onset of peripheral insulin resistance [6]. Also, studies have shown that other brain regions, such as the hypothalamus, become insulin resistant when the rats are in a high fat feeding condition [7]. However, the linkage between dietary fat and central insulin resistance and its molecular mechanisms are still unclear. The present dissertation investigated molecular mechanisms of dietary fat induced insulin resistance in the brain.

2. The roles of insulin on regulation of peripheral metabolism

Insulin is a peptide hormone of 5.8 kDa molecular weight secreted from beta cells of the endocrine pancreas [8]. It is composed of two peptide chains connected to each other by disulfide linkages [9]. Insulin release is regulated in response to the levels of blood glucose or other nutrients (e.g., amino acids, fatty acids). In peripheral tissues, insulin stimulates anabolic pathways through the regulation of carbohydrate, lipid and protein metabolism while it inhibits catabolism of these fuel reserves [10]. Insulin stimulates uptake of nutrients including glucose, fatty acids and amino acids into muscle and adipose tissue and promotes the storage and synthesis of these nutrients in the form of glycogen, lipids and protein respectively in liver, adipose tissue and muscle. Insulin
regulates blood glucose level by stimulating glucose uptake into multiple tissues including muscle and adipose.

In carbohydrate metabolism, insulin stimulates rapid glucose uptake, storage, and utilization of glucose by almost all tissues of the body particularly the muscle, liver, and adipose tissues. Insulin stimulates glucose uptake in muscle by the translocation of glucose transporters into the plasma membrane and this transported glucose is either metabolized or stored in the form of glycogen [11]. In the liver, insulin promotes storage and use of glucose while it inhibits glucose production through glycogenolysis and gluconeogenesis [12, 13]. After a meal, most of the glucose transported into liver cells is immediately converted to glycogen to be stored. Insulin activates glycogen synthesis and decreases liver phosphorylase (glycogen → glucose) activity, but increases activity of glucokinase (phosphorylates glucose to G-6-P to be trapped inside of liver cells) [12, 13]. Insulin also increases glycolysis and pentose pathways (production of NADPH and five sugar molecules) to increase glucose utilization but decreases gluconeogenesis by deactivating gluconeogenic enzymes [14].

Insulin also regulates lipid metabolism. When glucose level is high, insulin stimulates glucose uptake into adipose tissue cells similar to its function in muscles and the transported glucose is converted to glycerol and/or fatty acids which are used as substrates in the synthesis of triglyceride, a storage form of fat [15]. Insulin also decreases the break down and mobilization of fatty acids from triglycerides by inhibiting the activity of hormone sensitive lipase whereas by activating lipoprotein lipase in the wall of capillary vessels near adipocytes, it increases fatty acid translocation into adipocytes and storage in the form of triglycerides [15]. Insulin activates fatty acid
synthesis by activating acetyl CoA carboxylase which converts acetyl-CoA to malonyl-
CoA [16]. Malonyl-CoA is not only a key substrate in chain elongation of fatty acid
synthesis but it also allosterically inhibits fatty acid oxidation by preventing fatty acids
transport into mitochondria [16].

Insulin affects protein metabolism and growth. Insulin stimulates protein
synthesis and storage through increasing amino acid uptake, transcription, and mRNA
translation whereas insulin inhibits break down of proteins by inhibiting proteolysis,
gluconeogenesis and lysosome activity [17].

Insulin’s major function is to maintain glucose homeostasis by promoting
glucose uptake by tissues cells such as muscle and adipocytes and stimulating anabolic
pathways such as protein, glycogen, fatty acid and triacylglycerol synthesis [18]. Blood
glucose levels are regulated to stay within a narrow range, usually between 80 and
90mg/100ml of blood in the fasting person and 120 to 140mg/100ml blood after a meal
[19]. The level of glucose in the blood is a balance between input and removal. Glucose
appears in the blood from the gastrointestinal tract after digestion of food in the gut
lumen or is produced in the liver or kidney either from stored glycogen (glycogenolysis)
or the synthesis of glucose from lactate, pyruvate or amino acid precursors
(gluconeogenesis) [20]. Uncontrolled blood glucose concentrations cause 1) osmotic
pressure in the extracellular fluid and this can cause cell dehydration, 2) loss of glucose in
the urine, 3) loss of electrolytes and 4) damage to many tissues, especially to blood
vessels [21]. Too high concentration of glucose leads to coma and death [21]. The failure
to appropriately control blood sugar within the physiological range is diabetes. This can
occur from two major causes, either a lack of insulin secretion from the pancreas in
response to the blood glucose level (type 1 diabetes) or to the development of insulin resistance (type 2 diabetes) in the presence of high insulin levels. Figure 1-1 summarizes insulin’s physiological functions.

3. **Insulin signaling pathways (Metabolic pathways)**

   The metabolic functions of insulin which are described above are regulated through various signaling molecules involved in the insulin signaling pathways. Although insulin is involved in multiple networks of signal molecules and a wide array of effector systems, insulin’s function can be divided into two different major signaling pathways which are mitogenic (Ras/MAP kinases) and metabolic (PI3K/Akt) pathways [18]. The mitogenic signaling pathway plays a major role in cell growth and differentiation whereas the PI3K/Akt signaling pathways mediate most of insulin’s metabolic actions. Figure 1-2 summarizes the insulin signaling pathways for both mitogenic and metabolic effects.

*Insulin receptors and Insulin receptor substrates (IRSs)*

Insulin binds to the insulin receptor on the plasma membrane of cells. The insulin receptor is composed of two extracellular α-subunits and two transmembrane β-subunits linked together by disulfide bonds [23] (Figure 1-2). The α-subunits are located entirely at the extracellular face of the plasma membrane and contain insulin binding sites whereas β-subunits are transmembrane [24]. Insulin binding induces a conformational change of the insulin receptor resulting in autophosphorylation at several tyrosine residues present in the β-subunits of the receptor [18]. These phosphorylated residues are recognized by insulin receptor substrate (IRS). It is known that there are 4 members of the IRS family (IRS-1, IRS-2, IRS-3 and IRS-4) [18, 25]. Among the IRS family, the
function of IRS-1 and IRS-2 are well investigated and both proteins are expressed in most cells and tissues including adipocytes, liver, skeletal muscle and brain and their molecular weight and the structure are very similar [18, 26]. IRS-3 expression has been detected in adipose tissues and liver [27] whereas IRS-4 has been observed only in culture cells derived from embryonic kidney [25]. Phosphorylation at multiple tyrosine residues on IRS proteins creates binding sites for Src homology-2 (SH2) domain containing signaling molecules such as PI3K or the adapter molecule Grb-2. Experiments have shown that both IRS-1 and IRS-2 proteins have an important role in the insulin signal transduction. Deletion of IRS-1 and IRS-2 caused insulin resistance in peripheral tissues and growth retardation [28]. However, the function of IRS-3 and IRS-4 are not well known.

**Phosphatidylinositol 3 kinase (PI3K)**

The activated insulin receptor phosphorylates tyrosine residues on IRS-1/2 protein and these tyrosine phosphorylated residues are recognized by a subunit of PI3K [18]. Binding with the IRS-1/2 activates PI3K which catalyzes phosphorylation of phosphatidylinositol (4,5) bisphosphate [PtdIns (4, 5)P₂] (PIP2) to [PtdIns (3,4,5)P₃] (PIP3) [29]. It has been reported that PI3K exists as heterodimers which consist of an adaptor 85 kDa subunit tightly associated with a catalytic 110kDa subunit [30, 31]. PI3K plays an important role in several insulin stimulated responses such as increasing glucose uptake [32, 33], general and growth-specific protein synthesis [34] and cell growth and proliferation [35]. Treatment of adipocytes with PI3K inhibitors such as Wortmannin or LY294002, over-expression of dominant negative mutants of PI3K, and microinjection of neutralizing antibodies to the p100 catalytic subunits all result in the inhibition of insulin’s metabolic actions [36]. PI3K is also involved in regulation of the expression of
key genes in metabolism [e.g. insulin activated PI3K decreases gene expression of phosphoenol-pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase to inhibit glucose production] [37, 38]. Protein kinase B (PKB), p70 S6 kinase, and atypical protein kinase C (PKCs) have been reported to be downstream of PI3K and involved in several of insulin’s metabolic actions.

Protein kinase B (PKB/Akt)

Protein kinase B (PKB/Akt) is one of the major downstream signaling molecules that are activated by the increased PI3K. Akt is a PH domain containing serine/threonine kinase and has a similar structure to protein kinase A (PKA) and protein kinase C (PKC) [39]. There are three isoforms of Akt; Akt1 is expressed in a variety of tissues and regulates cell survival pathways by inhibiting apoptotic processes [40], Akt2 is highly expressed in metabolic tissues including adipose, muscle and liver and it is an important signaling molecule in insulin stimulated glucose uptake [41], and Akt-3 has been reported to be expressed in brain regions but the role of Akt-3 is less clear [42]. Activation of PKB/Akt requires interaction with PIP3 through the PH domain, which recruit Akt to the plasma membrane and result in its conformational change so that both serine and threonine residues of Akt can be phosphorylated by protein kinase 3-phosphoinositide-dependent protein kinase-1 and 2 (PDK1/2) respectively [43, 44].

Glycogen synthase kinase (GSK3) - Glycogen synthesis

Insulin stimulated phosphorylation of Akt is involved in the mediation of several signaling molecules to regulate insulin’s metabolic functions. After phosphorylation, Akt enters the cytoplasm where it induces the phosphorylation and inactivation of glycogen
synthase kinase 3 (GSK3) which is an important regulator of glycogen synthase. Since the phosphorylated form of GSK3 inhibits the activation of glycogen synthase, which is a major substrate of GSK3 and catalyses the final step in glycogen synthesis, the inactivation of GSK3 by Akt promotes glucose storage as glycogen [45-47].

**Mammalian target of rapamycin (mTOR)/P70 S6 kinase (p70S6K) – Protein synthesis**

Insulin signaling also stimulates protein translation and protein synthesis. The PI3K/Akt pathway phosphorylates and activates mammalian target of rapamycin (mTOR), which promotes protein synthesis through the activation of p70 S6 kinase (p70S6K) and inhibition of eukaryotic initiation factor-4E (eIF-4E) binding protein (4E-BP1) [48]. Inactivation of GSK3 by insulin stimulated Akt signaling leads to the dephosphorylation and activation of eIF2B and this activates protein synthesis and storage of amino acids [49]. The activation eIF2B is dependent on the PI3K/Akt pathway but independent of mTOR/p70 S6 kinase or MAPK pathways [50].

**Sterol regulatory element binding proteins (SREBPs) – Lipid synthesis**

Insulin signaling also promotes lipid synthesis while it inhibits lipolysis. It has been reported that lipid synthesis requires an increase of expression of a transcription factor, sterol regulatory element binding proteins (SREBPs). SREBPs regulate the expression of several genes needed for uptake and synthesis of cholesterol, fatty acids, phospholipids and triglycerides in animal cells [51, 52]. Although the exact molecular mechanism is unknown, insulin has been reported to enhance transcription of the gene encoding SREBPs and its activation [53, 54]. These activated SREBPs then bind to
specific sterol regulatory element DNA sequences and this up-regulates synthesis of enzymes involved in lipid biosynthesis [55, 56].

Glucose transporter 4 (GLUT4)

A key action of insulin is to stimulate glucose uptake into cells. Insulin stimulated Akt phosphorylation induces translocation of the glucose transporter 4 (GLUT4) from intracellular storage into the plasma membrane (e.g. muscle and adipose tissues) to stimulate glucose transport into cells [57]. Constitutively activated Akt resulted in persistent translocation of GLUT4 onto the plasma membrane in muscle, adipose, and cultured cell systems [58] while cellular introduction of a dominant negative mutant form of Akt blocked insulin stimulated GLUT4 translocation in adipocytes [59]. Particularly, insulin stimulated GLUT4 translocation and glucose uptake was accompanied by the activation of Akt-2 molecules [59]. These data indicate that Akt has an important role in insulin simulated glucose transport. However, this evidence is somewhat controversial because other studies demonstrated that a dominant negative mutant form of Akt inhibited insulin stimulated protein synthesis but did not inhibit GLUT4 translocation [60]. Furthermore, although insulin stimulated Akt phosphorylation was intact, gross glucose uptake was impaired in muscle tissues from insulin resistant diabetic subjects [61]. These controversial data about the role of Akt in insulin stimulated glucose transport suggests that there are other parallel signaling pathways (e.g. atypical PKCs and CAP/Cbl/TC10) which are working in concert with Akt pathway to stimulate GLUT4 translocation. IRS/PI3K mediated activation of atypical PKCs, PKCζ and PKCλ, have been reported to increase glucose transport and GLUT4 translocation [62]. Also,
evidence has been accumulated to support the PI3K independent CAP/Cbl/TC10 pathway as the complementary pathway for insulin stimulated glucose transport [63, 64].

4. Relation between high fat diet, obesity and insulin resistance

The increased prevalence of obesity is a huge health concern. Worldwide, at least 1 in 10 adults is obese, but > 25% of the populations in western countries are obese [5]. In addition, there has been a dramatic increase in the rate of juvenile obesity which greatly increases the risk of persistent obesity and obesity related health risks during adulthood [65]. The causal factors for obesity have been suggested to be a combination of excessive dietary calories, lack of physical activity, and genetic susceptibility. Particularly, the current western diet style, the high fat calorie dense diet, is considered a major cause of obesity (due to increased adiposity) which is closely linked to major health issues such as cardiovascular disease, hypertension, hyperlipidemia, neuro degenerative disease and Type 2 Diabetes [66, 67]. Among these disease conditions, the most devastating one may be Type 2 Diabetes. By 2000, approximately 171 million individuals were diagnosed with diabetes, and this number is expected to increase to 366 million by 2030 [68].

The development of insulin resistance, which is usually a gradual process beginning with excess weight gain and obesity, is believed to be the major underlying mechanism for the onset of Type 2 Diabetes [69]. Also, epidemiological and clinical studies show that both obesity and Type 2 Diabetes are associated with insulin resistance [69, 70]. However, most obese and insulin resistant individuals are able to maintain normal glucose tolerance and do not develop hyperglycemia because, under normal conditions, the pancreatic β-cells increase insulin secretion sufficiently to compensate for
the reduced insulin sensitivity [71-73]. But when β-cell dysfunction is present, impaired glucose tolerance, impaired fasting glucose and, at the extreme, Type 2 Diabetes results [74]. The interaction between obesity, insulin resistance and β-cell dysfunction is the main causal factor of Type 2 Diabetes [75].

Another important factor contributing to insulin resistance is the accumulation of omental fat (e.g. upper body fat) and fat in the intramyocellular compartment [10, 76, 77]. These fats can exist independent of the level of general adiposity [10]. Many studies have demonstrated that even though subcutaneous fat also contributes to the development of insulin resistance, increased abdominal fat distribution has greater adverse effects to induce insulin resistance [76]. It has been proposed that increased omental adipose tissue impairs insulin sensitivity through promoting the secretion of free fatty acids and adipocyte-derived inflammatory cytokines such as TNF-α or Interleukin-6 [10].

The molecular mechanism through which obesity is related to insulin resistance in insulin responsive tissues is not fully understood. Dietary fat has been implicated as a major cause of insulin resistance. Significant insulin resistance was observed from rats that were on high-fat diet for just 3 weeks [78]. Large quantities of dietary fats including saturated, monounsaturated and poly-unsaturated fats all appear to affect insulin sensitivity negatively to some degree, compared to high carbohydrate chow diet. High fat diet causes the accumulation of various lipids and fatty acids in the circulation and in tissues (muscle and liver) and this will disrupt insulin’s metabolic signaling pathway. However, among the fats, saturated fat appears to be the most harmful to the induction of insulin resistance [79]. According to many studies, fatty acid incubation of insulin sensitive cells, particularly saturated fatty acids such as palmitic acid, decreases
phosphorylation level of proteins involved in insulin signaling [74, 79, 80]. High saturated fat diet increases the levels of saturated fatty acids and intracellular fatty acid metabolites such as diacylglycerol (DAG), fatty acyl-Coenzyme A (fatty acyl-CoA), and ceramides [74] which in turn, induce dysfunction of several insulin signaling molecules including IRS-1/2, PI3K, and Akt.

In contrast to saturated fatty acids, polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) are found to be effective in enhancing insulin signaling pathways in peripheral cells. Particularly, it has been shown in a controlled feeding study that elevated polyunsaturated fatty acid consumption improved insulin resistance [81, 82]. Also, in a recent study, feeding this long chain n-3 fatty acid (PUFA) to insulin resistant rats increased insulin sensitivity, glucose metabolism, and reduced cardiovascular disease [81].

5. Role of brain insulin on maintaining energy homeostasis

The role of insulin in the brain is less clear than the role of insulin in peripheral tissues. Not only are there fewer studies on brain insulin but there is still debate on whether insulin is produced in brain or if it is only transported from its peripheral source [83]. However, there is much evidence to support the presence of insulin in the brain. Several studies provide evidence that insulin mRNA is localized within the olfactory regions, limbic regions, and the periventricular nucleus (PVN) of the hypothalamus [84, 85]. Although, the concentration of cerebrospinal fluid insulin level was much lower than plasma insulin concentration, the infusion of insulin into plasma significantly raised insulin concentration in the cerebrospinal fluid [86]. Similar to the pancreas, it has been reported that glucose regulates the production of brain insulin. Injection of glucose into
the hypothalamus increased the concentration of insulin in the extracellular space [2, 87]. Insulin receptors (IR) are expressed not only in insulin sensitive peripheral tissues (muscle, fat and liver) but also, they are expressed widely in the CNS [2].

It was originally thought that the brain is insulin independent but more recent evidence has shown that insulin plays a crucial role in the brain in the regulation of energy balance, glucose homeostasis, learning, memory, food intake, neuroprotection and neuroplasticity [2]. It is known that the altered insulin signaling in the brain has been linked to the pathogenesis of neurodegenerative disorders such as Alzheimer’s disease [88, 89]. In the hippocampus, the insulin/IR complex is abundantly distributed in synaptic membranes and they function to regulate synaptic activities that are required for learning and memory [90-93]. Brain insulin regulates peripheral glucose metabolism by increasing insulin secretion and regulating hepatic glucose production [2]. The physiological role of insulin signaling in the brain is illustrated by the effects of insulin receptor knockout. Mice with a neuron-specific disruption of the insulin receptor (IR) gene (NIRKO mice) [83] had normal brain development and neuronal survival. However, the mice showed increased food intake, and developed diet sensitive obesity with increased body fat and plasma leptin levels, mild insulin resistance, elevated plasma insulin levels, and hypertriglyceridemia [28]. Taken together these studies demonstrate that insulin functions not only as a peripheral regulator of nutrient storage to maintain proper glucose level in the circulation but also it is a key afferent signal to the brain for the control of energy homeostasis [94].
6. Energy homeostasis

There is a growing concept that obesity induced by high fat diet could arise in part from defects in the brain regulation of energy homeostasis as well as peripheral metabolism [3]. Although the prevalence of obesity has increased dramatically over the last 25 years in the United States, this does not indicate that body weight is not regulated. Numerous studies suggested that body weight is tightly regulated and that brain regulates energy homeostasis by processing information from adiposity signals, including insulin and the adipocyte-derived hormone leptin, both of which circulate proportionate to body adiposity level [95]. Changed adiposity or energy balance in the body induce modification of the circulating hormones including insulin and leptin, which in turn inform the brain about the nutritional status of the body [96]. Bernstein et al. [97] demonstrated that overfed or underfed animals who gained or lost considerable amounts of weight during controlled regulation of food intake rapidly recovered their body weights to a weight close to the control animals when they were allowed to free-feed. In addition, it has been observed that energy expenditure was also increased in overfed animals suggesting that increase nutritional status regulates both food intake and energy expenditure at the same time [98]. Such studies as these suggest that there is a system that regulates body weight and energy balance. This regulation is known as energy homeostasis. Energy homeostasis is a physiological process in which energy intake is matched to energy expenditure over time to maintain body weight and body composition by promoting the stability of body fat level. A current model of energy homeostasis suggests that the level of body adiposity is regulated through an endocrine negative feedback loop involving adiposity hormones insulin and leptin (Figure 1-3). Both
hormones function in a negative feedback manner to reduce food intake while increasing energy expenditure through activating signaling in the arcuate nucleus (Arc) of the hypothalamus [95] and elsewhere in the brain [99].

7. **Metabolic sensing neurons in CNS and insulin signaling**

Insulin functions in a negative feedback manner to reduce food intake while increasing energy expenditure via actions on target neurons located in brain regions, particularly in the arcuate nucleus of the hypothalamus [95]. The hypothalamus has been suggested to have a critical role in the regulation of energy homeostasis [1]. The arcuate nucleus, situated adjacent to the floor of the third ventricle in the mediobasal hypothalamus (MBH), lies immediately above the median eminence which is a site characterized by an incomplete blood-brain-barrier (BBB) [100, 101]. This incomplete BBB allows neurons to sense acute fluctuations of hormones such as insulin and leptin and other signals and nutrients in the blood. The arcuate nucleus contains two major metabolic sensing neurons which are orexigenic AgRP/NPY neurons and anorexigenic POMC/CART neurons. These neurons have been shown to be responsible for the regulation of food intake and energy expenditure, and both types of neuron are responsive to insulin [94, 102].

In addition to the hypothalamus, many other regions of the central nervous system are involved in the regulation of energy balance. The brain stem, amygdala and other limbic regions of brain form complex neuronal connections with the hypothalamus to regulate feeding behavior [103, 104]. As described in later chapters, lesions of the amygdala have been associated with obesity and increased food intake [105, 106] and many neuro-peptides such as enterostatin [107], mu-opioid receptor agonist [108] and
galanin [109] have been shown to alter food intake when it is administrated into the amygdala. Neuro-peptide Y (NPY) is also expressed in the amygdala and various limbic systems and it is associated with number of behaviors including anxiety, depression, as well as feeding [110-113].

**NPY/AgRP neurons**

The orexigenic AgRP/NPY neurons co-express neuro-peptide Y (NPY) and Agouti-related peptide (AgRP); these neuro-peptides potently stimulate food intake, reduce energy expenditure, and thus increase body weight gain [94]. NPY-mediated orexigenic action occurs through the activation of Y1 or Y5 receptors [114]. Increased hypothalamic NPY mRNA was observed in diabetic animals and insulin deficiency led to activation of production and release of NPY neuro-peptides [115]. Injection of NPY into cerebral ventricles or hypothalamic area of rats potently increased food intake and reduced energy expenditure [116]. Moreover, genetic knockout of NPY reduced hyperphagia and obesity in *ob/ob* mice [117].

Similar to NPY, AgRP which is co-expressed in most NPY neurons in the arcuate nucleus of the hypothalamus [118], stimulates food intake when it is centrally administrated [119] and its level is increased in the hypothalamus of fasted rats [120]. The orexigenic action of AgRP is mediated via antagonizing neuronal melanocortin receptors such as MC3R and MC4R [121]. Since melanocortin signaling inhibits food intake and increases energy expenditure, blocking of melanocortin receptors by AgRP significantly increases food intake and energy expenditure similar to NPY [121]. It has been suggested that NPY/AgRP neurons are activated by low (negative) body energy
status to restore normal energy status [118]. This might be done by increasing NPY signaling and AgRP induced inhibition of the melanocortin signaling pathway [122].

**POMC/CART neurons**

The arcuate nucleus also contains anorexigenic pro-opiomelanocortin (POMC) / cocaine and amphetamine regulated transcript (CART) neurons. These neurons produce proopiomelanocortin, which in turn, is cleaved to produce α-melanocyte stimulating hormone (α-MSH) by posttranslational processing [102]. α-MSH has a potent anorectic effect in the CNS through the activation of melanocortin signaling [123]. POMC/CART neurons also produce the cocaine and amphetamine-regulated transcript (CART) peptide. Although its specific role in the anorexic action in response to insulin or leptin has not been determined, it has been suggested to have similar role as α-MSH does [102].

The AgRP/NPY and POMC/CART neurons located in arcuate nucleus (first order neurons) project widely and innervate other regions of hypothalamus such as paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) and regulate the activation of “second order” neurons [124] as shown in figure 1-4. The α-MSH secreted from the POMC neurons activates melanocortin-4 (MC-4) receptors expressed on secondary (downstream) neuronal populations, located in the PVN and ultimately decrease food intake and increase energy expenditure [125, 126]. Whereas, NPY released from NPY/AgRP neurons activates NPY receptors on secondary (downstream) neurons located in perifornical area (PFA) and LHA region to increase food intake and decreased energy expenditure. AgRP directly blocks α-MSH mediated activation of the MC-4 receptors, thus inhibiting α-MSH action [119]. These “second order” neurons located in the PVN and LHA express receptors for both NPY and AgRP and are implicated as
critical sites for relaying AgRP and NPY mediated signaling to downstream targets that regulate food intake and energy expenditure [102].

*Insulin signaling on metabolic sensing neurons*

Since the mechanism of insulin signaling and the physiological effect of insulin have been investigated primarily in peripheral tissues such as muscle, liver and adipose tissues with respect to glucose uptake and metabolism, most knowledge regarding molecular signaling mechanisms and the biological effects of insulin was gathered from studies in classical insulin sensitive tissues [96]. Recent work has focused on elucidating the signaling pathways and underlying mechanisms for insulin’s anorectic effect in the CNS, particularly in the hypothalamus. Interestingly, homologues of the insulin receptor, IRS1/2, PI3K, and PKB/Akt proteins are present in the mammalian central nervous system in such regions as the arcuate nucleus of the hypothalamus [3]. Moreover, the major molecular structure and most of the properties of brain insulin receptors are identical to peripheral insulin receptors [127]. This evidence suggests that brain insulin may have similar signaling pathways to control energy homeostasis as it does in peripheral tissues [128].

Insulin activates very similar signaling pathways in the brain as it does in peripheral tissues through activation of insulin receptors as described earlier in this chapter. Briefly, insulin activates the intrinsic tyrosine kinase activity of the receptor, leading to tyrosine phosphorylation of IRS-1/2, activation of PI3K and serine/threonine phosphorylation of Akt [18] [29]. The phosphorylated Akt mediates multiple downstream insulin signaling molecules including the phosphorylation and exclusion of transcription factor forkhead box protein O1 (FoxO1), which in turn regulates neuropeptide gene
expression. Recent studies reported that the phosphorylation and exclusion of FoxO1 from the nucleus lead to increased POMC gene expression in POMC neurons whereas insulin decreased FoxO1 mediated AgRP gene expression in NPY/AgRP neurons [129].

Several studies have demonstrated that insulin receptors are widely expressed in various neuronal populations including the NPY/AgRP neurons and POMC/CART neurons within the arcuate nucleus of the hypothalamus [95, 130]. Insulin controls energy homeostasis through regulation of neuro-peptide gene expression and release of those neuro-peptides as well as neuronal activity [95]. It has been suggested that activation of insulin signaling in the orexigenic NPY/AgRP neurons decreases NPY/AgRP neuro-peptides gene expression and that induces hyper-polarization of the NYP/AgRP neurons, and thus decrease neuronal activity (reduced firing rate) and neuro-peptide release [131]. In contrast to NPY/AgRP neurons, insulin stimulates POMC/CART neurons to increase POMC/CART neuro-peptide gene expression and release [95]. As described above, neuro-peptide POMC increases production of α-MSH which binds to melanocortin receptors (MC3 or MC4) to activate melanocortin signaling and mediate anorexic effect of insulin [132].

Both inhibition of NPY/AgRP neurons and activation of POMC/CART neurons by insulin appear to be dependent to IRS/PI3K signaling and the opening of ATP-sensitive K⁺ channels [131]. Intracerebroventricularly administrated LY294002 (a PI3K inhibitor) inhibited insulin induced anorexia in rats, suggesting that the PI3K pathway has a key role in mediating the effect of insulin on food intake [3, 128]. It has been reported that insulin regulates ATP-sensitive K⁺ channel opening by activation of PI3K signaling and downstream signaling molecules such as Akt, PDK1, glycogen synthase kinase 3
(GSK3), or mammalian target of rapamycin (mTOR) [129, 133]. Although insulin has been suggested to activate PI3K signaling in both NPY/AgRP and POMC/CART neurons, it appears to induce different signaling events downstream of PI3K depending on the cell type [2]. Thus insulin activation of the same signaling cascade (IRS-PI3K pathway) can lead either to membrane depolarization (POMC/CART neurons) or membrane hyperpolarization (NYP/AgRP neurons) [131, 134]. However, it is not known how activation of the same cascade leads to these contrasting results.

8. Mechanisms for insulin resistance in peripheral tissues

In peripheral tissues, high fat diet induced elevation of fatty acids and lipid metabolites have been suggested to be the major causal factor of insulin resistance. Although it is not fully understood, several mechanisms have been proposed through which fatty acids and/or lipid metabolites mediate insulin resistance in peripheral tissues. Most of these proposed mechanisms have focused on altered activation and phosphorylation of insulin signaling molecules such as IRS 1/2, PI3K and Akt [28]. It has been suggested that the mechanisms for insulin resistance in brain might be similar to mechanisms for peripheral insulin resistance because brain insulin has similar insulin signaling pathways and that altered PI3K signaling in the brain has been observed to increase food intake and to become insensitive to insulin’s anorectic effect [1, 3, 135]. This suggests that elevated fatty acids and lipid metabolites might have an important role in the induction of insulin resistance in the brain as it does in peripheral tissues. In fact, several recent studies demonstrated that insulin signaling molecules in peripheral tissues including IRS 1/2, PI3K, and Akt are also altered in the brain in response to high fat feeding or intracerebroventricularly applied lipid and fatty acids [136]. Therefore, it is
helpful to understand the possible mechanisms for insulin resistance in peripheral tissues in order to understand brain insulin resistance.

*Mitochondrial dysfunction and increased oxidative stress*

Studies suggested that elevated FFA can cause mitochondrial dysfunction through the reduction of lipid oxidation and oxidative phosphorylation and the malfunction of mitochondria is a possible causal factor for insulin resistance [137-140]. Altered mitochondrial function causes decreased insulin stimulated glucose metabolism such as glycogen synthesis, and glucose oxidation resulting in reduced ATP generation [141]. The saturated fatty acid, palmitic acid significantly reduced muscle ATP production compared to unsaturated fatty acid when applied to muscle cells [141]. Since, like most cellular responses, insulin stimulated glucose uptake in muscle cell requires high energy such as ATP, reduced production of mitochondrial ATP due to mitochondrial dysfunction may induce insulin resistance [142-144]. Altered mitochondrial function also reduces fat oxidation resulting in the accumulation of cellular fatty acids and this can lead to insulin resistance although the detailed molecular mechanisms are not understood [145]. Mitochondria are the primary site for the production of reactive oxygen species (ROS). Increased mitochondrial oxidative phosphorylation due to increased nutrient input in absence of sufficient ATP consumption to generate ADP may accelerate mitochondrial ROS production because of the depleted ADP availability and increased occupancy of electron carriers [138]. Oxidative stress is caused from increased ROS which can damage DNA, proteins and lipids by oxidizing process and it is suggested to play a key role in inducing insulin resistance [146]. Both animal and human studies with lipid infusion or high fat diet have shown decreased mitochondrial ATP synthesis, mitochondrial
respiration, and oxidative phosphorylation [147-151]. In vitro experiments have shown that palmitic acid application to muscle cells increased ROS production, impaired FFA oxidation, decreased ATP synthesis and mitochondrial density in skeletal muscle [139, 152-154].

*Activation of proinflammatory NF-κB and mitogen-activated kinases*

Over nutrition is associated with chronic inflammation in metabolic tissues [155]. Although the molecular mechanism is not known, it has been suggested that chronic activation of pro-inflammatory signaling in peripheral tissues such as skeletal muscle and adipose might be one causal factor that contributes to elevated FFA induced metabolic disorders such as insulin resistance [156, 157]. This chronic inflammation is caused by an elevated circulating level of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and prolonged elevated free fatty acids level, particularly saturated fatty acids [158]. These cytokines can block insulin action whereas, knockout of these cytokines genes can disrupt the correlation between elevated FFA and insulin resistance [159]. These cytokines are mainly secreted from increased macrophages in adipose tissue. It has been reported that obesity increases the number of macrophages in adipose tissues and corresponding elevated cytokine levels. The expression and secretion of both TNF-α and IL-6, is increased and these impair glucose metabolism in hepatocytes, muscle and adipocytes by affecting various signaling pathways [158].

Both TNF-α and IL-6 are known to activate the inhibitor of nuclear factor κB kinase-β (IKK-β) which is a key pathway in tissue inflammation in adipocytes and hepatocytes [155]. Since IKK-β is a serine kinase, it has been reported that increased
level of IKK-β induces serine phosphorylation of IRS-1 with subsequent reduction of insulin dependent tyrosine phosphorylation of IRS-1, activation of PI3-kinase, and ultimately a reduced glucose uptake rate [69, 160]. In contrast, reduction of IKK-β or decreased IKK-β expression improved insulin sensitivity by decreasing IKK-β mediated serine phosphorylation of IRS-1 [161].

The saturated fatty acid palmitic acid, but not an unsaturated fatty acid, induced serine phosphorylation of IRS-1 and corresponding altered insulin signaling pathway through the activation of IKK-β when applied to peripheral cells. In contrast, the dominant negative construct of IKK-β reversed the saturated fatty acid induced serine phosphorylation of IRS-1 to tyrosine phosphorylation of IRS-1 [162]. IKK-β controls the activation of nuclear factor kappa-light-chain-enhancer of activate B cells (NF-κB), a family of transcription factor that regulate expression of pro-inflammatory genes [163]. The IKK-β/NF-κB pathway has a critical role in lipid-induced insulin resistance in L6 myocytes [163]. In this study, palmitic acid induced the inhibition of insulin stimulated glucose uptake and also stimulated nuclear translocation of NF-κB in a dose dependent manner, whereas inhibition of NF-κB nuclear translocation blocked the harmful effect of palmitate on insulin action [163]. Also it has been reported that NF-κB activates IκB kinase ε (IKKε), a direct transcriptional target of the NF-κB pathway, which regulates further inflammatory signals through phosphorylation of other transcription factors. Chiang et al. [164] showed that IKK-ε gene and protein expression and enzyme activity was increased in liver, adipocytes and adipose tissue macrophages after a high fat diet, which increased activation of NF-κB. IKK-ε knockout mice resist the deleterious effects of HFD on insulin sensitivity and glucose intolerance [164].
Palmitic acid induced activation of mitogen activated protein kinases (MAP kinase), p38 MAP, JNK and ERK is suggested to mediate the palmitic acid induced insulin resistance in muscle cells through the activation of the IKK-β and NF-κB proinflammatory signaling pathway [158, 165, 166]. Palmitic acid application to L6 skeletal muscle cells induced the activation of MAP kinase, particularly ERK, and the activated ERK regulated the activation of both IKK-β and NF-κB pathway; palmitate-induced insulin resistance in muscle cells reduces glucose uptake [158].

**ER-stress induced activation of JNK pathway**

Like other possible mechanisms that explain elevated fatty acid induced insulin resistance, lipotoxicity caused by increased saturated fatty acid level in the circulation has been shown to cause endoplasmic reticulum (ER) stress in peripheral tissues such as adipose, muscle, and liver. Increased ER stress is suggested as a critical intracellular response that induces insulin resistance under conditions of over nutrition [167, 168]. Elevated ER stress has been shown in adipose tissue of ob/ob mice and mice fed high-fat diets [167, 169, 170]. The ER is the major site for cellular protein and lipid synthesis as well as protein folding. When the influx of misfolded or unfolded proteins exceeds the ER folding capacity, the ER becomes stressed due to the accumulation of newly synthesized unfolded proteins and the ER activates the unfolded protein response (UPR) to alleviate ER stress [171]. The UPR includes three signaling pathways which are inositol-requiring enzyme (IRE)-1, PKR-like ER protein kinase (PERK), and activating transcription factor (ATF)-6. These three signaling pathways alleviate ER stress by slowing protein synthesis, increasing expression of protein chaperones, and promoting degradation of unfolded proteins [172, 173]. However, prolonged ER stress induces the
UPR to activate several inflammatory and stress signaling pathways including IKK-β/NF-κB and c-Jun N-terminal kinase (JNK)/Activator protein (AP)-1 pathways and these signaling pathways can impair insulin signaling and induce insulin resistance [171].

ER stress has been reported to activate several serine/threonine kinases including JNK. ER stress activates JNK to phospho-JNK (primarily to phospho JNK1) in cultured cells and animal models [169, 174-176] and the activation of JNK is coupled to ER stress induced UPR, IRE-1 activation [176]. JNK activation has been shown to produce insulin resistance and inflammation, while JNK inhibition improved insulin sensitivity in fat, liver and muscle of several animal models of obesity [169]. JNK activation inhibits insulin signaling transduction through increasing serine phosphorylation of insulin receptor substrate (IRS)-1 [169]. ER stress has been demonstrated to trigger another serine/threonine kinase, IκB kinase (IKK-β) and is a major source for the production of ROS, which not only produces insulin resistance (by serine phosphorylation of IRS-1) but also stimulates synthesis and release of proinflammatory cytokines and chemokines such as TNF-α and IL-6 [169, 177].

An accumulation of ceramide

Recently, ceramides, structural units and common precursors of complex sphingolipids have been suggested to act as signaling molecules and induce insulin resistance in peripheral tissues such as liver, muscle and adipose [178]. Elevated circulating saturated fatty acids induce activation of ceramide synthase and sphingomyelinase that are related to ceramide synthesis [179]. Also, long chain saturated fatty acids which are more poorly oxidized than long chain unsaturated fatty acids are likely to increase intracellular saturated fatty acyl chains such as palmitoyl-CoA, and this
accelerates the de novo synthesis of ceramide [179]. Ceramide level was increased in muscle and liver of female Zucker (fa/fa) rats [180], and administration of excess long chain saturated FFAs (e.g. palmitate) was shown to promote ceramide formation in cultured myocytes while unsaturated or shorter saturated FFAs (i.e. myristate) had no significant effect [181]. It has been reported that ceramide inhibits insulin stimulated glucose uptake, Glut4 translocation and glycogen synthesis [20, 182-184]. Although, the molecular mechanism of ceramide induced insulin resistance is not fully understood, several possible mechanisms have been proposed.

Ceramide is proposed to activate several serine/threonine kinases such as extracellular signal-regulated kinase 2, JNK, IκK-β and these can increase serine phosphorylation of IRS-1, instead of tyrosine phosphorylation of IRS-1, resulting in inhibition of subsequent recruitment and activation of PI3-kinase and this inhibits further insulin signaling pathways [185-187]. Several possible mechanisms have been suggested by which ceramide inhibits phosphorylation and activation of Akt; Ceramide induces the dephosphorylation of Akt by activating protein phosphatases 2A (PP2A) which is the primary phosphatase responsible for dephosphorylation of Akt [188-190]. Treating C2Cl2 myocytes and brown adipocytes with the PP2A inhibitor alleviated the inhibitory effect of ceramide on Akt phosphorylation [191, 192]. It is suggested that ceramide blocks the translocation of Akt from cytosolic to plasma membrane preventing its binding with PIP3 [193-195]. Recently, it has been suggested that serine/threonine kinase PKCζ, the atypical PKC isoform, is activated by ceramide and this inhibits Akt translocation by inducing threonine phosphorylation at Akt. This alters the function of the PH domain of Akt blocking its ability to interact with PIP3 [194, 196]. In L6 myocytes, ceramide
inactivation of Akt was reduced by the administration of PKCζ inhibitors or the expression of dominant negative PKCζ constructs [196].

An accumulation of diacylglycerol, leading to activation of PKCθ

It appears that the neutral lipid triacylglycerol is not likely to induce insulin resistance. Instead, the accumulation of the intermediate metabolite diacylglycerol (DAG) has been reported to induce insulin resistance in peripheral tissues such as muscle and liver [197]. DAG can be produced from multiple sources, de novo synthesis of DAG by esterification of two long-chain acyl-CoAs to glycerol-3-phosphate [198], breakdown of phospholipids, PIP2, by phospholipase C (PLC) [199] and the hydrolysis of triglyceride by recently discovered adipose triglyceride lipase (ATGL) [200-202].

It has been reported that increased fatty acids, particularly long chain saturated fatty acids, accelerate the accumulation of diacylglycerol in peripheral tissues especially in skeletal muscle cells [181]. Palmitate, but not unsaturated fatty acids such as oleate, significantly increased the accumulation of DAG and decreased glycogen synthesis in C2C12 myocytes [181]. Similarly, palmitate but not linoleate increased the level of DAG in L6 myocytes and decreased insulin stimulated glucose uptake [203]. These results suggest that saturated fatty acids enhance the accumulation of DAG in muscle cells whereas unsaturated fatty acids are likely converted into triglyceride [198].

DAG is not only synthesized and incorporated into the plasma membrane as glycerophospholipids, a main component of the cell membrane, but DAG also is an important second messenger in the plasma membrane involved in intracellular signaling. It is thought to alter insulin signaling through activation of protein kinase C (PKC)θ [204]. PKCθ, a member of novel PKCs (nPKCs; δ, ε, η, and θ) and a family of serine/threonine
kinases performs critical roles in the regulation of cellular differentiation as well as proliferation in various cell types and responds to diverse stimuli such as hormones, neurotransmitters, and growth factors [205-208]. PKCθ is activated in response to accumulation of DAG [209-211]. It has been shown that PKCθ is activated by DAG binding to its conserved domain 1 (C1), and this causes the translocation of PKCθ from the cytosol to the plasma membrane and the activation of PKCθ [212, 213]. One possible mechanism through which the activation of PKCθ can induce insulin resistance is through serine phosphorylation of the insulin receptor which inhibits tyrosine phosphorylation and kinase activity of the receptors. Serine residues in the β-subunit of the insulin receptor seem to be an important target for PKC phosphorylation [214]. Also, PKCθ has been reported to phosphorylate IRS-1 at serine residues inhibiting its tyrosine phosphorylation [215]. In vitro studies demonstrated that serine phosphorylation in either IR or IRS-1 inhibit binding between IR and IRS-1 and this results in decreased PI3-kinase activation and Akt phosphorylation [216, 217]. Ultimately, the serine phosphorylation in both IR and IRS-1 might result in altered insulin stimulated glucose transporter activation and corresponding glucose uptake. A study by Yu et al. [218] showed that infusion of a lipid emulsion in rats which elevated DAG level by three-fold, was associated with increased PKCθ activity, reduced insulin stimulated IRS-1 tyrosine phosphorylation and a corresponding 50 % reduced PI3-kinase activity. Similarly, studies with obese Zucker rats showed that both DAG mass, and PKCθ membrane localization were increased [219]. Furthermore, PKCθ knockout mice were protected from high fat diet induced insulin skeletal muscle insulin resistance showing no decreases in insulin
stimulated tyrosine phosphorylation of IRS-1 and corresponding PI3-kinase activity compared to wild-type mice [220].

The possible mechanisms through which saturated fatty acids induce insulin resistance are summarized in figures 1-5 and 1-6.

9. Overview of Aims

The primary goal of the research described in this dissertation was to investigate the molecular mechanism of the central insulin resistance caused by dietary fat and intracellular fatty acids. The overall hypothesis states that the dietary fat and elevated fatty acids induce the development of central insulin resistance in neuronal cells.

Fatty acid chain length and the degree of fatty acid saturation are known to influence insulin signaling. Among different kinds of fatty acid, saturated fatty acids such as palmitic acid have been identified to cause insulin resistance in peripheral tissues [222-224]. Thus, as described in chapter 2, I investigated the effect of different kinds of fatty acids (saturated fatty acid, mono-unsaturated fatty acid, and poly-unsaturated fatty acid) on insulin signaling in neuronal cells (GT1-7; hypothalamic cell lines and amygdala primary cells) as an in vitro approach. In insulin sensitive peripheral cells such as muscles and adipocytes, altered insulin signaling induces impaired glucose uptake. Thus, I investigated whether the alteration of insulin signaling in these neuronal cells is also associated with the level of glucose uptake as in peripheral cells.

Recent data from our laboratory showed that 3 days of high fat diet (HFD) abolished insulin’s anorectic effect when it was directly injected into the central nucleus of the amygdala (CeA) of rats suggesting that amygdala insulin regulates energy balance as it does in the hypothalamus. Thus, in chapter 3, I investigated whether the loss of
insulin’s anorectic effect in the amygdala is associated with a HFD induced alteration of insulin signaling in the amygdala and/or hypothalamus. As described in chapter 2, I have shown that only saturated fatty acid, palmitic acid, but not mono-unsaturated fatty acid nor poly-unsaturated fatty acid, potently impaired insulin signaling in neuronal cells as an *in vitro* approach. To test whether palmitic acid has a similar effect when it is applied in an *in vivo* experiment, the palmitic acid was directly infused into the CeA and the effect on insulin signaling in the amygdala and hypothalamus was also investigated.

Our laboratory recently has shown that a high fat diet significantly increased myristoylated alanine-rich C kinase substrate (pMARCKS), a substrate for PKCθ, in the rat’s amygdala and that PKCθ expression was significantly increased in the both amygdala and hypothalamic areas. Taken together with other recent reports, these data suggest that PKCθ might be a possible mechanism that underlies fatty acid induced insulin resistance in the central nervous system as well as in peripheral tissues. Thus, in Chapter 4, I investigated the effect of high fat diet and saturated fatty acid on PKCθ activation, and the effect of PKCθ activation on insulin signaling in both neuronal cells and brain tissues.

The findings and interpretations from all of these experiments is summarized in chapter 5. Study limitations and future directions are also addressed. The data presented suggests a possible mechanism for high fat diet induced brain insulin resistance and suggests future research directions for further understanding the mechanisms involved.

10. References


139. Lambertucci RH, Hirabara SM, Silveira Ldos R, Levada-Pires AC, Curi R, Pithon-Curi TC: *Palmitate increases superoxide production through*


Figure 1-1: Insulin’s physiological function. Insulin regulates diverse physiological processes. Figure 1 summarizes insulin’s many physiological effects. (1) It increases cellular uptake of glucose in muscle and adipocytes. (2) Insulin increases DNA replication and protein synthesis through uptake of amino acids. (3) In muscle and liver, insulin increases glycogen synthesis and induces lipid uptake to adipocytes and increases synthesis of fatty acids. (4) Insulin also increases ion flux into muscle and fat cells through increasing \((\text{Na}^+, \text{K}^+)-\text{pump}\) activity which lowers potassium level in the blood. (5) Insulin decreases apoptosis, gluconeogenesis, lipolysis and proteolysis [18, 22].
Figure 1-2: **Insulin signaling transduction pathway.** Insulin binding to the insulin receptor results in phosphorylation of tyrosine residues on the receptor and substrates such as IRS. Phosphorylated IRS activates both mitogenic and metabolic pathways. For the metabolic pathways, docking of the regulatory subunit of PI3-kinase to phosphotyrosine residues of IRSs activates its serine/threonine kinase activity and the phosphorylation cascade involving PDKs and Akt/PKB. PI3K/Akt pathway activates several anabolic signaling pathways to increase protein, lipid and glycogen synthesis and regulate blood glucose level. PI3K induced Akt phosphorylation and PI3K independent CAP-TC10 pathways enhance the translocation of glucose transporter (GLUT) in plasma membrane. Also IRSs activates Ras complex to activate MAP kinase pathways [23].
Figure 1-3: Model of central nervous system control of energy homeostasis. Adiposity signal Insulin and leptin are secreted proportional to body fat content and control both anabolic and catabolic pathways in central nervous system to regulate energy homeostasis. Both leptin and insulin act to repress brain anabolic pathways that stimulate eating and inhibit energy expenditure, while they activate catabolic pathways that inhibit food intake and increase energy expenditure. Figure adapted from Schwartz et al. [95].
Figure 1-4: Neuronal connection between first order neurons (NPY/AGRP and POMC/CART neurons in the arcuate nucleus) and second order and downstream neurons in PVN and LHA/PFA. Both insulin and leptin activated the first order NYP/AGRP and POMC/CART neurons project to the PVN and to the LHA and PFA to activate second order and downstream neurons to regulate food intake and energy balance. Figure adapted from Schwartz et al. [94, 95].
Figure 1-5: Signaling pathway for saturated free fatty acids (SFFAs) and saturated long chain fatty acyl-CoA (SLCFA-CoA) mediated insulin resistance. Insulin receptor (IR) and insulin receptor substrate (IRS) are induced to be phosphorylated at serine residues by activation of various serine/threonine kinases including PKCθ, IκK-β and JNK. Increased serine phosphorylation alters tyrosine phosphorylation of both IR and IRS, which, in turn, impair further insulin signaling transduction such as glucose uptake [191, 192, 215, 219, 221].
Figure 1-6: Summarized possible effects of saturated fatty acids on peripheral insulin resistance. Elevation of intracellular saturated fatty acid level has been suggested to activate signaling pathways: mitochondrial dysfunctions, inflammatory response, ER stress, accumulation of Ceramide and diacylglycerol and PKCθ.
CHAPTER 2
FATTY ACIDS INDUCED INSULIN RESISTANCE IN NEURONAL CELLS

1. Introduction

The accumulation of various lipid metabolites and fatty acids in peripheral tissues (such as liver, muscle and adipose) has been implicated in the insulin resistance seen in type 2 diabetes. In the insulin resistant state, the liver increases hepatic glucose production and adipose tissue increases lipolysis resulting in an increase in circulating free fatty acids. Also, skeletal muscle decreases insulin stimulated glucose uptake significantly in the insulin resistant state [1]. Many studies have demonstrated that fatty acids, particularly long chain saturated fatty acids such as palmitic acid, induce insulin resistance in peripheral cells [2-7]. Palmitic acid (PA, C16:0) is the most abundant saturated fatty acid, representing about 30% of the total non-esterified fatty acid (NEFA) in human plasma [2, 8]. Although the molecular mechanism through which saturated fatty acids induce insulin resistance in peripheral cells is not fully understood, a number of possible mechanisms have been proposed. These have been described in the previous chapter but briefly, these include:

*Mitochondrial dysfunction and increased oxidative stress*

Studies suggested that the accumulation of saturated fatty acid might induce insulin resistance through increasing mitochondrial reactive oxygen species (ROS) and corresponding mitochondrial dysfunction [9-11]. Increased ROS damages mitochondrial DNA, protein and lipids by oxidizing process, which in turn, alters mitochondrial function and decreases ATP-production [4]. Since ATP is essential energy source in
insulin stimulated glucose metabolism such as glycogen synthesis, glucose oxidation and glucose uptake, decreased mitochondrial ATP production can alter insulin action and induce insulin resistance [4, 9].

*Activation of proinflammatory NF-κB and mitogen-activated kinases*

Accumulation of saturated fatty acids induces production of proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and activation of mitogen activated protein kinases (MAPKs). These cytokines and MAPKs alter insulin signaling pathways through the activation of several serine kinases such as inhibitor of nuclear factor κB kinase-β (IKK-β) which is a key pathway in tissue inflammation [12-14]. Since IKK-β is a serine kinase, it has been reported that increased IKK-β level inhibited insulin stimulated phosphorylation of insulin receptor substrate (IRS)-1 at tyrosine residues by phosphorylating it at serine residues [15, 16].

*ER-stress induced activation of c-Jun N-terminal kinase*

Elevation of saturated fatty acids cause ER stress and prolonged ER stress induces the UPR to activate several inflammatory and stress signaling pathways including c-Jun N-terminal kinase (JNK) pathway which can induce insulin resistance [17]. JNK activation inhibits insulin signaling transduction through increasing serine phosphorylation of IRS-1 [18].

*An accumulation of ceramide*

Increased ceramide production due to elevated long chain saturated fatty acyl-CoA has been suggested to induce insulin resistance by inhibition of IRS-1 tyrosine
phosphorylation, inhibition of Akt (Protein kinase-B) phosphorylation and Akt translocation into plasma membrane [5, 19-24].

An accumulation of diacylglycerol, leading to activation of PKCθ

Elevated saturated fatty acids accelerate the production of lipid metabolites such as diacylglycerol (DAG) in peripheral tissues such as muscle and liver [25, 26]. The accumulation of DAG in plasma membrane induces the activation and translocation of PKCθ which is a serine/threonine kinase which can induce insulin resistance by increasing serine phosphorylation on both IRS-1 and the insulin receptor blocking insulin stimulated tyrosine phosphorylation and corresponding PI3K/Akt activity [27-29]. Ultimately, insulin stimulated glucose uptake and glucose metabolism is altered [26].

It is also evident that many of these mechanisms that are linked to saturated fatty acids-induced insulin resistance may be interdependent upon each other and interact to induce insulin resistance [30]. As described in chapter 1, expression and the phosphorylation level of insulin signaling molecules such as the insulin receptor, IRS-1 and phosphatidylinositol 3-kinase (PI3K) are critical in this insulin signaling transduction [31, 32]. Phosphorylation of Akt is downstream of PI3K. Most studies have focused on the phosphorylation of IRS-1, the activation of PI3K or the phosphorylation of Akt level on insulin resistance in peripheral cells. The serine and threonine phosphorylation of Akt through PI3K mediates most of insulin’s metabolic functions (glucose uptake, glycogen synthesis, lipid synthesis and protein synthesis). Particularly, insulin signaling to regulate glucose uptake in muscle is mediated through a pathway dependent on PI3K and Akt phosphorylation [33, 34]. There are three subtypes of Akt (Akt1, Akt2 and Akt3) in mammals. Akt1 is involved in the regulation of the protein synthesis pathway and the
cellular survival pathway by inhibiting apoptosis [35]. The function of Akt3 has been less identified; however, it has been reported that Akt3 function is related to brain development; Akt3 knockout mice have a reduced brain size [36]. Insulin resistance studies focus on Akt2 because activated Akt2 is the key protein in insulin signaling which affects insulin stimulated glucose uptake [37] in skeletal muscle and adipose cells to regulate blood glucose level through translocation of glucose transporter proteins into the plasma membrane. It has been reported that all Akt isoforms are expressed in the brain [38]. Akt2 is expressed in brain and has an important role in regulation of the expression of glucose transporter in brain to increase glucose supply into neurons [38]. The studies reported in this dissertation focused on the phosphorylation level of Akt2 in the brain or neuronal cells as a marker of insulin signaling action and the alteration caused by various fatty acids.

In contrast to saturated fatty acids, mono-unsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) are effective in enhancing insulin sensitivity in peripheral cells [3, 6, 39, 40]. MUFAs have a single double bond in the fatty acid chain. The common long chain MUFAs are oleic acid (OA, C18:1) and palmitoleate (PO, C16:1). In particular, OA represents about 90% of the MUFA and 30% of the total free fatty acids in human plasma. It enhances insulin sensitivity in skeletal muscle and adipocytes [6, 41]. The saturated fatty acid palmitic acid caused a significant increase in the activation of JNK, apoptosis and inhibition of insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation in skeletal muscle [42], whereas the unsaturated FFA oleate had no effect. In a second study which compared the effects of palmitate and the mono-unsaturated fatty acid palmitoleate on insulin action and glucose utilization in rat L6...
skeletal muscle cells, palmitoleate significantly increased insulin stimulated glucose uptake, glycogen synthesis and glucose oxidation whereas palmitate had an inhibitory effect [30].

Polyunsaturated fatty acids possess two or more double bonds in their hydrocarbon chains. Varying by the location of the first double bond near the methyl terminus, they can be classified as either n-6 or n-3 fatty acids. The most common n-6 PUFA is linoleic acid (LA, C18:2n-6). LA can be found in vegetable oils, seeds and nuts. The most common n-3 PUFA is α-linolenic acid (ALA, C18:3n-3), and it is found in leafy vegetables, walnuts, soybeans, flaxseed, and seed and vegetable oils [43]. Through desaturation and elongation processes, both LA and ALA can be converted into longer chain PUFAs. LA can be processed to produce arachidonic acid (AA, C20:4n-6), whereas, ALA can be converted to eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). Both n-6 and n-3 PUFAs are essential fatty acids since humans cannot synthesize them, therefore, they need to be consumed in the diet [43].

N-3 fatty acids, in particular, have been shown to have beneficial effects for several chronic diseases including cancer, by decreasing tumor growth and metastasis, and cardiovascular disease, by decreasing the thickness of the carotid arteries, plaque build-up in arterial walls and reducing very low density lipoproteins (VLDLs) [44-47]. In recent years, there has been a higher focus on the role of n-3 fatty acids in insulin resistance. Studies suggest that the anti-diabetic effects of n-3 fatty acids are elicited through increasing basal metabolic rate, fat oxidation and glucose metabolism [48, 49]. In a controlled feeding study to determine the effect of EPA and DHA on insulin resistance, body fat mass decreased and lipid oxidation was significantly increased in healthy
volunteers when dietary fat was substituted with fish oil [48]. Several studies have also shown that n-3 fatty acids improve insulin resistance in many rodent models of obesity and diabetes [50-52]. Although the underlying molecular mechanisms are not well understood, n-3 fatty acids are reported to repress inflammatory responses such as JNK signaling and enhance insulin sensitivity [53, 54]. JNK activation induces insulin resistance through induction of serine phosphorylation of IRS-1 protein, which then results in, altered insulin signaling [55, 56]. JNK activation also inhibits both serine and threonine phosphorylation of Akt [54].

Another possible mechanism for the n-3 fatty acid affect on insulin signaling pathways might be the activation of G-protein coupled receptors (GPCRs) that may crosstalk with the insulin signaling pathway and potentiate insulin stimulated insulin signaling pathways [57]. Recently, several groups have identified orphan G-protein coupled receptors (GPR40, GPR41, GPR43, GPR83, and GPR120) which can be activated by various free fatty acids [58-60]. This demonstrates that fatty acids are not only an important energy source and act mainly in the intracellular region, but may also act as signaling molecules as selective ligands for membrane receptors [61]. In particular, both GPR40 and GPR120 have become potential drug targets for diabetes because these two GPCRs regulate insulin secretion in response to fatty acids [61]. GPR40 is activated by medium- to long chain fatty acids and has a direct role in potentiating glucose stimulated insulin secretion in pancreatic β-cells in response to elevated free fatty acid [58-61]. GPR120 is activated mainly by long-chain unsaturated fatty acids and plays an important role in the secretion of glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK) from enteroendocrine cells [62-64].
GPCRs are members of a large family that share the common structural motif of seven transmembrane helices and the ability to activate heterotrimeric G proteins, such as \(G_{\alpha s}\), \(G_{\alpha i}\), and \(G_{\alpha q}\). The activation of GPCRs by ligand binding stimulates and induces a variety of cellular responses through several second messenger pathways such as cAMP production, phospholipase C (PLC), ion channels, and mitogen-activated protein kinases [65, 66]. Fatty acid binding to GPR40 and GPR120 initiates responses similar to those of cholinergic stimulation of GPCRs [67]. It activates heterotrimeric GPCRs containing the \(\alpha\)-subunit \(G_q\) \((G_{\alpha q})\) which activates PLC mediated hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). The two second messengers (DAG and IP3) activate protein kinase C (PKC) and increase intracellular calcium concentration \([Ca^{2+}]\) by mobilization from the ER [62, 68].

In contrast to n-3 PUFAs, the effect of n-6 PUFAs on insulin resistance is controversial. Several studies have suggested that LA may have beneficial effects on insulin resistance, glucose metabolism, body composition and the prevention of type-2 diabetes [69, 70]. However, other studies have reported that n-6 fatty acids may induce insulin resistance through the activation of proinflammatory signaling pathways. CLA has been reported to induce a proinflammatory response by increasing secretion of cytokines such as TNF-\(\alpha\), and it has been shown that CLA activates the NF-\(\kappa B\) pathway in primary cultures of human adipocytes [71, 72].

Intensive research has identified multiple mechanisms that might contribute to the effect of fatty acids on insulin resistance in peripheral tissues as described previously. However, there have been few investigations on the effects of insulin and fatty acids in the brain because the brain was considered, until recently, to be insulin independent.
Insulin receptors and insulin receptor mRNA expression have been found in various regions of the brain including the olfactory bulb, hypothalamus, hippocampus, cerebellum, cerebral cortex and amygdala [73, 74], and the molecular structure and the properties of brain insulin receptors are identical to the peripheral insulin receptors [75]. Also, glucose transporters including Glut4 have been found in brain regions [76]. Diverse experimental approaches have contributed to our recognition that the brain is insulin sensitive and that insulin actions in the CNS have an important role in the regulation of body weight and peripheral metabolism. Intracerebroventricular (icv) insulin reduced food intake and activated insulin stimulation of PI3K/Akt signaling pathway in rat’s hypothalamus [77]. The neuron specific insulin receptor knockout mice (NIRKO), mice lacking insulin receptors in the CNS become obese with elevated circulating insulin levels, and develop moderate insulin resistance suggesting an important role of brain insulin and insulin receptors in the regulation of body weight and peripheral metabolism [78]. It has been suggested that increased dietary fat has a critical role in the onset of insulin resistance in both peripheral and brain tissues. The anorectic effect of icv insulin administration disappeared when the rats were on high a fat diet and insulin stimulation of hypothalamic PI3K and Akt phosphorylation was blunted [77]. Rats on high a fat diet increased saturated long chain fatty acid levels (palmitoyl and stearoyl CoA) in the hypothalamic region suggesting accumulation of saturated fatty acids in hypothalamus might be a critical factor causing hypothalamic insulin resistance as in peripheral tissues [77]. The effects of icv palmitic acid infusion further support the inhibitory role of elevated saturated fatty acid on hypothalamic insulin resistance. After palmitic acid infusion into the hypothalamic region, palmitoyl-CoA level is significantly
increased and insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation was significantly decreased compared to the icv vehicle-infused rats [77, 79]. Furthermore, studies showed that only palmitic acid and not oleic acid or n-3 poly-unsaturated fatty acids induced this harmful effect [77, 80].

The majority of studies have focused on the hypothalamus because it has a central role in the regulation of body weight and peripheral metabolism [81]. Many other regions of the brain such as nucleus accumbens, the amygdala and the brain stem as well as the hypothalamus are also involved in the regulation of energy homeostasis [82, 83]. The amygdala, a brain region that has been previously studied for its function in processing emotions, such as fear and anxiety has recently become of interest for its role in influencing ingestive behaviors [84, 85]. Our laboratory has demonstrated that the insulin has a strong anorectic effect when injected into the central bed nucleus of the amygdala (CeA) and that the CeA becomes rapidly insulin resistant in animals fed a high-fat diet (HFD) [86]. This suggests the possibility that insulin may act directly in the amygdala to regulate energy balance as hypothalamus or possibly through its connections to the hypothalamus. Also, similar to the hypothalamus, HFD-induced saturated fatty acid accumulation might inhibit the insulin action in amygdala region.

The experiments discussed in this chapter were designed to identify and characterize the insulin signaling transduction pathway in neuronal cells and the changes induced by various types of fatty acid. We sought to determine whether similar signaling pathways are activated in neuronal cells as in the classically insulin sensitive peripheral cells and the effects of elevated levels of saturated fatty acids.
2. Hypotheses

1) Neuronal cells may activate similar signaling pathways in response to insulin stimulation as insulin sensitive peripheral cells.

2) Among different kinds of fatty acids, only saturated fatty acids, specifically palmitic acid, will inhibit insulin signaling pathways in neuronal cells.

To test these hypotheses, the protein expression and phosphorylation level of insulin signaling molecules were determined in response to insulin stimulation when neuronal cells were incubated with or without fatty acids in culture. In addition, the changes in insulin stimulated glucose transport levels were measured as a functional end point of insulin action.

3. Materials and Methods

Cell culture

GT1-7 cells: The GT1-7 mouse hypothalamic neuronal cell line was obtained from Dr. P. Mellon (University of California, San Diego) and was derived originally from immortalized fetal hypothalamic neurons. GT1-7 cells were cultured on 6 well plates and maintained in Dulbecco’s media modified Eagle’s medium (Hyclone, Logan, UT) containing 10% fetal bovine serum (Hyclone, Logan, UT) at 37 °C in 5% CO₂ atmosphere. Cells with 60 ~ 80% confluent and below passage 20 were used.

L6 skeletal muscle cells: The L6 rat skeletal muscle cell line was obtained from American Tissue Culture Collection (Manassas, VA). L6 cells were cultured on 6 well plates and maintained in DMEM containing 10% fetal bovine serum, penicillin (100 unit/ml) and streptomycin (100ug/ml) (MP Biomedical, Solon, Ohio) at 37 °C in 5% CO₂ atmosphere. Cells with 60 ~ 80% confluent and below passage 20 were used.
**Amygdala primary neuronal cells:** The procedures used for preparation of amygdala primary neuronal cells were based on the methods of Brouard *et al* [87] and Cratty and Birkle [88]. Pregnant Sprague-Dawley rats containing embryonic day (19–20) pups were killed by administration of CO₂. The uterus was dissected out and placed in cold 1X PBS (NaCl, 137 mM; Na₂HPO₄, 21 mM; KH₂PO₄, 29 mM; KCl, 1.2 mM, pH 7.3), and the embryos were removed and decapitated. The brains were removed and amygdala dissected. The tissue was sliced, placed in PBS and cells were dissociated for 10–15 minutes at 37 °C in 0.25% trypsin (Mediatech, Manassas, VA) containing 75 U/ml of DNase I in serum-free medium (SFM) consisting of a mixture of DMEM and Ham’s F-12 (1:1, V/V, Caisson Laboratories Inc. Logan, UT) supplemented with 14 mM glucose, 15 mM NaHCO₃, 5 mM Hepes, Penicillin (20 unit/ml) and streptomycin (20 μg/ml). Cells were collected by centrifugation (500g, 5 minutes), resuspended in SFM supplemented with 7.5% (v/v) FCS. Cells were counted using a hemacytometer (Hausser Scientific Partnership, Horsham, PA) and plated at a density of 2 x 10⁵ cells per well on 6 well plates coated with 1.5 μg/ml of Poly-L-Ornithine (Sigma, St. Louis, MO) for 1 hour in room temperature. Following plating, cells were incubated at 37°C in a 5% CO₂ atmosphere. After 24 hours, the medium was replaced by fresh medium containing cytosine arabinoside (AraC; 20 μM) to limit the proliferation of glial cells [89]. After 2 days, half of the medium was replaced with medium that did not contain AraC. After 24 hours, half of the medium was again replaced with the medium that did not contain AraC. These cultured cells were used for studies at day 5. The amygdala primary cell culture prepared in this way contained about 80% neuronal cells and 20% glial cells.
**Fatty acids preparation**

**Palmitic acid preparation:** Sodium palmitate (Sigma, St. Louis, MO) preparation and application to neuronal cells followed the methods of Mayer and Belsham (2010). A 100 mM solution of palmitate was prepared by dissolving 27.8 mg sodium palmitate in 1 ml sterile water by alternating heating and vortexing until the palmitate was dissolved completely. Immediately after the palmitate solution is dissolved, 200 μl of the 100 mM palmitate solution were added into 3.8 ml of serum-free DMEM containing 5 % fatty acid-free bovine serum albumin (FA-free-BSA; Roche, Indianapolis, IN), creating a 5 mM palmitate solution. The 5 mM palmitate solution was shaken at 140 rpm at 40 °C for 1 hour and was then immediately used to treat the neuronal cells. Serum-free DMEM with 5 % FA-free-BSA was used as the vehicle control.

**Oleic acid, linoleic, linolenic acid preparation:** Oleic acid (OA), linoleic acid (LA), and linolenic acid (LN) from Sigma (St. Louis, MO) were dissolved into 100% ethanol for stock solutions (OA; 8.834 mM, LA; 8.929 mM, LN; 35 mM) and stored -20 °C under nitrogen to prevent oxidation. For the preparation of fatty acid-BSA conjugates, 68 μl of 8.83 mM OA, 67.3 μl of 8.93 mM LA, and 17.2 μl of 35 mM LN stock solution was dissolved into 12 ml of serum-free DMEM containing 5 % FA-free BSA to obtain 50 μM fatty acid-BSA conjugates. The 50 μM fatty acid-BSA conjugates were shaken at 140 rpm for 1 hour at 40 °C and then immediately used to treat the neuronal cells. Serum-free DMEM with 5 % FA-free-BSA was used as the vehicle control.
**Fatty acid incubation and insulin stimulation**

All cells were serum deprived for 2 hours by changing media to serum-free DMEM containing 0.05% FA-free-BSA. After 2 hours of serum deprivation, various fatty acid-BSA conjugate doses were prepared by dilution of fatty acid with FA-free-BSA and were added and cells incubated for a further 12 or 24 hours. Control cells received an equivalent amount of FA-free-BSA. 50 nM of insulin (Sigma, St. Louis, MO) was then applied and cells harvested after 15 minutes incubation. Finally, the protein was extracted and stored at -20 °C for protein analysis.

**Phosphotidyl-inositol-3 kinase (PI3-kinase) inhibitors application**

Both Wortmannin and LY294002 were purchased from Calbiochem (San Diego, CA). GT1-7 neuronal cells were pre-incubated with either Wortmannin (1 μM) for 15 or 30 minutes or LY294002 (10 or 20 μM) for 45 minutes before insulin [50 nM] application.

**Akt inhibitor application**

An inhibitor of kinase activity of Akt, triciribine was purchased from Santa Cruz biotechnology (Santa Cruz, CA). Before insulin [50nM] application, GT1-7 neuronal cells were pre incubated with triciribine (1 μM or 2 μM) for 2 hours.

**Cell harvesting and Protein extraction**

Cells were washed twice with cold Dulbecco’s phosphate buffered saline (D-PBS; Invitrogen, Carlsbad, CA) after incubation. 200 μl of ice-cold whole cell lysis buffer containing anti-protease and anti-phosphatase agents (50 mM KCL, 1% NP-40, 25 mM HEPES-pH 7.8, 10 μl/ml Leupeptin, 20 μg/ml Aprotonin, 125 μM DTT, 1 mM PMSF
and 1 mM Orthovanadate) was added and the cells were recovered using a spatula. Cells were sonicated and centrifuged at 4 °C and 13,000 rpm for 15 minutes to obtain total protein. Protein concentration was measured using a Pierce BCA protein assay kit (ThermoScientific Inc., Rockford, IL).

**Western blot**

40-50 μg of protein samples were added to 6 x SDS loading buffer and heated to 100 °C for 7 minutes, followed by a brief centrifugation. Each sample was loaded on a 8 % SDS-polyacrylamide gel and separated in 1 x TG-SDS electrophoresis buffer (0.025 M Tris, 0.192 M Glycine and 0.1% Sodium Dodecyl Sulfate) at 80 V until the 10 kD reference band reached the bottom of the gel. Following electrophoresis, the proteins were transferred on to PVDF membranes (Thermo Scientific Inc., Rockford, IL) for 3 hours at 80 V using a Bio-Rad Trans-blot apparatus with transfer buffer (0.5 M Tris base; pH 7.4, 3.9 M Glycine and 20% methanol). The membrane was blocked for 2 hours with 5% non-fat dry milk in TBS-T buffer (1 M Tris [pH7.4], 5 M NaCl and 0.1% Tween 20). After blocking the membrane, it was incubated overnight at 4 °C with each primary antibody diluted in 1% nonfat milk buffer. Following incubation, the membranes were washed five times with TBS-T and then incubated with the appropriate horse radish peroxidase conjugated secondary antibody for 1 hour at room temperature. After washing the membrane five times, protein expression and phosphorylation level were measured using ECL Western Blotting Substrate (Thermo Scientific Inc., Rockford, IL) and blue autoradiograph film (Bio Express, Kaysville, UT). Image density was assessed on a Bio-Rad XR/Geldoc Imaging system using Bio-Rad Quantity One software.
The following primary antibodies were used: Rabbit anti-phosphorylated Akt-1/2/3\textsuperscript{Ser473} (sc-7985-R), rabbit anti-Akt-1/2/3 (sc-8312), rabbit anti-phosphorylated IRS-1\textsuperscript{Tyr989} (sc-17200), rabbit anti-PKC\(\theta\) (sc-212), and mouse anti-\(\beta\)-Actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit anti-phosphorylated mTOR\textsuperscript{Ser2448} (ab51044) was purchased from Abcam Inc.(Cambridge, MA).

**Glucose uptake assay**

When cells reached 60 ~ 80% confluence in 6 well plates, each well was washed three times with glucose free PBS (Invitrogen, Grand Island, NY). Cells were incubated with 1% FFA-free BSA for 2 hours then washed two times with glucose free PBS. This was replaced with glucose free DMEM containing various insulin doses (20 nM ~ 200 nM) and cells were incubated for 30 minutes at 37 °C in 5% CO\(_2\). After 30 minutes, 100 \(\mu\)l glucose free DMEM containing 0.1 mM 2-deoxyglucose (D8375, Sigma, St. Louis, MO) and 1 \(\mu\)Ci \(^{3}\)H-2-deoxyglucose (TRK672, Amersham) were added to every well and incubation continued for 5 minutes at 37 °C in 5% CO\(_2\) atmosphere. Cytochalasin B (20 \(\mu\)M) was used as a negative control to inhibit transporter mediated glucose transport. Each well was washed 3 times in cold PBS to stop the reaction and placed on ice. 0.5 ml of 1% SDS was added into each well and placed in room temperature for 10 minutes for cell lysis. If the cell lysate was very viscous, 400 \(\mu\)l of 1% SDS was added additionally. Three hundred micro-liter of Cell lysates were transferred into 5 ml scintillation cocktail (Fisher Scientific, Pittsburgh, PA) and CPM were counted in a liquid scintillation counters (Beckman LS6000TA Scintillation Counter, Fullerton, CA). The protein
concentration for each sample was measured using a Pierce BCA protein assay kit (Thermo Scientific Inc., Rockford, IL).

**RNA isolation and purification**

Total RNA for both GT1-7 neuronal cells and amygdala primary cells were isolated using TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH). 1 ml of TRI Reagent was added into each well and each cell lysate was transferred into a 1.5 ml tube after 5 minutes of incubation at room temperature. One hundred micro-liter of 1-bromo-3-chloropropane (BCP, Molecular Research Center, Inc., Cincinnati, OH) was added for the phase separation. After samples were centrifuged (15 minutes, 13,000 rpm, 4°C), the clear RNA-rich aqueous phase was transferred to a new 1.5 ml tube and 500µL isopropanol added. After centrifugation at 10,000 rpm at 4°C for 8 minutes, the RNA pellet was washed with 1mL 75% (v/v) ethanol and then air-dried at room temperature for 10 minutes. The pellet was resuspended in RNase-free water. Using a TURBO DNA-free™ kit (Ambion, Inc., Austin, TX), extracted RNA was purified. After cleaning, RNA quality of each sample was quantified by spectrophotometric analysis (A260/A280) and visually assessed using 1% agarose gel electrophoresis.

**Semi-quantitative RT-PCR**

Purified total RNA was primed using oligo (dT) and reverse transcribed using superscript First-Strand Synthesis system (Invitrogen, Carlsbad, CA) for the generation of cDNA. The resultant cDNA was analyzed by semi-quantitative PCR using the following mouse specific primer sets. *Gpr40*: Forward, 5’- TTG GTC ATC ACT GCC TTC TG-3’, Reverse, 5’- CTA GCC ACA TTG GAG GCA TT-3’; *Gpr120*: Forward, 5’- GTT CAG
GAA CGA ATG GAG GA-3’, Reverse, 5’- AGA TGC CTG CTG TTG GAA GT-3’; and peptidyl-prolyl cis-trans isomerase B (Ppib): Forward, 5’-GCT GGA TGG CAA GCA TFT G-3’, Reverse, 5’-TGT CTT GGT GCT CTC CAC CTT-3’.

PCR was carried out under the following parameters for Gpr40 and Gpr120: 1 cycle of 5 minutes at 95 °C, 40 cycles of 1 minute at 95 °C, 45 seconds at 56°C, 1 minute at 72 °C and 1 cycle of 5 minutes at 72 °C; for Ppib: 1 cycle of 5 minutes at 95 °C, 28 cycles of 1 minute at 95 °C, 45 seconds at 57°C, 1 minute at 72 °C and 1 cycle of 5 minutes at 72 °C. PCR product was run on 3 % agarose gel and stained with ethidium bromide. The intensity of each band on the gel was measure by Quantity One (Bio-Rad, Hercules, CA). As an internal control, Ppib (cyclophinlin B) was used.

Calcium imaging

Neuronal cells were cultured on a Petri dish (30 mm) with sterile cover slides (1.5 mm) until they reached 30 % confluence. For serum deprivation, media was changed to serum free DMEM for 1 hour. The media was aspirated and neurons were loaded with 5 μl of fura-2/AM (Molecular Probes, Eugene, OR) for 1 hour in Tyrode’s saline solution with 10% pluronic acid at 37 °C in the dark. Neurons were rinsed twice with Tyrode’s saline buffer and the cover slides were mounted into an imaging chamber (RC-25F and RC-26Z, Warner Instruments, Hamden, CT) and viewed on an inverted microscope (Nikon, Eclipse TS100, Japan) while perfused continuously with Tyrode’s solution. Neurons were illuminated using a 100-watt xenon lamp. Excitation wavelengths (340/380 nm) were delivered by a monochromator (Bentham FSM150, Intracellular Imaging Inc., Cincinnati, OH) at a rate of 20 excitations per minute. A CCD camera (pixelFly, Cooke, MI) attached to a microscope, controlled by imaging software (Incyt Im2TM,
Intracellular Imaging) was used to measure fluorescence. Using a standard curve that was produced for the imaging system using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR), the ratio of fluorescence (340/380 nm) was directly converted to Ca\(^{2+}\) concentrations. BSA-conjugated fatty acids (30 μM of palmitic acid, Oleic acid and linoleic acid) were used extracellularly with a bath perfusion system at a flow rate of 4 ml/minutes allowing complete exchange of the extracellular solution in less than 20 seconds.

**Statistical analysis**

Data are presented as Mean ± SEM and analyzed using GraphPad Prism 5 (GraphPad, La Jolla, CA). Two-way ANOVA (± insulin x diets) was used to study the interaction between diets and the presence or absence of insulin on expression and phosphorylation level of insulin signaling molecules. Individual groups were compared with either an unpaired t-test or a one-way ANOVA followed with Tukey’s multiple comparison test. Values of p < 0.05 were considered statistically significant.

4. Results

The initial experiments were designed to characterize the insulin signaling responses in GT1-7 and amygdala primary neuronal cells.

*Insulin stimulates insulin signaling pathways in neuronal cells*

In this study, two neuronal cell types, mouse hypothalamic GT1-7 cells and amygdala primary cells, were used to explore their sensitivity to insulin. As a first approach, GT1-7 cells were incubated with insulin and the phosphorylation levels of specific proteins involved in the insulin signaling pathway were measured using Western
blot analysis. The insulin doses and time frame that were chosen were based on the methods of Thrush et al. [90] and Sinha et al. [91]. As shown in figure 2-1, insulin incubation for 15 minutes increased the phosphorylation level of Akt\textsuperscript{2 Ser\textsuperscript{473}} in GT1-7 cells in a dose dependent manner. The half maximum insulin stimulation of Akt\textsuperscript{2 Ser\textsuperscript{473}} phosphorylation was approximately 50 nM insulin. In subsequent experiments, the time course of the insulin stimulation of phosphorylation of Akt\textsuperscript{2 Ser\textsuperscript{473}} was investigated. As shown in figure 2-2, maximum stimulation of phosphorylation of Akt\textsuperscript{2 Ser\textsuperscript{473}} was observed at 10 or 15 minutes in GT1-7 cells. Subsequently, amygdala primary neuronal cells were tested to see if these cells were also insulin sensitive. As shown in figure 2-3, 50 nM insulin incubation for 15 minutes increased phosphorylation level of Akt\textsuperscript{2 Ser\textsuperscript{473}} nearly 3-fold, indicating that the amygdala primary cells are insulin sensitive as well as the GT1-7 neuronal cell lines.

*Insulin stimulated phosphorylation Akt\textsuperscript{2 Ser\textsuperscript{473}} occurs through PI3-Kinase activation*

To determine the dependency of insulin stimulated phosphorylation of Akt\textsuperscript{2 Ser\textsuperscript{473}} on the activation of PI3-Kinase, which is located upstream of Akt, two known PI3-Kinase inhibitors, Wortmannin and LY294002, were used. Wortmannin is a fungal metabolite that specifically inhibits PI3-kinase [92]. LY294002 is a selective PI3-kinase inhibitor [93]. These two different PI3-kinase inhibitors have been reported to block PI3-kinase activity in several types of cells, as well as in different animal systems [94, 95]. Wortmannin 1 μM for 15 minutes completely inhibited insulin stimulation of phosphorylation Akt\textsuperscript{2 Ser\textsuperscript{473}} in GT1-7 cells as shown in figure 2-4. Likewise, LY294002 also inhibited insulin stimulated phosphorylation of Akt\textsuperscript{2 Ser\textsuperscript{473}} in a similar manner to Wortmannin. These results indicate that insulin stimulated phosphorylation of Akt\textsuperscript{2 Ser\textsuperscript{473}}
in neuronal cells requires the activation of PI3-kinase just as the classical insulin response in peripheral cells (e.g. muscle cells). To confirm the involvement of PI3-kinase in insulin stimulated Akt phosphorylation, the effects of insulin were compared in the L6 myocyte cell line (a positive control) and GT1-7 cells. Figure 2-5 (panel A) shows that insulin stimulated phosphorylation of Akt$^{2\text{Ser}473}$ in L6-myocytes was inhibited by PI3-kinase inhibitors, Wortmannin (1 μM for 15 minutes) as it was in GT1-7 cells. In contrast, while LY294002 10 μM for 45 minutes totally inhibited phosphorylation of Akt$^{2\text{Ser}473}$ in GT1-7 cells, it was a less potent inhibitor in L6 myocyte cell lines because it only reduced the Akt$^{2\text{Ser}473}$ phosphorylation by 70%.

Insulin stimulation increased glucose uptake in GT1-7 cell line

One of the most important functions of insulin is the activation of glucose uptake in peripheral cells to regulate blood glucose level. The phosphorylation of Akt promotes for glucose uptake. Since insulin stimulates the phosphorylation of critical signaling molecule Akt$^{2\text{Ser}473}$ through the activation of PI3-Kinase in GT1-7 cells, I investigated whether insulin activation of Akt$^{2\text{Ser}473}$ phosphorylation was also associated with stimulations of glucose uptake in GT1-7 cell lines as it is in adipocytes and muscle cells. To do this, a glucose uptake assay was performed using $[^{3}\text{H}]$ 2-deoxy-D-glucose. Figure 2-6 shows the increase of glucose uptake in GT1-7 cells in response to insulin stimulation. Over the dose range used, 20 to 100 nM insulin dose had a significant effect. Cytochalasin B (Cyto) was used as a negative control to inhibit transporter-mediated glucose transport.
Data shown in the previous section showed that neuronal cells are insulin sensitive as are peripheral tissues, insulin activating PI3-kinase to enhance Akt$^{\text{Ser473}}$ phosphorylation and glucose transport. To determine the effect of saturated fatty acids on insulin signaling in neuronal cells, GT1-7 cells were incubated with palmitic acid. **Figure 2-7** shows that the palmitic acid had an inhibitory effect on insulin signaling in the GT1-7 cells. The experiments were performed with an insulin dose [50 nM] that gave half-maximum stimulation of Akt$^{\text{Ser473}}$ phosphorylation (**figure 2-1**). Palmitic acid decreased insulin stimulated phosphorylation of Akt$^{\text{Ser473}}$ in a dose dependent manner with half inhibition dose at [13.7 μM]. In a second experiment (**figure 2-8**), the palmitic acid effect on insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation was again demonstrated. However, palmitic acid had no effect on Akt$^{\text{Ser473}}$ phosphorylation in the absence of insulin. In a subsequent experiment, the effect of palmitic acid on amygdala primary neuronal cells was investigated to show that palmitic acid had similar effects in amygdala primary cells as in GT1-7 cells. **Figure 2-9** shows that all concentrations of palmitic acid used (5 μM to 50 μM) significantly inhibited the phosphorylation level of Akt$^{\text{Ser473}}$ in response to insulin. Thus primary amygdala neuronal cells appear to be more sensitive to the inhibitory effect of palmitic acid on insulin signaling than neuronal cell lines. There was no evidence of toxicity of PA dose that was used to amygdala primary cells in these experiments.

**Palmitic acid administration attenuates insulin stimulated glucose uptake in GT1-7 cells**

Previously, it was shown that insulin stimulates glucose uptake in GT1-7 neuronal cells (**figure 2-6**). In this experiment, the effect of the saturated fatty acid palmitic acid
on insulin stimulated glucose uptake was investigated. GT1-7 neuronal cells were incubated with BSA-palmitic acid (5 to 25 μM) conjugates for 12 hours in the absence of serum. Palmitic acid had no significant effect on basal glucose uptake in comparison with the controls (figure 2.10). However, when the cells were stimulated with 50 nM insulin for 30 minutes, there was a significant (approximately 4-fold) increase in glucose uptake. Prior incubation of GT1-7 cells with palmitic acid for 12 hours completely blocked this insulin stimulation of glucose uptake (figure 2-10).

*Effects of mono-unsaturated and poly-unsaturated fatty acids on the insulin stimulation of phosphorylation of Akt2Ser473 in GT1-7 cells*

To investigate the effects of mono-unsaturated and poly-unsaturated fatty acids on insulin signaling in neuronal cells, dose response effects of both oleic and linoleic acid on insulin stimulated Akt2Ser473 phosphorylation level were studied. Figure 2-11 shows that the mono-unsaturated fatty acid oleic acid (OA) had no effect on insulin stimulated phosphorylation of Akt2Ser473 at doses of 5–50nM. Similarly, Figure 2-12 shows the lack of effect of the n-6 polyunsaturated fatty acid linoleic acid (LA) on insulin stimulated AktSer473 phosphorylation when it was compared to the cells treated with insulin alone. Similarly, the incubation of cells with various doses of the n-3 poly-unsaturated fatty acid linolenic acid (LN) did not elicit significant inhibition of the insulin stimulated Akt2Ser473 phosphorylation compared to cells treated only with insulin (figure 2-13). In a subsequent experiment (figure 2-14), the effects of PA, OA and LA at a dose of 10 μM on insulin stimulated Akt2Ser473 phosphorylation level were compared. Only palmitic acid significantly decreased insulin stimulated Akt2Ser473 phosphorylation level. Neither oleic nor linoleic acids had any effect on insulin stimulated Akt2Ser473 phosphorylation. Finally
(figure 2-15), the effects of PA and LN on insulin signaling in GT1-7 cells were compared. Whereas palmitic acid (10 μM) significantly decreased the insulin stimulated phosphorylation of Akt2$^{\text{Ser}473}$, 10μM linolenic acid had no effect on the phosphorylation level of Akt2$^{\text{Ser}473}$ in the absence or presence of insulin.

**Effects of fatty acids on insulin stimulated phosphorylation of Akt2$^{\text{Ser}473}$ in amygdala primary neuronal cells**

After testing various types of fatty acid for their effect on phosphorylation of Akt2$^{\text{Ser}473}$ in GT1-7 cells, the effect of linolenic acid on insulin signaling in amygdala primary cells was studied using the same protocol that was used for GT1-7 cells. Previous experiments (figure 2-9) showed that palmitic acid concentrations from 5 to 50 μM significantly decreased insulin stimulated phosphorylation of Akt2$^{\text{Ser}473}$. The effects of 5 μM and 25 μM linolenic acid were tested in primary amygdala cells. However, as shown in figure 2-16, incubation with linolenic acid significantly increased insulin stimulated phosphorylation of Akt2$^{\text{Ser}473}$ at both doses compared to only insulin stimulated cells. To confirm the linolenic acid stimulation of insulin signaling, a second experiment was performed in which the response to both linolenic acid and palmitic acid were directly compared at the 10 μM dose in the presence and absence of insulin (figure 2-17). Neither fatty acid had any effect on Akt$^{\text{Ser}473}$ phosphorylation in the absence of insulin. However, similar to the GT1-7 cells, palmitic acid decreased insulin stimulated phosphorylation of Akt2$^{\text{Ser}473}$ significantly in amygdala primary cells although the response was smaller than that previously observed (figure 2-9). Conversely, unlike GT1-7 cells, the poly-unsaturated fatty acid, linolenic acid significantly enhanced insulin stimulated phosphorylation of Akt2$^{\text{Ser}473}$ in comparison to the insulin-only stimulated
cells. Thus amygdala primary cells also appear to be more sensitive or differentially sensitive to linolenic acid compared to the GT1-7 cells.

*Expression of G-protein coupled receptors and effect of fatty acids on intracellular calcium mobilization in neuronal cells*

To investigate if neuronal cells express G-protein coupled receptors (Gpr40 and Gpr120), RT-PCR for *Gpr40* and *Gpr120* mRNA transcripts was performed on total RNA extracted from GT1-7 and amygdala primary cells. As shown in figure 2-18, both GT1-7 and amygdala primary neuronal cells express both GPR40 and GPR120. In a subsequent experiment, the cell-based assay of ratio metric calcium imaging was used to investigate the effect of different types of fatty acids on calcium mobilization in two neuronal cell types, GT1-7 and amygdala primary cells. As shown in figure 2-19 and figure 2-20, both GT1-7 and amygdala primary cells responded to the four different fatty acids (saturated fatty acid; palmitic acid, mono-unsaturated fatty acid; oleic acid, and two poly-unsaturated fatty acids; linoleic and linolenic acid) and each fatty acid stimulated a rapid increase in intracellular calcium concentration. The application of palmitic and oleic acid elicited rapid and biphasic rises in $[\text{Ca}^{2+}]_i$ in both GT1-7 and amygdala primary cells, although the shape of the response to PA differed in the 2 cell types. Both poly-unsaturated fatty acids, linoleic and linolenic acids elicited rapid, but not biphasic rises in $[\text{Ca}^{2+}]_i$ in both GT1-7 and amygdala primary cells.
Effect of palmitic acid and insulin on mTOR signalling in GT1-7 hypothalamic neuronal cells and amygdala primary neuronal cells

The palmitic acid and insulin effect on mTOR signalling in GT1-7 neuronal cells and amygdala primary cells was also investigated as an in vitro model. To test the effect of insulin stimulation on mTOR signalling, an insulin dose response experiment was performed. As shown in figure 2-21, insulin increased the phosphorylation level of mTOR\textsuperscript{Ser2448} significantly in a dose-dependent manner in GT1-7 cells ($p < 0.01$ or $p < 0.05$). The effect of palmitic acid and insulin on mTOR\textsuperscript{Ser2448} phosphorylation was also investigated. As shown in figure 2-22, both palmitic acid (5 μM and 25 μM) alone and insulin alone significantly increased mTOR\textsuperscript{Ser2448} phosphorylation compared to control samples ($p < 0.01$). However, there was no additive effect between insulin and palmitic acid.

In amygdala primary cells, insulin also significantly increased mTOR\textsuperscript{Ser2448} phosphorylation level compared to samples incubated without insulin ($p < 0.05$ or $p < 0.001$ respectively) as shown in figure 2-23. However, in contrast to GT1-7 hypothalamic cells, palmitic acid alone did not significantly increase the phosphorylation level of mTOR\textsuperscript{Ser2448} in amygdala primary cells (figure 2-23). This indicates that there might be different mTOR signalling between hypothalamus and amygdala in response to palmitic acid.

Effect of Akt inhibitor (triciribine) on insulin stimulated Akt and mTOR signaling in GT1-7 hypothalamic neuronal cells

In order to investigate whether insulin stimulated mTOR signalling in neuronal cells is independent to the activation of Akt signaling, GT1-7 neuronal cells were pre-
incubated with triciribine (1 μM or 2 μM), an Akt kinase inhibitor, for two hours before insulin [50 nM] application. **Figure 2-24-A** shows significant inhibition of insulin stimulated Akt phosphorylation level which occurred with both 2 μM and 4 μM of triciribine application in GT1-7 neuronal cells ($p < 0.01$). However, triciribine application did not have an effect on insulin stimulated mTOR signaling (**figure 2-24-B**).

5. Discussion

The present study focuses on two neuronal cell types, GT1-7 mouse hypothalamic cells and amygdala primary neuronal cells. As there is no amygdala neuronal cell line available, hypothalamic GT1-7 cells were chosen for most of the studies as a neuronal model. These studies were then validated in amygdala primary neuronal cells, although the cost and time to produce sufficient cell number somewhat limited the range of experiments that could be completed. The response of the cells to insulin was assessed by the stimulation of Akt$^{2\text{Ser}473}$ phosphorylation. Like insulin sensitive peripheral cells, insulin stimulated Akt$^{2\text{Ser}473}$ phosphorylation in both cell types. The ability to inhibit this activation with two different PI3-kinase inhibitors (Wortmannin and LY294002) showed that insulin activation of Akt signaling was modulated through the activation of PI3K. In the brain, as well as in insulin sensitive peripheral cells, intact insulin signaling via the PI3K/Akt pathway is responsible for most of metabolic actions of insulin which regulate peripheral energy and glucose homeostasis [96-98]. It is also responsible for the alteration of insulin action in brain, and it also has a direct influence in metabolic diseases such as obesity, diabetes or metabolic syndrome [6]. For example, inhibition of insulin-dependent activation of PI3K blocks insulin action in the arcuate nucleus, which results in a decreased ability of circulating insulin to suppress endogenous glucose production in
liver [4, 5]. Our results suggest that both hypothalamic neuronal cells and amygdala primary cells respond to insulin and mediate insulin’s metabolic function through similar signaling pathways as in peripheral cells.

One of the main functions of insulin that is related to the overall metabolic homeostasis is the regulation of plasma glucose level. Insulin sensitive peripheral cells especially, muscle cells, increase glucose uptake in response to insulin stimulation [33, 99]. Insulin binds to its receptor and activates insulin signaling molecules such as Akt phosphorylation, and this results with translocation of insulin sensitive glucose transporter (Glut4) into plasma membrane to accelerate glucose uptake [81]. It is reported that various regions of brain such as olfactory bulb, cortex, hippocampus, hypothalamus, and cerebellum have Glut4 mRNA and protein expression similar to the peripheral tissues [100-103]. Although Glut4 is highly expressed in brain regions, there is little information about the function of Glut4 on insulin stimulated glucose transport in the brain. To determine whether insulin stimulated Akt phosphorylation in neuronal cells will also elicit a functional change in glucose uptake in a similar way as in peripheral cells, we investigated the insulin stimulated glucose uptake in GT1-7 hypothalamic cells. Treating GT1-7 neuronal cells with various doses of insulin significantly increased insulin stimulated glucose uptake compared to basal glucose uptake level. This further demonstrates that insulin may have a similar metabolic role in neurons as in peripheral cells. Thus the two cell types used in these studies appeared to be responsive to insulin from both a signaling end point and the functional end point of glucose transport.

Numerous studies have focused on the effects of fatty acids on insulin signaling in peripheral tissues showing that increased level of free-fatty acids and various lipid
metabolites may impair insulin action [40]. Saturated fatty acid-induced insulin resistance has been extensively studied in vitro using skeletal muscle cells [6, 104]. In L6 myocytes, palmitate (300 μM) rapidly decreased insulin-stimulated glucose uptake [40]. However, little information is available to date on free fatty acid actions in the brain and only a few studies have examined the central effects of increased free fatty acids level on brain insulin signaling. Although the brain has been known to mainly respond and depend on glucose, recent studies demonstrated that the brain is also sensitive to fatty acids [105]. Fatty acids can cross the blood-brain barrier and enter into the brain regions [106]. It has been found that palmitic acid level is elevated in hypothalamic regions of animals fed a high fat diet [77]. The hypothalamus is sensitive to elevated free fatty acids, and their presence affects hypothalamic regulation of energy and glucose homeostasis.

Interestingly, our neuronal cells were able to only withstand a maximum 50 μM concentration of palmitic acid for 24 hours. Higher concentrations and increased incubation times were toxic and eventually killed the cells. Hence all of the studies were undertaken with FA concentrations at or below 50 μM. Nevertheless, the other insulin sensitive peripheral cells such as adipocytes, myocytes and β-cell cell lines are able to withstand fatty acid concentration up to 1 mM, for a maximum of 48 hours [107-109] without killing the cells. This indicates that neuronal cells, at least under the incubation conditions used in this studies, are more sensitive to the toxic effects of free fatty acids than peripheral tissue cells.

The present studies demonstrated that neuronal cells, like insulin sensitive peripheral cells, become insulin resistant when exposed to the saturated fatty acid palmitic acid. We investigated the effect of fatty acid type [saturated fatty acid (palmitic
acid, PA), mono-unsaturated fatty acid (oleic acid, OA) and poly-unsaturated fatty acids (linoleic and linolenic acid, LA and LN) on insulin stimulated Akt2^{Ser473} phosphorylation in GT1-7 cells. Our result demonstrated that only the saturated fatty acid PA, but not mono- or poly-unsaturated fatty acids, significantly inhibited insulin signaling in GT1-7 cells decreasing insulin stimulated phosphorylation of Akt2^{Ser473}. This result supports recent in vivo work by Posey et al. [77]. They demonstrated that icv injections of palmitate blunted insulin stimulated PI3K activation and Akt phosphorylation in the hypothalamus. The results described in this chapter also support a recent in vitro study, which established that the prolonged high-dose palmitate exposure causes ER stress and insulin resistance in the cultured hypothalamic neuronal cell lines [110].

Additionally, our data has shown that palmitic acid induced attenuation of Akt2^{Ser473} phosphorylation is linked to a functional inhibition of insulin stimulated glucose transport in GT1-7 neuronal cells. Palmitic acid (5 µM and 25 µM) incubation for 12 hours completely inhibited insulin stimulated glucose uptake in GT1-7 neuronal cells. This result suggests that saturated fatty acid induced insulin resistance and corresponding inhibition of glucose uptake occurs in brain cells as in other insulin sensitive peripheral cells. Glut4 levels in the brain are responsive to insulin; Glut4 mRNA levels are altered in several brain areas in hyperglycemic and hyperinsulinemic rats suggesting that impaired insulin action represses Glut4 gene expression in the brain [100].

Another important finding reported in this chapter is that like hypothalamic neuronal cells, the amygdala primary cells are also insulin sensitive. They also become insulin resistant when the saturated fatty acid, palmitic acid, is applied. Insulin actions in hypothalamus are important in the regulation of body weight and peripheral metabolism.
Recently, it has been suggested that amygdala as well as other parts in the brain are involved in regulation of energy homeostasis and feeding behavior. The amygdala, as well as other regions of the brain, has a high expression level of insulin receptors [111] suggesting the probability that insulin has significant effects on the functional activity of the amygdala as it does in the hypothalamus. This is supported by recent studies in our lab which reported that insulin injections in the amygdala have an inhibitory effect on food intake and that 3 days feeding of a high fat diet completely blocks this insulin anorectic effect, before there are any evident changes in peripheral insulin sensitivity [86]. While recognizing that the primary amygdala cells used in our studies were principally but not completely of a neuronal rather than glial nature, the demonstration that they were insulin sensitive (insulin stimulated Akt2Ser473 phosphorylation) and that this insulin response was blocked by the saturated fatty acid palmitic acid, suggests they are a good cell model for the in vivo amygdala response to insulin. However, in contrast to GT1-7 neuronal cells, amygdala primary cells differed in their response to linolenic acid which, significantly increased insulin stimulated Akt2Ser473 phosphorylation suggesting there is an insulin sensitizing effect of linolenic acids.

N-3 PUFAs including linolenic acid, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) enhance insulin sensitivity and have anti-diabetic effects by increasing basic metabolic rate and fatty acid oxidation in peripheral cells [48, 49]. The molecular mechanisms underlying the effect of n-3 fatty acids on insulin sensitivity are poorly understood although several possible mechanisms have been proposed. These include: 1) modulation of cell membrane function by increasing polyunsaturated fatty acid composition in cell membrane leading to increased insulin receptor population and
enhanced insulin receptor activity [112, 113]; 2) the prevention of decreased PI3-kinase activity and depletion of glucose transport GLUT4 level in muscle and adipose tissues; 3) activation of peroxisome proliferator activated receptor (PPAR)-γ system which stimulates insulin dependent glucose uptake [114]; 4) repression of both expression and activity of glucose-6-phosphatase in liver to reduced hepatic glucose production [115].

In addition, it has been reported that n-3 fatty acids have anti-inflammatory effects, and may enhance insulin sensitivity by the inhibition of proinflammatory signaling pathways [116-118]. According to one study, the expression of inflammatory marker such as cytokine TNF-α was significantly reduced in overweight women when the diet is supplemented with EPA and DHA for 12 weeks [119]. Another study demonstrated that n-3 fatty acids such as DHA and EPA, but not saturated fatty acids, inhibited inflammatory signaling by effects on phosphorylation of JNK and IKKβ, cytokine secretion and inflammatory gene expression in a mouse adipocyte cell line [53]. These studies suggested that n-3 fatty acids mediated these anti-inflammatory effects through the activation of the G-protein coupled receptor GPR120. siRNA mediated knockdown of Gpr120 in this cell line completely abrogated the anti-inflammatory effect of n-3 fatty acids demonstrating that these anti-inflammatory effects are selectively exerted through GPR120 [53].

Studies also have revealed that DHA application into adipocyte cells increased both insulin stimulated glucose uptake and GLUT4 translocation into plasma membrane, whereas knockdown of either Gpr120 or the α-subunit of G-protein-q (G_αq) abolished the effect of DHA on insulin stimulated glucose uptake and GLUT4 translocation. These data
suggests that GPR120 is a functional n-3 fatty acid receptor and mediates the insulin sensitizing effect through \( G_{\alpha q} \)-mediated activation of PLC signaling pathways [53].

In relation to the studies reported in this chapter, linolenic acid enhanced insulin stimulated Akt2\(^{Ser473} \) phosphorylation in amygdala primary cells, but not in GT1-7 cells. This may reflect the activation of GPR120, and \( G_{\alpha q} \) - PLC signaling pathways. According to the semi-quantitative PCR data, both \( Gpr40 \) and \( Gpr120 \) mRNA expressions were detected in the neuronal cells (GT1-7 and amygdala primary cells), consistent with a study, which reported the presence of \( Gpr40 \) and \( Gpr120 \) mRNA expression in CNS of adult monkey which included the cerebral cortex, hippocampus, amygdala, hypothalamus, cerebellum, spinal cord [120, 121]. Furthermore, calcium imaging experiments showed that linolenic acid elicited a rapid increase in intracellular calcium concentration in both amygdala primary cells and GT1-7 neuronal cells. Other studies have also reported that the increasing intracellular calcium concentration in response to n-3 fatty acids are caused by the activation of GPR120 and \( G_{\alpha q} \) mediated PLC [61, 121]. Furthermore, GPR120 and \( G_{\alpha q} \) mediated PLC activation induced the activation of atypical PKC isoforms (ζ, λ/ι) and Akt phosphorylation which enhanced insulin stimulated glucose uptake in adipose cell lines [61, 62, 122, 123] suggesting the presence of possible crosstalk between GPCR signaling pathways and insulin signaling pathways [57]. However, it is not clear why the linolenic acid enhancement of insulin signaling was not observed in GT1-7 cells despite the presence of GPR120 in these cells and ability to functionally affect calcium signaling. It is possible that the effect might have been observed at a lower insulin concentration or that GPR120 levels in GT1-7 cells are insufficient to transport sufficient levels of linolenic acid into the cells.
According to our data and other studies, it is possible to hypothesize that the enhanced insulin stimulated Akt$_2^{\text{Ser473}}$ phosphorylation in amygdala primary cells in response to linolenic acid might be due to the activation of the GPR120 pathway and the crosstalk between the insulin signaling pathways, similar to the mechanisms suggested in regards to the peripheral cells. Nonetheless, our data is not sufficient to demonstrate the mechanism and calls for further investigations that are necessary to explore the involvement of GPCR signaling pathway mediated by fatty acids and the relation between insulin signaling pathways in neuronal cells.

Similar to other studies, our in vitro experiments using GT1-7 hypothalamic neuronal cell lines also demonstrated that insulin activates mTOR signaling (figure 2-21). In peripheral cells, mTOR signaling is identified to be downstream of Akt signaling [124]. For that reason, if Akt signaling is blocked, mTOR signaling should be also inhibited. In spite of this, our data has shown that palmitic acid application significantly inhibited insulin stimulated Akt signaling, but mTOR signaling remained intact in response to both palmitic acid and insulin application. In addition, our in vivo data has shown a similar result in which insulin stimulated Akt signaling was inhibited but mTOR signaling was intact in both amygdala and hypothalamus of rat fed high fat diet (HFD) for 3 days (see Chapter 3). These results suggest the existence of different signaling pathways for insulin to activate mTOR signaling in neuronal cells other than Akt signaling. To investigate this hypothesis further, the Akt inhibitor, triciribine, was applied to GT1-7 cells and measurements of insulin stimulated Akt and mTOR phosphorylation level were taken. Insulin stimulated Akt signaling was significantly inhibited in response to triciribine while mTOR signaling remained intact in response to insulin and triciribine application in
GT1-7 neuronal cells. The results further demonstrate that there must be different signaling pathways for insulin to activate mTOR signaling in neuronal cells other than through Akt signaling pathway.

Furthermore, mTOR signaling was significantly enhanced by palmitic acid in GT1-7 neuronal cells (figure 2-22). However, there was no synergistic effect of insulin and palmitic acid on mTOR$^{\text{Ser2448}}$ phosphorylation in GT1-7 cells; the mTOR$^{\text{Ser2448}}$ phosphorylation level in response to combined insulin and palmitic acid was not significantly different compared to either insulin alone or palmitic acid alone conditions (figure 2-22). Since it has been demonstrated that increased amino acids level in the hypothalamus such as leucine significantly increased mTOR signaling in hypothalamus region and induced an anorectic effect [124], palmitic acid induced mTOR signaling in GT1-7 cell might have a similar signaling role as does the amino acid leucine. However, at this time it is not clear whether the palmitic acid and leucine activate mTOR signaling through the same signaling pathways or whether palmitic acid induced mTOR signaling in the brain induces a similar anorectic effect as leucine does.

In summary, the results of current studies have demonstrated the effect of free fatty acids on insulin action in neuronal cells. Neuronal cells appear to have a similar insulin signaling pathway to peripheral cells. Importantly, only the saturated fatty acid palmitic acid but not mono- and poly-unsaturated fatty acids significantly inhibited the insulin stimulated Akt2 phosphorylation and glucose uptake in these neuronal cells. This suggests that the elevation of saturated fatty acid that induces insulin resistance in neuronal cells might have similar mechanisms as those suggested for fatty acid induced insulin resistance in peripheral cells, such as the liver, adipose and muscle cells. Whether
the alteration of intracellular signaling in neuronal cells is due to the accumulation of free fatty acids and/or lipid metabolites in the intracellular regions, or through actions as signaling molecules at the extracellular membrane receptors cannot be identified from the current studies. It is possible to hypothesize that the free fatty acids might act as ligands for selective G-protein coupled membrane receptors and affect insulin signaling pathway via the GPCR signaling pathway. More investigations and studies on the molecular mechanisms of fatty acid effects on insulin action in the central nervous system, both in hypothalamus and extra hypothalamic areas such as amygdala, will contribute to the understanding of how dietary fat acts in the CNS to modulate the onset of metabolic diseases such as insulin resistance and type 2 diabetes.

6. References


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Figure 2-1: Effects of insulin on Akt$_2^{\text{Ser}473}$ phosphorylation in GT1-7 neuronal cells. Insulin induced Akt$_2^{\text{Ser}473}$ phosphorylation in GT1-7 neuronal cells. Representative Western blot analysis (upper band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt$_2^{\text{Ser}473}$ phosphorylation level relative to $\beta$-Actin as a loading control in GT1-7 cells. Data are shown with Mean $\pm$ SEM for three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control in the absence of insulin.
Figure 2-2: Insulin time course experiment in GT1-7 cells. Exposure of cells to insulin [50 nM] for 15 minutes induced the maximum phosphorylation of Akt2$^{Ser473}$. Representative Western blot analysis (upper band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2$^{Ser473}$ phosphorylation level related to β-Actin as the loading control in GT1-7 cells. Data are shown as Mean ± SEM for three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control in the absence of insulin.
Figure 2-3: Effects of insulin on Akt2Ser473 phosphorylation in Amygdala primary neuronal cells. Insulin induced phosphorylation of Akt2Ser473 in amygdala primary cells. Representative Western blot analysis (upper band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2Ser473 phosphorylation level and β-Actin as a loading control in amygdala primary cells. Data are shown with Mean ± SEM for two independent experiments. *p < 0.05 compared to control in the absence of insulin.
Figure 2-4: Effect of Wortmannin and LY294002 on phosphorylation of Akt2Ser473 in GT1-7 neuronal cells. Wortmannin and LY294002 inhibited insulin stimulation of Akt2Ser473 phosphorylation in GT1-7 cells. A: representative Western blot analysis (bottom band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2Ser473 phosphorylation level and β-Actin as a loading control in GT1-7 cells in presence or absence of Wortmannin 1 μM. B: representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of Akt2Ser473 phosphorylation level and β-Actin as a loading control in GT1-7 cells in presence or absence of LY294002). Data are shown as Mean ± SEM for two independent experiments. *p < 0.05, **p < 0.01 compared to only insulin [50nM] treated sample.
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Figure 2-6: Insulin stimulated glucose uptake in GT1-7 neuronal cells. Insulin stimulated glucose uptake in GT1-7 neuronal cells. Glucose uptake was determined with different dose of insulin as indicated. Cytocholasin B (Cyto) was used as a negative control. Data are shown with Mean ± SEM for three independent experiments. *p < 0.05 compared to control in the absence of insulin.
Figure 2-7: Effects of palmitic acid (PA) on insulin stimulated Akt2^{Ser473} phosphorylation in GT1-7 neuronal cells. Palmitic acid inhibited insulin stimulated Akt2^{Ser473} phosphorylation in GT1-7 neuronal cells with dose dependent manner. Representative Western blot analysis (upper band indicates pAkt2) and a corresponding line graph demonstrating the quantitative measure of Akt2^{Ser473} phosphorylation level with half inhibition rate at 13.7 μM of PA. β-Actin was used as a loading control in GT1-7 cells exposed to 12 h of palmitic acid doses followed by insulin [50 nM]. Data are shown with Mean ± SEM for three independent experiments. *p < 0.05, ***p < 0.001 compared to only insulin [50 nM] treated sample.
Figure 2-8: Effects of palmitic acid (PA) on insulin stimulated Akt2<sup>Ser473</sup> phosphorylation in GT1-7 neuronal cells. Palmitic acid inhibited insulin stimulated Akt2<sup>Ser473</sup> phosphorylation in GT1-7 neuronal cells. Representative Western blot analysis (bottom band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2<sup>Ser473</sup> phosphorylation level and β-Actin as a loading control in GT1-7 cells exposed to 12 h of palmitic acid doses (10 and 25μM) followed by insulin [50 nM]. Data are shown with Mean ± SEM for two independent experiments. *p < 0.05, **p < 0.01 compared to only insulin [50 nM] treated sample.
Figure 2-9: Effect of palmitic acid (PA) in insulin stimulated phosphorylation of Akt2 in amygdala primary neuronal cells. Palmitic acid inhibited insulin stimulated Akt2\textsuperscript{Ser473} phosphorylation in amygdala primary neuronal cells. Representative Western blot analysis (upper band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2\textsuperscript{Ser473} phosphorylation level and β-Actin as a loading control in amygdala primary cells exposed to 12 h of palmitic acid doses followed by insulin [50 nM]. Data are shown with Mean ± SEM for two independent experiments. ***p< 0.001 compared to only insulin [50 nM] treated sample.
Figure 2-10: Palmitic acid effect in insulin stimulated glucose uptake in GT1-7 neuronal cells. Palmitic acid significantly reduced insulin stimulated glucose uptake compared to only insulin treated cells. Data are shown with Mean ± SEM for two independent experiments. **p < 0.005, ***p < 0.001 compared to only insulin 50 nM treated sample.
Figure 2-11: Effect of oleic acid (OA) on insulin stimulated phosphorylation of Akt2 in GT1-7 cells. Oleic acid has no significant effect on insulin stimulated Akt2$^{\text{Ser473}}$ phosphorylation in GT1-7 neuronal cells. Representative Western blot analysis (upper band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2$^{\text{Ser473}}$ phosphorylation level and β-Actin as a loading control in GT1-7 cells exposed to 12 h to oleic acid doses followed by insulin [50 nM] for 15 minutes. Data are shown as Mean ± SEM for two independent experiments. *$p < 0.05$ compared to only insulin 50 nM treated sample. There was no significant effect of any oleic acid dose on the insulin response.
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**Figure 2-13: Effect of linolenic (LN) acid on insulin stimulated phosphorylation of Akt2 in GT1-7 cells.** Linolenic acid had no significant effect on insulin stimulated Akt2<sup>Ser473</sup> phosphorylation in GT1-7 neuronal cells. Representative Western blot analysis (upper band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2<sup>Ser473</sup> phosphorylation level and β-Actin as a loading control in GT1-7 cells exposed to 12 h of linolenic acid doses followed by insulin [50 nM] for 15 minutes. Data are shown as Mean ± SEM for two independent experiments. *p < 0.05 compared to only insulin 50 nM treated sample. There was no significant difference between only 50 nM insulin treated sample and any sample with different linolenic acid doses.
Figure 2-14: The comparison of the effects of different fatty acids on insulin stimulated Akt2^Ser473 phosphorylation in GT1-7 cells. Only palmitic acid (PA) but oleic acid (OA) and linoleic acid (LA) significantly inhibited insulin stimulated Akt2^Ser473 phosphorylation in GT1-7 cells. Representative Western blot analysis (upper band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2^Ser473 phosphorylation level and β-Actin as a loading control in GT1-7 cells exposed to 12 h of fatty acids (PA, OA, LA) followed by insulin [50 nM]. Data are shown as Mean ± SEM for two independent experiments. **p < 0.01 compared to only insulin 50 nM treated sample.
Figure 2-15: The comparison between palmitic acid (PA) and linolenic acid (LN) effects on insulin stimulated phosphorylation of Akt2Ser473 in GT1-7 cells. Only palmitic acid (PA) but not linolenic acid (LN) significantly inhibited insulin stimulated Akt2Ser473 phosphorylation in GT1-7 cells. Representative Western blot analysis (bottom band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2Ser473 phosphorylation level and β-Actin as a loading control in GT1-7 cells exposed to 12 h of fatty acids (PA and, LN) followed by insulin [50 nM]. Data are shown as Mean ± SEM for two independent experiments. *p < 0.05, ***p < 0.001 compared to only insulin [50 nM] treated sample.
Figure 2-16: Effect of linolenic acid (LN) on insulin stimulated phosphorylation of Akt$_{2\text{Ser}^{473}}$ in amygdala primary cells. Linolenic acid significantly increased insulin stimulated Akt$_{2\text{Ser}^{473}}$ phosphorylation in amygdala primary neuronal cells. Representative Western blot analysis (upper band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt$_{2\text{Ser}^{473}}$ phosphorylation level and β-Actin as a loading control in amygdala primary cells exposed to 12 h of linolenic acid doses followed by insulin [50 nM]. Data are shown as Mean ± SEM for two independent experiments. **$p < 0.01$ compared to only insulin [50 nM] treated sample.
Figure 2-17: The comparison between palmitic acid (PA) and linolenic acid (LN) effects on insulin stimulated phosphorylation of Akt2<sup>Ser473</sup> in Amygdala primary cells. Whereas palmitic acid (PA) significantly inhibited insulin stimulated Akt2<sup>Ser473</sup> phosphorylation, linolenic acid (LN) significantly increased insulin stimulated Akt2<sup>Ser473</sup> phosphorylation in amygdala primary cells. Representative Western blot analysis (bottom band indicates pAkt2) and corresponding bar graphs demonstrating the quantitative measure of Akt2<sup>Ser473</sup> phosphorylation level and β-Actin as a loading control in amygdala primary cells exposed to 12 h of fatty acids (PA and, LN) followed by insulin [50 nM]. Data are shown as Mean ± SEM for two independent experiments. *p < 0.05, ***p < 0.001 compared to only insulin [50 nM] treated sample.
Figure 2-18: mRNA expression of GPR40 and GPR120 in GT1-7 and amygdala primary neuronal cells. Total RNA from GT1-7 and amygdala primary cells were analyzed by semi-quantitative RT-PCR and the products separated on agarose gels and visualized with ethidium bromide. A representative gel from two independent experiments is shown.
Figure 2-19: Effects of fatty acids on intracellular calcium mobilization in GT1-7 neuronal cells. GT1-7 cells were loaded with fura-2/AM and single cells were analyzed for changes in fluorescence every 3 seconds for 1 minute (baseline), every 3 seconds for 3 minutes after application of each fatty acid. Bath application of (A) 30 μM palmitic acid (19 out of 69 cells; 27.5 %), (B) 30 μM oleic acid (42 out of 109 cells; 38.5 %), (C) 30 μM linoleic acid (32 out of 61 cells; 51.6 %), and (D) linolenic acid (26 out of 58 cells; 44.8 %) induce increase in intracellular calcium concentration. Both palmitic and oleic acid elicit a typical rapid biphasic [Ca^{2+}]_i increase in GT1-7 neuronal cells compared to both poly-unsaturated fatty acids, linoleic and linolenic acids.
Figure 2-20: Effects of fatty acids on intracellular calcium mobilization in amygdala primary cells. Amygdala primary cells were loaded with fura-2/AM and single cells were analyzed for changes in fluorescence every 3 seconds for 1 minute (baseline), every 3 seconds for 3 minutes after application of each fatty acids. Bath application of (A) 30 μM palmitic acid (18 out of 39 cells; 46.1 %), (B) 30 μM oleic acid (15 out of 33 cells; 45.5 %), (C) 30 μM linoleic acid (16 out of 30 cells; 53.3 %), and (D) linolenic acid (14 out of 29 cells; 48.3 %) induce increase in intracellular calcium concentration. Both palmitic and oleic acid elicit a typical rapid biphasic \([\mathrm{Ca}^{2+}]\), increase in amygdala primary cells compared to both poly-unsaturated fatty acids, linoleic and linolenic acids.
Figure 2-21: Effect of insulin on mTOR$_{\text{Ser}2448}$ phosphorylation in GT1-7 neuronal cells. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of mTOR$_{\text{Ser}2448}$ phosphorylation level and β-Actin as a loading control in GT1-7 neuronal cells. Data are shown as Mean ± SEM for two independent experiments. *$p < 0.05$, **$p < 0.01$ compared to control samples.
Figure 2-22: Effect of palmitic acid on the insulin stimulation of mTOR<sub>Ser2448</sub> phosphorylation in GT1-7 neuronal cells. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of mTOR<sub>Ser2448</sub> phosphorylation level and β-Actin as a loading control in GT1-7 neuronal cells. Data are shown as Mean ± SEM for three independent experiments. *p < 0.05, **p < 0.01, compared to control samples.
Figure 2-23: Effect of palmitic acid and insulin on mTOR^{Ser2448} phosphorylation in amygdala primary cells. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of mTOR^{Ser2448} phosphorylation level and β-Actin as a loading control in amygdala primary cells. Data are shown with Mean ± SEM for two independent experiments. *p < 0.05, ***p < 0.001 compared to both control and only palmitic acid incubated samples.
Figure 2-24: Effect of Tricibirin (Akt inhibitor) on insulin stimulated Akt2\textsuperscript{Ser473} and mTOR\textsuperscript{Ser2448} phosphorylation in GT1-7 hypothalamic neuronal cells. Representative Western blot analysis and corresponding bar graphs demonstrate the quantitative measure of mTOR\textsuperscript{Ser2448} and Akt2\textsuperscript{Ser473} phosphorylation level and β-Actin as a loading control in GT1-7 neuronal cells. Data are shown with mean ± SEM for two independent experiments. *$p < 0.05$, **$p < 0.005$ in comparison to both control and only insulin stimulated samples.
CHAPTER 3
THE EFFECT OF HIGH FAT DIET AND SATURATED FATTY ACIDS ON INSULIN SIGNALING IN THE AMYGDALA AND HYPOTHALAMUS OF RATS

1. Introduction

In animals and humans, ingestion of nutrients induces metabolic and endocrine changes which are integrated by the brain through a complex series of interactions. As a result, the brain can regulate appetite, nutrient intake, peripheral metabolism and energy expenditure and thus determine body weight and energy balance [1, 2]. The hypothalamus has a central role in the regulation of energy homeostasis by detecting and integrating information on the circulating level of nutrients and nutrient induced hormones, such as insulin and leptin. These activate specific metabolic sensing neurons in the hypothalamus and other brain regions [2]. NPY/AgRP coexpressing neurons and POMC expressing neurons are examples of metabolic sensing neurons. They are located in the arcuate nucleus (ARC) of the mediobasal hypothalamus [3]. Agouti-related peptide (AgRP) / neuro-peptide Y (NPY) neurons produce the orexigenic neuro-peptides AgRP and NPY whereas, pro-opiomelanocortin (POMC) / cocaine- and amphetamine-related transcript (CART) neurons produce the anorexigenic neuro-peptides POMC, POMC-derived melanocortins such as α-melanocyte stimulating hormone (α-MSH), and cocaine and amphetamine regulated transcript (CART) [2, 4, 5]. These neuro-peptides are secreted from axonal projections to other regions of hypothalamus such as paraventricular nucleus (PVN), perifornical area (PFA), and lateral hypothalamic area (LHA) and regulate food intake and body weight.
Insulin has a complex interplay with other catabolic signals (e.g. leptin, serotonin, and melanocortins) to regulate both orexigenic and anorexigenic neurons in the arcuate nucleus of hypothalamus to control feeding behavior, body weight, energy and glucose homeostasis [1]. Insulin binds to its receptor on AgRP/NPY and POMC/CART neurons, stimulating receptor autophosphorylation and activating its signal cascade [1]. Insulin decreases AgRP expression in the AgRP/NPY neurons [6] thus inhibiting food intake, whereas insulin stimulates POMC expression in POMC neurons to increase α-MSH production, which reduces food intake and increases energy expenditure [7-9]. As in peripheral tissues, intact insulin signaling through IRS/PI3K pathways is essential for energy and glucose homeostasis in these orexigenic and anorexigenic neurons [1]. Insulin stimulated IRS-2 associated PI3K activation and Akt phosphorylation has a key role in the activation of anorexigenic neurons [3]. IRS-2-associated PI3K activation was observed within minutes following icv insulin administration in rat’s hypothalamus and administration of PI3K inhibitors prevented the insulin stimulated suppression of food intake [10].

As described above, it has been well accepted that insulin stimulated IRS-PI3K-Akt signaling in specific neuronal populations in the hypothalamus has a critical role in the regulation of food intake. However, mammalian target of rapamycin (mTOR) recently has been recognized as a crucial regulator of this response [11-15]. mTOR signaling integrates cellular nutrient status and hormonal signals in specific populations of neurons such as POMC and AgRP/NPY in the CNS and regulates food intake and energy balance [13]. The activation of mTOR requires both nutrient signals (e.g. amino acids and glucose) and hormonal signals such as leptin and insulin [16, 17]. Cota and
colleagues [13] have shown that the increased mTOR signaling in response to intracerebroventricular (icv) administration of leptin induced an anorectic response, whereas icv rapamycin (an inhibitor of mTOR) injection significantly inhibited mTOR signaling and leptin’s anorectic effect. The icv injection of the amino acid leucine, also reduced food intake in rats and this leucine induced anorectic response was accompanied by significantly reduced mRNA levels of NPY [13, 18] and increased mRNA levels of POMC [13] within the hypothalamic area, particularly in the ARC [19].

mTOR is a serine-threonine kinase and is known to integrate nutrient signals and hormonal signals to control growth and development in peripheral tissues [16, 17, 20]. The primary pathways by which insulin or leptin activate mTOR in both peripheral tissues and CNS appears to be the PI3K/Akt pathway [21]. Insulin mediated PI3K/Akt signaling is upstream of the mTOR signaling activation [22, 23]. The phosphorylated Akt inhibits tuberous sclerosis complex (TSC) 1/2 by phosphorylation, resulting in Ras homolog enriched in brain (Rheb) activation, and activated Rheb then binds to and activates mTOR [24]. Two targets of mTOR are the ribosomal S6 kinase (S6K1 and S6K2) and the eukaryotic initiation factor 4E (eIF4E) – binding protein 1 (4E-BP1) [25-28]. The mTOR mediated phosphorylation and activation of S6K1/2 and 4E-BP1 leads to an increase in ribosomal biogenesis which activates cells to increase protein synthesis and regulate growth and proliferation [20, 24]. Although the activation of mTOR signaling is essential for cell growth and proliferation, chronic activation of mTOR by insulin or excess nutrients has been linked to the development of several diseases including insulin resistance in peripheral tissues and diabetes [29, 30]. Activation of the mTOR pathway is significantly increased in the liver and skeletal muscle of insulin resistant obese rats
maintained on a HFD [31]. The serine/threonine kinase activity of mTOR and S6K1/2 increase serine phosphorylation of IRS-1, and this impairs the activity of the PI3K/Akt signaling pathway [32-34]. The mTOR and S6K1/2 pathways have been proposed as a physiological feedback mechanism that negatively regulates insulin signaling transduction [31, 35]. According to studies described above, it appears that in peripheral tissues, the aberrant mTOR signaling associated with an overabundance of energy may cause insulin resistance, whereas, the increased mTOR signaling in CNS due to overabundance of nutrients may reduce food intake and energy balance [13, 29, 36].

As mentioned in chapter 2, studies have shown that elevated lipid metabolites and free fatty acids, particularly saturated fatty acids, appear to induce insulin resistance and impair insulin signaling in the hypothalamic region as well as in peripheral tissues [37]. Rats fed high fat diet become obese and centrally insulin resistant as shown by the loss of insulin’s anorectic effect when insulin was intracerebroventricularly (icv) administrated [37, 38]. Feeding HFD induced the accumulation of saturated fatty acids (palmitoyl-, stearoyl-CoA) in the hypothalamic region [37]. Free fatty acids enter the brain from the circulation and are rapidly incorporated into brain lipids, 40% of $[^{14}\text{C}]$-palmitate being incorporated within 45 seconds [39]. These data suggest that the accumulation of saturated fatty acids in the hypothalamus might be a critical factor leading to hypothalamic insulin resistance as it is in peripheral tissues [37].

A direct demonstration of fatty acid induced insulin resistance in the brain has been reported. Intracerebroventricular (icv) injection of palmitate impaired the insulin stimulation of Akt$^{\text{Ser473}}$ phosphorylation in the hypothalamus. This effect was linked to changes in PKCθ subcellular localization [37, 40]. The studies demonstrated that icv
administration of palmitic acid significantly increased palmitoyl-CoA, whereas *icv* insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation level was significantly inhibited in rat’s hypothalamus when compared to *icv* vehicle-infused rats [37, 40].

Although, the hypothalamus is thought to have a central role in the regulation of body weight and peripheral metabolism [1], many other regions of the brain such as limbic regions (nucleus accumbens and amygdala) and the brain stem are also involved in the regulation of energy homeostasis [41, 42]. The amygdala, a brain region which has been studied for its role in regulating emotions, such as fear and anxiety, has recently become of interest to study for its role in influencing ingestive behaviors [43, 44]. The administration of several neuro-peptides/hormones into the central nucleus of the amygdala (CeA) affects food intake, particularly the ingestion of diets rich in fats. A significant reduction of dietary fat intake has been observed after administration into the CeA of enterostatin [45] or the melanocortin-4 agonist MT-II [46, 47], whereas mu-opioids [44, 48, 49], as well as melanocortin 4 antagonists such as SHU9119 [46, 47, 50] increased intake of dietary fats. Also, as well as other regions of the brain, the amygdala highly expresses insulin receptors [51], which suggests a possible role of insulin in the amygdala in the regulation of food intake similar to its role in the hypothalamus.

Our laboratory recently investigated the effects of insulin directly administered into the amygdala on food intake using a SD rat model [52]. Insulin had a strong anorectic effect when injected into the central nucleus of the amygdala (CeA) and rats fed HFD for 3 days lost the sensitivity to amygdala insulin, thus the insulin’s anorectic effect was suppressed. Furthermore, this acute (3 days) HFD induced CeA insulin resistance occurred before any evident changes in peripheral insulin sensitivity [52]. This result
suggested that the rapid loss of the insulin anorectic response in the amygdala may be a significant factor in the normal hyperphagic response to the introduction of HFDs and that dietary fat impaired insulin signaling pathways in the amygdala as well as in hypothalamus.

In chapter 2, using neuronal cell lines, I demonstrated that saturated fatty acids significantly inhibited the insulin signaling pathway in neuronal cells (mouse hypothalamic cell lines) and in rat amygdala primary cells. In this chapter, the studies were extended to show that similar responses occurred in vivo. Specifically, the experiments in this chapter investigated the effect of dietary fat and saturated fatty acids on insulin signaling pathways in both the amygdala and hypothalamus of rats. Since previous studies had shown that the insulin anorectic response of the amygdala was lost within 3 days, the same time point was chosen to show that dietary fat abolished the insulin stimulation of its signaling pathways. Subsequently, the effects of direct infusion of saturated fatty acid onto the CeA on insulin signaling are described.

2. Hypotheses

1) Three days high fat diets will inhibit insulin stimulated Akt\textsuperscript{Ser473} phosphorylation and phosphorylation of mTOR in the amygdala.

2) Three days CeA palmitic acid infusion will inhibit insulin stimulated phosphorylation of Akt\textsuperscript{Ser473} and mTOR in the amygdala.

3) Changes in insulin signaling in the amygdala in response to HFD and CeA palmitic acid infusion will also change the activity of Akt/mTOR signaling pathways in the hypothalamus.
3. Materials and Methods

Animals and diet

A total of 48 male Sprague-Dawley rats (body weight 220-240 g; Charles River) were used in two separate experiments. They were individually housed in hanging wire mesh cages in a temperature (22-24 °C) and light controlled (lights off 19:00-07:00 h) room. All rats were initially fed chow diet (11 % energy as fat; 3.30 kcal/g; Harlan Teklad 8604, Madison, WI) unless specified otherwise and water was available ad libitum. The Institutional Animal Care and Use Committee of Utah State University approved the animal protocols.

Cannula implantation

Rats anesthetized with pentobarbital sodium (Nembutal; 50 mg/Kg body weight, ip) were stereotaxically implanted with one unilateral stainless steel cannula (Plastics One, Roanoke, VA) aimed to the central nucleus of the amygdala: [coordinates (AP/L/DV to bregma) -2.4/-3.8/-7.5 mm] according to Paxinos and Watson [53] and previous experiments [45, 52]. Each cannula was secured in place with 3 anchor screws and dental acrylic and occluded with a 26 gauge wire stylet. The injector was designed to project 0.5 mm beyond the guide cannula tip. Each rat received an injection of the analgesic drug Carprofen (Rimadyl® 5 mg/kg, s.q.) before returning to their home cage.

Insulin administration

After adaptation to diets (LFD or HFD) for 3 days or after 3 days infusion of palmitic acid or vehicle, rats were weight matched and assigned to experimental groups. They rats were injected with either 0.9% (w/v) saline vehicle (0.5μl) or insulin (0.5μl,
8mU, Humulin R; Eli Lilly, Indianapolis IN) into the CeA over a 1 minute time period and sacrificed after 10 minutes for rapid removal of brain tissues. The dose of insulin used in these experiments was selected based on previous experiments [52]. Solutions were prepared fresh before injection. The injector was left in place for an additional 1 min to prevent backflow.

Sacrifice and protein extraction

Rats were sacrificed by decapitation. The brain was rapidly removed and the hypothalamus, cortex and both amygdala (injected and non-injected sides) were dissected and immediately frozen in liquid nitrogen and stored at -80 °C until processed. Both hypothalamus and amygdala tissues were placed in 1.5 ml tubes and homogenized in 600 μl of whole cell lysis buffer containing anti-protease and anti-phosphatase agents (50 mM KCl, 25 mM HEPES-pH 7.8, 10 μl/ml leupeptin, 20 μg/ml aprotonin, 125 μM DTT, 1 mM PMSF and 1 mM orthovanadate) using a Teflon pestle. Each homogenate was divided into two tubes. Half of the homogenate was used to extract total protein, the other half was used to extract both cytosolic and plasma membrane fraction proteins for the later analysis of PKCθ distribution. For total protein extraction, 2 % Nonidet P-40 (NP-40; nonionic detergent; United States Biochemical Corp, Cleveland, OH) containing whole cell lysis buffer was added to the homogenate and this was sonicated and centrifuged at 4 °C and 13,000 rpm for 15 min. Protein concentration was measured using a Pierce BCA protein assay kit (ThermoScientific Inc., Rockford, IL).
**Palmitic acid preparation and osmotic pump implantation**

The day before osmotic pump implantation, palmitic acid (PA) (Sigma, CA) was dissolved and complexed in hydroxypropyl β-cyclodextrin [45% (w/v), HPC; Sigma, CA] to make a 17 mM PA-HPC solution. The solution was diluted in artificial cerebrospinal fluid (a-CSF; Harvard Apparatus, MA) to make a final concentration of 0.05 mM PA-HPC solution. Mini osmotic pumps (Alzet mini osmotic pumps, 1007D) were filled with either 0.05 mM PA-HPC solution or a-CSF-HPC solution as vehicle and primed overnight under sterile conditions. On the day of implantation, the prepared pumps were installed subcutaneously while rats were under isofluorane anesthesia and the pumps were connected to the infusion cannula via sterile vinyl tubing for continuous infusion of either a-CSF-HPC or PA-HPC into central nucleus of amygdala through the cannula. The infusion rate was 0.5 μl / hour for 3 days. Total infused amount was 36 μl (1.8 nmol of PA).

**Cell culture, fatty acids application and insulin administration**

The procedures for GT1-7 neuronal cell and amygdala primary cell culture, palmitic acid and insulin administration were described in chapter 2.

**Western blot**

The procedures were described in chapter two and followed exactly in this chapter. Additional antibody, rabbit anti-phosphorylated mTOR<sup>Ser2448</sup> (ab51044), was purchased from Abcam Inc (Cambridge, MA).
Experimental designs

Experiment 1: Effect of 3 days of either HFD or LFD on insulin signalling in amygdala and hypothalamus

Twenty-three male SD rats initially fed a regular chow diet were fitted with unilateral amygdala cannula (day 1), and the diet was changed to LFD (10 % energy as fat; 3.85 kcal/g D12450B; Research Diets) for 5 days. On the basis of their body weight, the rats were assigned to two experimental groups (day 6): LFD (n = 11) and HFD (n = 12) and each group fed either LFD or HFD (45 % energy as fat, 4.73 kcal/g D12451 Research Diets, New Brunswick, NJ) for 3 days (Food was weighed and changed on the second day because of the potential oxidation of HFD). During the 3 days, food intake and body weight were recorded every day. After 3 days of each diet, each diet group was separated into two sub-groups again based on their body weight (LFD-saline; n = 5, LFD-insulin; n = 6, HFD-saline; n = 6, and HFD-insulin; n=6). All rats were injected with either vehicle (saline, 0.5 μl) or insulin (8 mU) into the CeA. Rats were sacrificed 10 minutes after the insulin injection and brain tissues (hypothalamus and amygdala) were collected and immediately frozen in liquid nitrogen to be stored at -80°C until they were processed.

Experiment 2: Effect of 3 days of continuous palmitic acid infusion into the amygdala on insulin signalling pathways in the amygdala and hypothalamus

Twenty-two male SD rats initially fed a regular chow diet were fitted with a unilateral amygdala cannula (day 1), and the diet was changed from chow to LFD. On the basis of their body weight, the rats were assigned to two experimental groups (day 6):
Vehicle (VC; n = 11) and palmitic acid (PA; n = 11) and each group of rats were implanted with osmotic pumps filled with either PA-HPC or a-CSF-HPC solution which were connected to the infusion cannula. After 3 days of infusions, the two groups (VC and PA) of rats were weighed and subdivided into two groups again (VC-saline, VC-insulin, PA-saline, and PA-insulin) based on body weight. Rats were injected with either insulin (8 mU) or saline (0.5 μl) into amygdala. Rats were sacrificed 10 minutes after the insulin injection and brain tissues (hypothalamus and amygdala) were collected and immediately frozen in liquid nitrogen to be stored at -80°C until they are processed.

4. Results

*Three days of HFD blunted insulin stimulated Akt phosphorylation in rat amygdala and hypothalamus*

In this experiment, each group of rats was fed with either LFD or HFD throughout the experimental period (3 days) and was injected with insulin (8mU) or saline into the amygdala 10 minutes before the rats were sacrificed. The brain tissues (amygdala and hypothalamus) were collected to study the effects of diet and amygdala insulin on the insulin signaling pathway in both amygdala and hypothalamus.

*Body weight and food intake:* During the 3 days diets (LFD or HFD), the body weight and food intake were recorded every day. As shown in figure 3-2 (A and B), rats fed a HFD gained significantly more body weight in the 3 days compared to LFD fed rats ($p < 0.05$). The food intake (g) was converted into kilocalories (kcal) and the results are shown in the figure 3-2 (C and D). Total calorie intake was significantly increase in HFD fed rats compared to LFD fed groups ($p < 0.05$). There were no significant differences in
either body weight or food intake between the groups that were used for insulin or vehicle injections on either diet.

**Insulin signaling pathways in amygdala and hypothalamus:** According to a previous report from our laboratory [52], 3 days HFD fed rats did not show any anorectic response after being injected with insulin to the central nucleus of the amygdala (CeA). Thus, the aim was to show that the insulin resistance was expressed in alterations in the activity of the insulin signaling pathway in the CeA after 3 days of HFD feeding. In order to investigate the hypothesis that 3 days HFD blocks insulin stimulated signaling in the amygdala, the amygdala (insulin injected side) tissues of each group of rats were accessed to analyze the change in phosphorylation level of Akt\(^{\text{Ser473}}\) using Western blots. As shown in figure 3-3, amygdala insulin (8 mU) injection elevated Akt\(^{\text{Ser473}}\) phosphorylation in the amygdala of rats fed LFD (LFD-insulin) compared to the LFD-saline group \((p < 0.001)\). In contrast, amygdala insulin injection had no significant effect on Akt\(^{\text{Ser473}}\) phosphorylation in the HFD fed (HFD-insulin) group compared to the saline injected rats (HFD-saline). After eating an HFD for 3 days, rats had completely lost the Akt\(^{\text{Ser473}}\) phosphorylation response to amygdala insulin. There was no significant effect of diet alone on Akt\(^{\text{Ser473}}\) phosphorylation (LFD-saline versus HFD-saline), whereas there was a significantly different Akt\(^{\text{Ser473}}\) phosphorylation level between LFD-insulin and HFD-insulin group \((p < 0.001)\) as shown in figure 3-3. According to two-way ANOVA analysis, there was interaction between diets and injections (insulin and saline; \(p < 0.01\)).

It was also considered possible that a HFD and amygdala insulin injections might affect the hypothalamic insulin signaling pathway through neuronal pathways from the amygdala. To investigate the hypothalamic response to HFD feeding and to amygdala
insulin injections, the hypothalamic tissue of each group of rats was assayed to measure the Akt<sup>Ser473</sup> phosphorylation level using Western blots. As shown figure 3-4, insulin injection into the amygdala induced a significant increase of Akt<sup>Ser473</sup> phosphorylation in the hypothalamus of rats fed LFD (LFD-insulin) within 10 minutes ($p < 0.001$). This result suggests that 10 minutes of CeA insulin stimulation affected hypothalamic Akt signaling pathways. Amygdala Western blot data did not show a significant difference of Akt<sup>Ser473</sup> phosphorylation level between LFD-saline and HFD-saline (figure 3-3); however, 3 days HFD (HFD-saline) induced a significantly increased Akt<sup>Ser473</sup> phosphorylation in hypothalamus compared to LFD-saline group ($p < 0.01$). In addition, amygdala insulin injection in HFD fed rats (HFD-insulin) induced a significant decrease in Akt<sup>Ser473</sup> phosphorylation in hypothalamic tissues compared to the hypothalamus of the amygdala saline injected (HFD-saline) group ($p < 0.05$). These data show that 3 days of HFD elicited different responses in the hypothalamus and amygdala. Further, they show that activating insulin signaling in the amygdala also activates Akt<sup>Ser473</sup> phosphorylation in the hypothalamus in rats fed a LFD whereas an inhibitory effect was observed in rats fed the HFD. According to two-way ANOVA analysis, there was a strong interaction between diets and injections (insulin and saline; $p < 0.001$).

*Effect of palmitic acid infusion onto the amygdala for 3 days on insulin stimulation of Akt phosphorylation in the amygdala and hypothalamus*

In this experiment, each group of rats was infused with either vehicle (artificial-CSF) or palmitic acid (PA) onto the amygdala (CeA) throughout the 3 days experimental period and then injected with either insulin (8mU) or saline (0.5 μl) into the amygdala.
minutes before the rats were sacrificed. Brain tissues (amygdala and hypothalamus) were collected to study the effects of saturated fatty acid infusion into the CeA on the insulin signaling pathway in both amygdala and hypothalamus.

**Body weight and food intake:** During the experimental period (3 days) all the rats were fed with LFD. The body weight and food intake were recorded at day 0, and 3. As shown in figure 3-5 (A and B), there were no significant differences in body weight gain during the 3 days between vehicle and palmitic acid infused groups. Neither were there any differences in food intake (figure 3-5 C and D).

**Insulin signaling pathways in amygdala and hypothalamus:** The previous experiment showed that in 3 days, HFD induced insulin resistance in the amygdala and hypothalamus. Because it has been reported that HFD increases palmitoyl-CoA levels in brain regions and elevated palmitic acid induces insulin resistance in both brain and peripheral tissues, we investigated whether direct CeA infusion of palmitic acid would mimic the effects of HFD on both amygdala and hypothalamic insulin signaling pathways. As in the previous experiment, both amygdala and hypothalamic tissues of each group or rats were assayed to measure the Akt^{Ser473} phosphorylation level using Western blot after rats had been injected with either insulin (8 mU) or saline (0.5 μl) into CeA.

Similar to previous data, insulin injections into the amygdala induced a significant increase of Akt^{Ser473} phosphorylation in the amygdala of rats infused with a-CSF vehicle (vehicle-insulin versus vehicle-saline, \( p < 0.001 \)) (Figure 3-6). In contrast, palmitic acid infusions reduced amygdala Akt^{Ser473} phosphorylation (PA-saline versus vehicle-saline, \( P < 0.05 \); Figure 3-6) and abolished the insulin stimulation of Akt^{Ser473} phosphorylation
(PA-saline versus PA-insulin; figure 3-6). These data show that 3 days of amygdala palmitic acid infusion blocked the signaling response to insulin.

The effects of CeA palmitic acid infusions and CeA insulin injections on insulin signaling pathways of hypothalamus were also investigated. Similar to the previous experiment, CeA insulin injection activated not only amygdala Akt$^{\text{Ser473}}$ phosphorylation but also Akt$^{\text{Ser473}}$ phosphorylation in the hypothalamus ($p < 0.01$) as shown in figure 3-7. In contrast to the effects of 3 days HFD feeding, in which CeA insulin reduced the level of Akt$^{\text{Ser473}}$ phosphorylation in the hypothalamus, 3 days of CeA palmitic acid infusion had no significant inhibitory effect on Akt$^{\text{Ser473}}$ phosphorylation levels in the hypothalamus as shown in figure 3-7. Also, the result of 3 days HFD experiment showed that 3 days HFD significantly increased Akt$^{\text{Ser473}}$ phosphorylation level in rat’s hypothalamus in absence of CeA insulin injection (HFD-saline) compared to LFD-saline group, whereas, 3 days CeA palmitic acid infusion had no such effect between vehicle-saline and palmitic acid-saline groups. The differing effects of HFD from amygdala PA infusions indicate that dietary fat may have both direct effects at the hypothalamus as well as indirect effects mediated through signaling from the CeA.

**Effect of amygdala palmitic acid and insulin on mTOR signaling in the amygdala and hypothalamus**

CeA insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation in hypothalamus was blunted by 3 days of HFD feeding (figure 3-4), whereas CeA insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation in the hypothalamus was not impaired by CeA palmitic acid infusion (figure 3-7). In contrast to the hypothalamic responses, both 3 days HFD and CeA palmitic acid infusion impaired CeA insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation in the
amygdala (Figure 3-3 and 3-6). This differential hypothalamic response to HFD and CeA palmitic acid suggests that there may be an alternative insulin signaling pathway that activates neuronal input from the amygdala to hypothalamus and this signaling might be independent of Akt signaling since amygdala Akt phosphorylation is impaired by 3 days CeA palmitic acid infusion. As described in the introduction to this chapter, insulin also activates mTOR signaling in brain regions (hypothalamus and extra-hypothalamic regions) and the activation of mTOR signaling in metabolic sensing neurons appears to regulate food intake and energy balance [13, 19]. Cell culture experiment described earlier also showed that palmitic acid inhibited insulin stimulation of Akt signaling but not insulin stimulation of mTOR signaling. Therefore, I hypothesized that mTOR signaling in the amygdala may be important for the hypothalamic response to amygdala insulin. Hence, mTOR signaling in both amygdala and hypothalamus was investigated to test the effects of HFD, amygdala insulin and palmitic acid. The data should show if the differential response to HFD and CeA palmitic acid is dependent on mTOR signaling in either amygdala or hypothalamus.

As shown in Figure 3-8, CeA insulin significantly increased amygdala mTOR\textsuperscript{Ser2448} phosphorylation levels in both the vehicle and the palmitic acid infused rats compared to saline injected groups (Vehicle-insulin vs. Vehicle-saline; \( p < 0.01 \) and PA-insulin vs. PA-saline; \( p < 0.05 \)). These data indicate that insulin stimulates mTOR\textsuperscript{Ser2448} phosphorylation in the amygdala and that the CeA palmitic acid infusion appears to have no effect on this response. In contrast to the amygdala response, there was no significant difference of mTOR\textsuperscript{Ser2448} phosphorylation in the hypothalamus in any of the treatment
groups (figure 3-9). Neither CeA insulin nor CeA palmitic acid infusion had any effect on hypothalamic mTOR$_{\text{Ser2448}}$ phosphorylation.

Effect of 3 days of LFD - HFD and amygdale insulin

on mTOR signaling in the amygdala and hypothalamus

Both amygdala and hypothalamus tissues were removed from rats fed either LFD or HFD for 3 days 10 minutes after injection with either vehicle or insulin (8mU) on to the CeA. As shown in figure 3-10, similar to previous 3 days CeA palmitic acid infusion experiment, amygdala mTOR signaling was increased by CeA insulin injection (LFD-Ins and HFD-Ins) compared to CeA saline injected samples (LFD-Sal and HFD-Sal; $p < 0.001$ and $p < 0.05$ respectively) on both diets. There were no statistical effects of diet alone on mTOR$_{\text{Ser2448}}$ phosphorylation (LFD-saline versus HFD-saline). Taken together with previous data, neither amygdala palmitic acid nor dietary fat (LFD and HFD) appear to have any effect on CeA mTOR signaling or its response to insulin in the amygdala.

Similar to the previous CeA palmitic acid infusion data, hypothalamic mTOR signaling was not affected by either diet composition or CeA insulin as shown in figure 3-11. There was no significant difference between all the groups.

5. Discussion

Recently our lab demonstrated that insulin has a strong anorectic effect when it was injected into the central nucleus of the amygdala (CeA) and that rat’s fed 3 days HFD lost this insulin anorectic response [52]. The present study extended previous experiments in cell culture models to investigate the effects of 1) a HFD and 2) CeA
palmitic acid infusion on insulin signaling pathways in the amygdala and hypothalamus in response to CeA insulin stimulation.

A recent study [37] demonstrated that rats fed HFD failed to suppress food intake in response to $icv$ (hypothalamic) insulin administration compared to LFD control rats. Also, they observed a blunted hypothalamic $Akt^{Ser473}$ phosphorylation (marker of PI3K activity) in rat’s fed HFD in response to $icv$ insulin stimulation suggesting that, as in peripheral tissues, diet induced central insulin resistance occurs and it is mediated through effects on the IRS/PI3K signaling pathway.

Similarly, CeA insulin administration significantly activated $Akt^{Ser473}$ phosphorylation in amygdala of rats fed LFD; however, 3 days HFD abolished the CeA insulin stimulated $Akt^{Ser473}$ phosphorylation consistent with the loss of anorectic response in this situation. The activation of $Akt^{Ser473}$ phosphorylation is a marker of the activation of IRS/PI3K signaling pathway which mediates most of insulin’s metabolic functions in classical insulin sensitive peripheral tissues [54, 55]. Also, numerous studies have demonstrated that a HFD induces the inhibition of IRS/PI3K signaling pathway in insulin sensitive peripheral tissues [56-58]. Our data suggests that the amygdala has similar insulin signaling pathways to those in peripheral tissues and that the IRS/PI3K-Akt pathway is similarly inhibited in rats fed HFD.

The present study also investigated the effect of 3 days of HFD on hypothalamic signaling pathways in response to CeA insulin administration. As described above, $icv$ (hypothalamic) insulin injection rapidly increased $Akt^{Ser473}$ phosphorylation in hypothalamus of rat’s fed LFD but not in rats fed HFD [37]. In our experiment, although insulin was injected into the central nucleus of the amygdala, interestingly, a robust
increase of Akt$^{\text{Ser473}}$ phosphorylation was observed in the hypothalamus in rats fed LFD (LFD-insulin) compared to LFD-saline group, whereas the CeA insulin reduced hypothalamic Akt$^{\text{Ser473}}$ phosphorylation in rats fed 3 days of HFD (HFD-insulin) compared to the HFD-saline group (Figure 3-4). These data suggests that there are neuronal connections between the CeA and the hypothalamus that are responsive to insulin signaling in the CeA.

Insulin has been described as a long-term regulator of energy balance acting via interactions with orexigenic and anorexigenic neuro-regulators in the ARC-PVN system [1]. When insulin is given into the third ventricle, it suppresses the orexigenic signals from the NPY/AgRP neurons and favors the anorexigenic signals from POMC/CART neurons, which induces the inhibition of food intake [1, 2, 59, 60]. According to recent data in our lab, insulin injections into the CeA induced $c$-Fos expression not only in amygdala regions but also in several regions of the brain that are involved in the regulation of feeding behavior, including the arcuate nucleus (ARC) and PVN in the hypothalamus, indicating that those areas were activated in response to amygdala insulin injection [52]. Taken together these data provide further evidence to support the possibility that the amygdala may regulate anorectic control on food intake through its neuronal output to other regions of the brain, particularly the hypothalamus. It is suggested that the CeA insulin activates a neuronal pathway that stimulates POMC neurons in the ARC-PVN axis, and that this might be responsible for the inhibition of food intake in response to CeA insulin [52]. Furthermore, HFD feeding induced insulin resistance in the amygdala and its inability to stimulate hypothalamic POMC neurons might be explained by the inability of CeA insulin to increase Akt$^{\text{Ser473}}$ phosphorylation.
in hypothalamus after 3 days HFD feeding. The amygdala is connected to other regions of the brain through numerous projections from and toward the cortex, other areas of the limbic system, areas of the hypothalamus or the brain stem [61]. However, the precise neuronal connections between the amygdala and PVN that enable these responses within the hypothalamus to insulin signaling in the CeA are not understood.

Another observation from this experiment was that even though $\text{Akt}^{\text{Ser473}}$ phosphorylation was significantly blunted in the hypothalamus of HFD fed rats (HFD-insulin versus HFD-saline; figure 3-4), HFD alone significantly increased $\text{Akt}^{\text{Ser473}}$ phosphorylation in the hypothalamus (HFD-saline versus LFD-saline; figure 3-4). Similar data was reported in the study from Posey and colleagues [37]. In this study, icv (hypothalamus) insulin stimulated hypothalamic $\text{Akt}^{\text{Ser473}}$ phosphorylation was significantly blunted in rats fed HFD compared to LFD, whereas HFD alone exhibited slightly higher baseline hypothalamic $\text{Akt}^{\text{Ser473}}$ phosphorylation compared to LFD-saline group. Taken together these observations suggest that the HFD stimulates Akt phosphorylation in the hypothalamus independently of effects in the CeA. However, possible mechanisms are not understood at this point.

Our laboratory has shown that HFD induced a loss of insulin’s anorectic effect within 3 days while peripheral insulin signaling was not altered with 3 days of HFD feeding [52]. Another study showed that a single day of HFD feeding blunted both insulin signaling in the hypothalamus and the ability of hypothalamic insulin to suppress hepatic glucose production, while several weeks of HFD feeding was required to induce insulin resistance in muscle and adipose tissue [62, 63]. Taken together, these data suggest that the development of a central insulin resistance is established far earlier than
peripheral insulin resistance and may have an important role in the subsequent
development of obesity and peripheral insulin resistance [52]. In the present study, the
suppression of CeA insulin signaling (phosphorylation of Akt\textsuperscript{Ser473}) in both amygdala and
hypothalamus within 3 days of HFD further supports the idea described above. Our data
is also consistent with insulin’s anorectic effect in the amygdala depending upon intact
PI3K mediated Akt signaling and that alteration of this signaling pathway due to HFD
may impair the regulation of food intake.

Saturated fatty acids, more specifically palmitic acid, induce insulin resistance in
the brain [37] as well as in peripheral tissues. Also, an elevated palmitoyl-CoA level in
hypothalamus has been reported after HFD feeding [64, 65]. The second experiment in
this chapter investigated if palmitic acid infusion into the CeA would mimic the effect of
HF feeding on insulin signaling in both amygdala and hypothalamus. Similar to the effect
of 3 days HFD on amygdala insulin signaling, 3 days of palmitic acid infusion blunted
CeA insulin stimulated Akt\textsuperscript{Ser473} phosphorylation in the amygdala. This result suggests
that insulin signaling in the amygdala, as well as the hypothalamus and peripheral tissues
is affected by accumulation of saturated fatty acids. Furthermore, palmitic acid infusion
reduced the level of Akt\textsuperscript{Ser473} phosphorylation alone in the amygdala, suggesting that the
basal levels of Akt\textsuperscript{Ser473} phosphorylation observed might reflect the effects of endogenous
insulin signaling.

Numerous studies have investigated the effects of saturated fatty acids on insulin
resistance in peripheral tissues and proposed several possible mechanisms (described in
chapter 1). Which of these mechanisms is important in amygdala insulin resistance
cannot be identified from this current research. However, recently, it has been reported
that both HFD and icv palmitic acid infusion blunted the PI3K-Akt signaling pathway in the hypothalamus through activation of inflammatory signaling pathways mediated by IKK-β [37]. Other studies have suggested that icv palmitic acid infusion impaired Akt\textsuperscript{Ser473} phosphorylation through activation of PKCθ and subsequent alteration of IRS phosphorylation [40].

After 3 days HFD, a decreased hypothalamic Akt\textsuperscript{Ser473} phosphorylation in response to CeA insulin was still observed. The effect of CeA palmitic acid infusion on the hypothalamic Akt signaling response to CeA insulin was investigated. As expected from previous studies, CeA insulin stimulation induced a robust increase in Akt\textsuperscript{Ser473} phosphorylation in the hypothalamus in vehicle infused rats presumably through neuronal connections between the amygdala and hypothalamus. In the CeA palmitic infusion rats, a similar result was expected to the HFD experiment; however, there was no inhibition of Akt\textsuperscript{Ser473} phosphorylation in hypothalamus of palmitic acid-insulin group compared to palmitic acid-saline group (figure 3-7). Although the amygdala again became insulin resistant, CeA insulin still stimulated Akt\textsuperscript{Ser473} phosphorylation in the hypothalamus (figure 3-7). These results suggest that a different insulin signaling pathway from Akt must be responsible for activation of the neuronal connections between amygdala and hypothalamus and that this alternative pathway appears to be independent of the effect of palmitic acid signaling. If this were the case, then CeA insulin might be able to activate the amygdala mediated hypothalamic insulin signaling pathway.

CeA palmitic acid infusion had no effect on hypothalamic Akt signaling pathways, whereas 3 days HFD appear to have an inhibitory effect in the hypothalamic region. This might reflect a direct effect of HFD-related signals onto the hypothalamus
that inhibit the response to amygdala inputs. Three days HFD appear to induce insulin resistance both in amygdala and hypothalamus since HFD induce an increase of saturated fatty acid level systemically affecting both hypothalamus and amygdala at the same time whereas, 3 days of CeA palmitic acid infusion appears to affect only the amygdala region (local effect) so that hypothalamus might be not be affected by elevated saturated fatty acids as compared to the systemic effect of HFD.

According to a number of studies, the mTOR signaling pathway might be one possible alternative signaling pathway activated by insulin that may underlie the differential response to HFD and amygdala PA infusions. As described in the introduction in this chapter, the mammalian target of rapamycin (mTOR) protein signaling plays a role in the hypothalamus regulating energy homeostasis in response to nutrient availability as well as hormonal signals such as leptin and insulin [13]. This suggests that mTOR signaling in hypothalamus may be one of the critical mediators in the regulation of food intake and energy balance in response to insulin as well as insulin stimulated PI3K-Akt signaling pathway.

Insulin and leptin activate mTOR signaling through PI3K-Akt signaling pathways in the hypothalamus [10, 29, 66] as well as in peripheral tissues [67]. However, it is not clear how nutrients activate mTOR signaling, although 5’ AMP-activated protein kinase (AMPK) [68] and Ste20-related mitogen-activated protein kinase 4 (MAP4K3) [69] have been suggested as candidate mediators. Activation of Ste20 family member, MAP4K, is required for activation of mTOR signaling [69]. MAP4K activity is regulated by amino acids, but not by growth factors including insulin, suggesting that nutrients signal to mTOR through the activation of MAP4K [69].
Since the mTOR expression and activity has been detected in various regions of the brain including the hippocampus, thalamus and cortex [18], amygdala mTOR signaling may also have a similar function in the regulation of food intake and/or energy balance. Also it is possible that amygdala mTOR signaling may indirectly regulate such functions through the mediation of hypothalamic insulin signaling via the neuronal interconnection between amygdala and hypothalamus. Hypothalamic Akt signaling was increased in response to amygdala insulin even when insulin activation of Akt signaling was inhibited in the amygdala by palmitic acid infusions. This suggests the possibility of an alternative insulin signaling pathway in the amygdala that is not inhibited by palmitic acid infusions. The mTOR signaling pathway in amygdala and hypothalamus was studied as a possible mediator of these effects.

According to the data shown in figure 3-8, insulin significantly increased amygdala mTOR$^{\text{Ser2448}}$ phosphorylation level compared to non-insulin injected groups (vehicle-saline and palmitic acid-saline) and this response was not inhibited by 3 days of palmitic acid infusion. Also, insulin significantly increased mTOR$^{\text{Ser2448}}$ phosphorylation level in amygdala primary cells and this response was not affected by palmitic acid (chapter 2). This suggests that mTOR signaling in amygdala is insulin dependent and independent of palmitic acid at least in short term (3 days) experiments. Similar to 3 days CeA palmitic acid infusion experiment, CeA insulin injection significantly increased mTOR$^{\text{Ser2448}}$ phosphorylation level in amygdala of both LFD and HFD fed rats compared to non insulin injected animals (figure 3-10). This again suggests that mTOR signaling in amygdala is activated by insulin and is independent of any inhibitory effects of either palmitic acid or HFD. Thus, it is possible that insulin activation of mTOR signaling in the
CeA activates a pathway that regulates Akt signaling in the hypothalamus. Such a response would explain the activation of hypothalamic Akt signaling after insulin and palmitic acid infusions into the CeA.

However, there was no significant elevation of mTOR phosphorylation in the hypothalamus of either amygdala vehicle-insulin or amygdala palmitic acid-insulin groups of compared to non-CeA insulin injected groups (figure 3-9). This suggests that amygdala insulin and insulin stimulated mTOR signaling in amygdala does not affect hypothalamic mTOR signaling. Similar to this, there was no significant difference of hypothalamic mTOR phosphorylation level between LFD-saline and LFD-insulin; HFD-saline and HFD-insulin CeA groups (figure 3-11). Since hypothalamic mTOR signaling was not affected by either HFD or CeA PA infusions, hypothalamic mTOR signaling seems not to be responsible for the differential response of hypothalamic Akt signaling in response to CeA insulin between rats fed HFD or infused with CeA palmitic acid. It appears that CeA insulin stimulates mTOR signaling in amygdala and this amygdala response is not affected by 3 days of HFD and CeA palmitic acid infusion. Although underlying molecular mechanism is not known at this point, the amygdala mTOR signaling may enhance hypothalamic Akt signaling through neuronal connections; however this response seems not to be mediated through the hypothalamic mTOR signaling because hypothalamic mTOR signaling was not affected by CeA insulin and insulin stimulated amygdala mTOR signaling. Since elevated hypothalamic Akt$_{\text{Ser473}}$ phosphorylation was only observed in rats of CeA palmitic acid infused (local effect) but not in hypothalamus of rat fed HFD (systemic effect), it suggests that the systemic effect of HFD might impair neuronal signaling in the connection between amygdala and
hypothalamus. It is also possible that HFD prevents the hypothalamic response to amygdala input. Figure 3-1 summarizes possible signaling pathways between the amygdala and hypothalamus in response to CeA insulin, HFD and amygdala palmitic acid infusion.

It is not clear at this time how the activation of amygdala mTOR signaling enhances the phosphorylation of Akt\textsuperscript{Ser473} in the hypothalamus or which cell types this Akt activation occurs. Further investigations are necessary to identify and understand the mechanisms involved in the connections between the amygdala and hypothalamus in the regulation of CNS metabolism in response to insulin and the effects of HFD or elevated saturated fatty acids. Another question is how mTOR signaling is regulated independently of Akt signaling since mTOR is normally regulated by Akt. Further studies are required to identify this Akt-independent regulation of mTOR.

In summary, the present study extended previous data from our laboratory to explain the observations that 1) CeA insulin induced an anorectic response and 2) that short term feeding of a HFD induced loss of the anorectic effect. Similar to insulin sensitive tissues such as skeletal muscles and adipose tissues, the insulin appears to activate the Akt signaling pathway in the amygdala and this is inhibited in response to lipid signals. Taken together with recent studies of HFD induced hypothalamic insulin resistance, our results suggest that diet induced insulin resistance is also present in multiple regions of the CNS and the corresponding molecular mechanisms might be similar to those shown in peripheral insulin resistance. However, our data suggest that insulin stimulation of the mTOR pathway is independent of lipid signaling. Also, it appears that the induction of insulin resistance in amygdala is far earlier than it is in
peripheral tissues. The CeA insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation in both amygdala and hypothalamus suggests that there might be neuronal connections between amygdala and hypothalamus. This also suggests the possibility that insulin may act in the amygdala in regulating energy balance as well as in the hypothalamus or possibly through its connections to the hypothalamus. CeA infusion of palmitic acid mimicked the effect of HFD to induce insulin resistance at the level of Akt$^{\text{Ser473}}$ phosphorylation in the amygdala suggesting that the HFD induced the accumulation of saturated fatty acids in the CNS and that the direct action of saturated fatty acids on insulin signaling pathways might be a critical factor in the induction of CNS insulin resistance. Also, the different response of hypothalamic insulin signaling pathways to the HFD and CeA palmitic acid infusion suggest that there might be additional signaling pathways in the connections between the amygdala and hypothalamus. Our data suggest that mTOR could be the one such possible signaling pathway. The present study suggests that there is an important signaling link between the amygdala and the hypothalamus that might be important for understanding not only the insulin anorectic effect but also the significance of insulin resistance in differing parts of the brain. Further identification of the molecular basis for amygdala insulin resistance may provide novel approaches to the treatment of metabolic disorders such as type 2 diabetes.

6. References


50. Balthasar N: Genetic dissection of neuronal pathways controlling energy homeostasis. *Obesity (Silver Spring)* 2006, 14 Suppl 5:222S-227S.


Figure 3-1: Signaling pathways induced by insulin in the CeA. The model illustrates that central nucleus of the amygdala (CeA) insulin activates Akt signaling in both amygdala and hypothalamus through pathway (1), which then regulates catabolic (POMC) and anabolic (AgRP/NPY) neurons in the hypothalamus to stimulate an anorectic response. Three days HFD abolished CeA insulin stimulated Akt signaling in both amygdala and hypothalamus. However, 3 days of palmitic acid infusion significantly decreased CeA insulin stimulated Akt signaling in the amygdala, but not in hypothalamus. This comparable result suggests that there must be alternative pathways to activate hypothalamic Akt signaling in response to CeA insulin stimulation. One possible alternative pathway (2) may be mTOR signaling as shown in this figure.
Figure 3-2: Effect of 3 days LFD or HFD on body weight gain and food consumption (kilocalories) in male SD rats. Both body weight and food intake were measured at day 0, 1, 2, and 3. 24 rats were divided into two groups (LFD or HFD) and daily body weight gain (g) (Panel A) and food intake (kcal) (Panel C) were measured. Panel B and D show that LFD and HFD groups are further divided into four subgroups (LFD-Saline, LFD-Insulin, HFD-Saline, and HFD-Insulin) to show their daily body weight gain (g) and food intake (kcal). Values represent Means ± SEM for 6 or 12 rats per group. *p < 0.05 compared to LFD group.
Figure 3-3: Effect of 3 days high fat diet on insulin signaling in rat’s amygdala. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of Akt<sup>Ser473</sup> phosphorylation level and β-Actin as a loading control in rat’s amygdala tissue. Data are shown with Mean ± SEM (n = 5-6). +++<i>p</i> < 0.001 compared to LFD-saline injected group. ***<i>p</i> < 0.001 compared to LFD-insulin injected group.
Figure 3-4: Effect of 3 days HFD and amygdala insulin stimulation on Akt signaling in the hypothalamus. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of Akt$^{\text{Ser473}}$ phosphorylation level and β-Actin as a loading control in rat’s hypothalamus tissue. Data are shown with Mean ± SEM (n = 5-6). $^{++} p < 0.01$, $^{+++} p < 0.001$ compared to LFD-saline injected group. $^{***} p < 0.001$ compared to LFD-insulin injected group. $^{#} p < 0.05$ compared to HFD-saline injected group.
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**Figure 3-5: Effect of 3 days palmitic acid infusion into the amygdala on body weight gain and food consumption (in kilocalories) in male SD rats.** The cumulative body weight gain and food intake for 3 days were measured. Panel A and C show that 24 rats were divided into two groups (vehicle or palmitic acid; n=12) and cumulative body weight gain (g) and food intake (kcal). Panel B and D show that each group (vehicle or palmitic acid; n = 12) are further divided into four subgroups (vehicle-saline, vehicle-insulin, palmitic acid-saline, and palmitic acid-insulin; n = 6) to show cumulative body weight gain (g) and food intake (kcal). Values represent Means ± SEM for 6 or 12 rats per group.
Figure 3-6: Effect of palmitic acid infusion into the amygdala for 3 days on insulin signaling pathways in rat amygdala. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of Akt^{Ser473} phosphorylation level and β-Actin as a loading control in rat’s amygdala tissue. Data are shown as Mean ± SEM (n = 5-6). +++$p < 0.001$, +$p < 0.05$ compared to vehicle-saline injected group. ***$p < 0.001$ compared to vehicle-insulin injected group.
Figure 3-7: Effect of insulin injection into the CeA of rats infused with PA into the CeA on Akt signaling in the hypothalamus. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of Akt\textsuperscript{Ser473} phosphorylation level and β-Actin as a loading control in rat’s hypothalamus tissue. Data are shown as Mean ± SEM (n = 5-6). ++\textit{p} < 0.01 compared to vehicle-saline injected group. +++\textit{p} < 0.001 compared to palmitic acid-saline injected group.
Figure 3-8: Effect of 3 days amygdala palmitic acid infusion on insulin signaling pathways in rat Amygdala. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of mTOR<sup>Ser2448</sup> phosphorylation level and β-Actin as a loading control in rat’s amygdala tissue. Data are shown as Mean ± SEM (n = 5). ++p < 0.01 compared to vehicle-saline injected group. *p < 0.05 compared to palmitic acid-saline injected group.
Figure 3-9: Effect of CeA insulin injection on hypothalamic mTOR signaling in rats infused with PA to the CeA for 3 days. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of mTOR$^{\text{Ser2448}}$ phosphorylation level and β-Actin as a loading control in rat’s hypothalamus tissue. Data are shown as Mean ± SEM (n = 5). There is no significant difference among all the samples.
Figure 3-10: Effect of high fat diet for 3 days and amygdala insulin on mTOR signaling in rat amygdala. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of mTOR$^{\text{Ser2448}}$ phosphorylation level and β-Actin as a loading control in rat’s amygdala tissue. Data are shown as Mean ± SEM (n = 4). $^{+++}p < 0.001$ compared to LFD-saline injected group. $^{*}p < 0.05$ compared to HFD-saline injected group.
Figure 3-11: Effect of high fat diet for 3 days and amygdala insulin on mTOR signaling in rat hypothalamus. Representative Western blot analysis and corresponding bar graphs demonstrating the quantitative measure of mTOR$^{\text{Ser2448}}$ phosphorylation level and β-Actin as a loading control in rat’s hypothalamus tissue. Data are shown as Mean ± SEM (n = 4). There is no significant difference among all the samples.
CHAPTER 4
LIPID ACTIVATION OF PROTEIN KINASE C-THETA (PKC\(\theta\)) AND ITS ROLE IN INSULIN RESISTANCE IN THE BRAIN

1. Introduction

As described in chapter 1, multiple mechanisms have been proposed for the development of peripheral insulin resistance in response to elevated fatty acids [1-4]. Among the proposed molecular mechanisms, the activation of protein kinase C-theta (PKC\(\theta\)) has been suggested to induce insulin resistance in peripheral tissues [5, 6]. PKC\(\theta\), an isoform of the novel class of PKCs (nPKCs; \(\delta\), \(\varepsilon\), \(\eta\), and \(\theta\)), is a serine/threonine kinase involved in signal transduction pathways that regulate a wide range of physiological processes, including cellular differentiation and proliferation, gene expression, membrane transport, and the organization of cytoskeleton and extracellular matrix protein [7]. It is thought that PKC\(\theta\) is activated when it is translocated from the cytosolic pool to the inner surface of the cell membrane where it interacts with receptors and other proteins to modify cell signaling [6, 8]. Recently, it has been reported that brain PKC\(\theta\) can be activated by lipid infusions [8, 9], a high fat diet [10], or by direct stimulation with saturated fatty acids [11, 12].

Although the molecular mechanism through which saturated fatty acids induce PKC\(\theta\) activation is not understood, it is thought that elevated diacylglycerol (DAG) has an important role in the translocation of PKC\(\theta\) to the membrane and its activation [5, 8, 13]. As described in chapter 1, it appears that the neutral lipid, triacylglycerol is not likely to induce insulin resistance. Instead, the accumulation of lipid metabolites such as diacylglycerol has been reported to induce insulin resistance in peripheral tissues such as
muscle and liver [14]. Increased fatty acids, particularly long chain saturated fatty acids, accelerate the accumulation of diacylglycerol in peripheral tissues especially in skeletal muscle cell [15]. Palmitate but not unsaturated fatty acids (e.g. oleic acid) significantly increased the accumulation of DAG and decreased glycogen synthesis in C2C12 myotubes [15]. The DAG is thought to alter insulin signaling through activation of PKCθ [16].

Although there is conflicting data, several studies have focused on PKCθ and its role in induction of peripheral insulin resistance [17-19]. A study of dominant negative PKCθ in muscle reported that activation of PKCθ is necessary for the maintenance of muscle insulin sensitivity and that without PKCθ activation, there is a significant decrease in the insulin activation of the PI3K/Akt pathway [20]. Also, PKCθ knockout mice become obese and insulin resistant when fed a high fat diet [21]. However, in in vivo studies, fatty acid induced PKCθ activation has been reported to impair insulin stimulated glucose uptake in skeletal muscle cells and adipocytes by decreasing Akt phosphorylation [11, 12, 22]. Also, increased PKCθ expression in C2C12 skeletal muscle cells decreased IRS-1 protein levels and the cells become insulin resistant [23]. Moreover, PKCθ null mice were protected from acute fatty acid induced insulin resistance [24].

The molecular mechanism of insulin resistance induced by activation of PKCθ is not understood clearly, although several possible mechanisms have been proposed. Since PKCθ is a serine/threonine kinase, PKCθ can disrupt insulin signaling via serine or threonine phosphorylation of the insulin receptor [25, 26], insulin receptor substrate-1 (IRS-1) [27, 28] and potentially other proteins such as glycogen synthase [29]. Increased serine phosphorylation inhibits tyrosine phosphorylation and kinase activity of insulin
receptors and IRS-1 resulting in impaired insulin signal transduction [30]. The serine residues in the β-subunit of the insulin receptor seem to be an important target for PKC phosphorylation [31].

Although only a few studies have focused on the mechanisms of insulin resistance in the brain. Benoit and colleagues [6] recently demonstrated that activation of PKCζ is a critical mediator in fatty acid-induced central insulin resistance in the hypothalamus in a similar manner as in peripheral tissues. They showed that PKCζ was expressed in neurons of the arcuate nucleus, particularly in NPY and AgRP coexpressing neurons. High saturated fat diets (HFS) attenuated the anorexic response to third-ventricular (icv) insulin in rats compared to control animals maintained on either a LFD or a high fat oleic acid diet. The attenuated anorectic response in the HFS diet was related to a reduction of insulin stimulated Akt phosphorylation and the activation of PKCζ [6]. Knockdown of PKCζ in the arcuate nucleus with shRNA improved glucose tolerance, decreased body weight gain, and enhanced hypothalamic insulin signaling pathways [6]. These data suggest that activation of PKCζ mediates the deleterious effects of a high saturated fat diet on insulin signaling in the CNS. Similar to its role in the peripheral tissues, icv palmitic acid infusion significantly increased hypothalamic diacylglycerol levels and induced translocation and activation of CNS PKCζ, and insulin resistance [6, 12, 20, 32].

Our laboratory recently showed that the levels of several phosphorylated proteins were dramatically changed in the amygdala and hypothalamus of rats fed a high fat diet (Table 4-1) [33]. The level of pMARCKS (Myristoylated alanine-rich C-kinase substrate), a substrate of PKCζ, was increased 116% in amygdala of high fat diet fed rats but reduced in the hypothalamus. Immunohistochemical staining of brain sections confirmed
an increased PKCθ expression in the amygdala of rats fed a HFD compared to rats fed a LFD [33]. These data are supported by a study [6] that showed that icv palmitic acid increased hypothalamic phosphorylation level of MARCKS. Since Cytosolic myristoylated alanine rich C kinase substrate (MARCKS) is phosphorylated by activation and translocation of many members of the PKC family, including PKCθ [34], these data suggested a possible role of PKCθ and its downstream signaling pathway on brain insulin resistance.

The present chapter investigated the possible role of PKCθ on HFD and saturated fatty acid induced insulin resistance in the hypothalamus and amygdala as well as in neuronal cells in culture.

2. Hypotheses

1) High fat feeding and amygdala saturated fatty acid infusion increase activation and expression of PKCθ in the amygdala and hypothalamus, and this will lead to inhibition of Akt signaling in the amygdala and hypothalamus.

2) Saturated fatty acids will increase activation and expression of PKCθ and inhibit Akt signaling in GT1-7 neuronal cells.

3) Knockdown of PKCθ will prevent the fatty acid inhibition of insulin stimulated Akt signaling in GT1-7 neuronal cells.

4) PKCθ over-expression in the amygdala will inhibit CeA insulin stimulated Akt signaling in the amygdala.

To investigate these hypotheses, the hypothalamic and amygdala tissues from rats used in previous experiments (3 days LFD-HFD and palmitic acid infusion) were analyzed for the PKCθ protein expression level. Also, the effect of PKCθ expression on
insulin stimulated Akt phosphorylation level was investigated in rat’s brain in vivo and in the GT1-7 neuronal cell line.

3. Methods

Animals and diets

Subcellular PKCθ expression experiment (3 days HFD and CeA PA infusion): The animal care and experimental protocols for the 48 Sprague Dawley rats used in previous experiments (3 days LFD-HFD and 3 days CeA PA infusion) were described in chapter 3.

PKCθ lentivirus experiment: Male, Sprague Dawley (SD) rats (Charles River, Wilmington, MA) at 8-10 weeks of age at the start of the studies were used. All rats were initially fed a rat chow diet (11% energy as fat; 3.30 kcal/g; Harlan Teklad 8604, Madison, WI) and were fitted with brain cannulas targeted bilaterally at the central nucleus of the amygdala before adaptation to a low fat/high carbohydrate (LFD; 10% energy as fat 3.85 kcal/g; D12450B; Research Diets, New Brunswick NJ) for at least 10 days before experimentation. After adaptation, rats were maintained in low fat diet (LFD) for 4 weeks. The rats were individually housed in hanging wired mesh cages under a controlled temperature (22–24 °C) and lighting (lights off 1900–0700 h) conditions in a room at the Laboratory Animal Research Center (LARC) at Utah State University. Animals used for lentivirus injection experiments were housed in the Biosafety 2 level facility according to CDC and NIH guidelines. Experimental protocols involving the animals were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee and Biosafety Committee of Utah State University.
**Preparation of lentiviral constructs**

Lentiviral constructs (LV-PKCθ and LV-GFP) were prepared by Dr. MieJung Park-York.

**Cannula implantation and lentiviral injection**

Stereotaxic cannula placement was performed as previously described [35], implanting cannulas aimed at the CeA using the following coordinates (AP/L/DV mm: CeA –2.4/4/5.5; 3rd ventricle -2.4/0/8.0) from bregma according to the atlas of Paxinos and Watson [36]. Rats were fitted with bilateral cannulas to the CeA. In order to keep the injection volume to a minimal to prevent wide diffusion 2.0µl injections over a 4 minutes period (3X10^7 TU/ml) were injected on 3 occasions, each 2 days apart, through an injection cannula that projected 0.5mm (CeA) beyond the guide cannula. This was left in place for a further 4 minutes before careful withdrawal. All animals were monitored for the appearance of any aberrant behaviors or evidence of malaise (e.g. weight loss, anorexia) associated with the carrier vehicle. This experiment was done in collaboration with Dr. Stephane Boghossian who performed all of the cannulations and with Dr. Miejung Park-York who prepared both the LV-PKCθ and LV-GFP constructs and supervised the BSL2 level work.

**Titration of LV-PKCθ**

LV-PKCθ stock was diluted 10 fold in complete culture medium (DMEM plus 10% FBS) and transduced into GT1-7 neuronal cells (at about 30% confluence) and incubated overnight at 37°C in 5% CO2 atmosphere. After approximately 18 hours of incubation, the medium was changed to fresh complete culture medium. Three days post-transduction,
the medium was changed to the fresh complete culture medium containing 2.5μg/ml Blasticidin (Invitrogen, Carlsbad, CA). The media containing antibiotic was changed every 3-4 days. When the Blasticidin resistant colonies formed several colonies were selected from each well and verified by PCR.

**Insulin administration**

The procedures were described in chapter 3 and followed exactly in this chapter.

**PKCθ siRNA transfection in GT1-7 cells**

GT1-7 cells were grown in six well plates and maintained in Dulbecco’s modified eagle’s medium (Hyclone, Logan, UT) containing 10% fetal bovine serum. Small interfering RNA (siRNA) for PKCθ (sc-36247) and control scrambled RNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and siRNA transfection procedures were followed as described in the manufacturer’s instructions. When GT1-7 cells were 60-80% confluent, siRNA duplex solutions [6 μl (60 pmol) of PKCθ or control scrambled siRNA in transfection media] and transfection reagent solution (sc-29528, Santa Cruz Biotechnology Inc.) were mixed for 45 minutes at room temperature. After GT1-7 cells were washed once with 2 ml of siRNA transfection medium (sc-36868), they were incubated with the prepared transfection mixture (siRNA duplex + transfection reagent solution) for 5 hours at 37°C in 5% CO₂ atmosphere. After 5 hours transfection, 2x normal growth media (supplemented with 2 times FBS concentration) was added without removing the transfection mixture and the cells were incubated for an additional 18 hours at 37°C in 5% CO₂ atmosphere. The media was
aspirated and replaced with fresh 1x normal growth medium. After 24 hours of incubation, cells were ready for experimental procedures.

*Cell culture, palmitic acid incubation, and insulin administration in GT1-7 cells*

The procedures of GT1-7 cell culture, palmitic acid incubation and insulin administration were previously described in chapter 2.

*RNA isolation and semi-quantitative PCR*

PCR The procedures for RNA isolation and semi-quantitative PCR were identical to those described in chapter 2 using the following mouse specific primer sets. *Prckq*: Forward, 5’- GGA ATC AGA AAA TGG GCA GA - 3’, Reverse, 5’- ATT GTT CTT GCG GCA TCT CT - 3’; and *Ppib* (Cyclophilin B): Forward, 5’-GCT GGA TGG CAA GCA TGT G-3’, Reverse, 5’-TGT CTT GGT GCT CTC CAC CTT-3’

*Protein extraction*

**Protein extraction**: Total protein extraction protocol for both cell and tissues were described in chapters 2 and 3.

**Membrane and cytosol fractionation**: In chapter 3, each homogenate of rat’s brain tissues (amygdala and hypothalamus) used in two experiments (3 days LFD-HFD and 3 days CeA PA infusion experiment) was divided into two separated lysates: 1) the total protein fraction for the analysis of AktSer473 phosphorylation, 2) the cytosolic and membrane fractions for the analysis of the subcellular PKCθ expression. The method for total protein extraction was described in chapter 3. For preparation of cytosolic and membrane fractions, a modification of the method of Benoit et al. [6] was used. The lysates for cytosolic and membrane fractionation were sonicated and were centrifuged at
700 rpm at 4 °C for 5 minutes and the pellet was discarded. The supernatant was collected in a new 1.5 ml tube and centrifuged at 13,000 rpm for 15 minutes at 4 °C. The resulting supernatant was then collected as the cytosolic fraction. The remaining pellet was solubilized in the above buffer with 2% Triton X-100 for 30 minutes on ice and centrifuged at 25,000 rpm for 20 minutes at 4 °C. The final supernatant was collected as the membrane fraction. Protein concentration was measured for both the cytosolic and plasma membrane fractions.

**Western blot**

The procedures were described in chapter 2 and 3 and followed exactly in this chapter. Additional antibody, rabbit anti- PKCθ (sc-212), was purchased from Santa Cruz biotechnology (Santa Cruz, CA).

### 4. Results

*Effect of dietary fat on PKCθ expression in the amygdala and hypothalamus*

To investigate the effect of HFD on PKCθ protein expression in the amygdala and hypothalamus, protein samples of amygdala and hypothalamus of rat’s fed either LFD or HFD for 3 days were used for Western blot analysis. As shown in figure 4-1, HFD significantly increased total PKCθ expression in the amygdala ($p < 0.05$) compared to amygdala from rats fed LFD for 3 days. Because the PKCθ activation is thought to be associated with membrane translocation, both cytosolic and plasma membrane fractions of amygdala were also assayed for PKCθ expression. Similar to the total fraction, 3 days HFD significantly increased PKCθ expression on both cytosolic and membrane fractions.
as shown in figure 4-1. Similar to the amygdala data, HFD significantly increased PKCθ expression in total, cytosolic and membrane fractions of hypothalamus compared to LFD fed rats (figure 4-2; \( p < 0.001 \), and \( p < 0.05 \), respectively). After analysis of amygdala PKCθ protein expression level, the effect of HFD on \textit{Prkcq} gene expression in the amygdala was also investigated. As shown in figure 4-1, 3 days of HFD significantly increased \textit{Prkcq} mRNA expression in the amygdala compared to LFD fed rats \( (p < 0.05) \).

\textit{Effect of amygdala palmitic acid infusion on PKCθ expression in the amygdala and hypothalamus}

To investigate the direct effect of saturated fatty acids in the amygdala on PKCθ protein expression, amygdala and hypothalamic protein samples of rat’s infused with palmitic acid or artificial-CSF (vehicle) into the CeA were used for Western blot analysis. As shown in figure 4-3, CeA palmitic acid infusion significantly increased PKCθ protein expression in both amygdala total and membrane protein fractions compared to CeA vehicle infused amygdala. In contrast, there was no significant change of PKCθ expression level in the cytosolic fraction (figure 4-3). Figure 4-4 shows the effect of PA infusion into the CeA on PKCθ expression in the hypothalamus. CeA palmitic acid infusion had no effect on PKCθ expression in the hypothalamus or in the distribution of PKCθ between cytosolic and membrane fractions (figure 4-4).

\textit{Effect of over-expression of PKCθ in the central nucleus of the amygdala (CeA)}

A PKCθ containing lenti-viral (LV-PKCθ) construct or Green Fluorescent Protein (GFP) containing lentiviral (LV-GFP) construct (control) was injected bilaterally
stereotaxically into the CeA of rats. Bilaterally injected PKCθ rats gained more weight, had increased food intake and hepatic, but not serum, triglyceride levels and lost the anorectic response to CeA insulin compared to the control rats that received a LV-GFP construct [33]. The amygdala tissues of these rats, injected either with LV-PKCθ construct (bilateral) or LV-GFP construct, were assayed for PKCθ protein expression using Western blot analysis. As shown figure 4-5, bilateral LV-PKCθ injection to the CeA significantly increased PKCθ protein expression level in both total and membrane fractions of the amygdala ($p < 0.05$) compared to LV-GFP injected samples, whereas there was no significant difference of PKCθ expression level on cytosolic fractions of amygdala samples (figure 4-5).

Akt signaling in PKCθ injected rats

Our data have shown that both high fat diet and CeA palmitic acid infusion increased PKCθ activation and inhibited CeA insulin stimulated Akt signaling in amygdala and/or hypothalamus. Furthermore, rats lost the anorectic response to CeA insulin when LV-PKCθ was injected into CeA. These data suggests that the activation of PKCθ has a potential role in the inhibition of insulin signaling, particularly Akt signaling. To test the direct effect of PKCθ and exclude the HFD effect on insulin stimulated Akt signaling in rat’s amygdala and hypothalamus, LV-GFP or LV-PKC were bilaterally injected into CeA and rats were maintained under LFD for four weeks. Then amygdala and hypothalamus tissues were assayed by Western blot analysis. As shown in figure 4-6, CeA insulin injection significantly increased Akt phosphorylation in CeA LV-GFP injected rats, whereas this response was abolished in CeA LV-PKCθ injected rats. This suggests that PKCθ activation has an important role in the alteration of insulin stimulated
Akt signaling in the rat’s amygdala. CeA insulin injection also significantly increased hypothalamic Akt signaling in CeA LV-PKCθ injected rats. However, in contrast to the amygdala data, CeA LV-PKCθ injection had no effect on the inhibition of hypothalamic Akt signaling (figure 4-7), again suggesting an Akt-independent pathway from the amygdala to hypothalamus.

**Effect of over-expression of PKCθ on insulin signaling
in GT1-7 neuronal cells in response to palmitic acid**

To investigate the effect of saturated fatty acids on PKCθ protein expression on neuronal cells, GT1-7 hypothalamic neuronal cells were incubated with palmitic acid and PKCθ protein expression level was assayed using Western blot analysis. As shown in figure 4-8, 12 hours of palmitic acid incubation (5 μM and 25 μM) significantly increased PKCθ expression compared to control samples ($p < 0.01$ and $p < 0.05$ respectively). This 12 hour time point was used in previous experiments (chapter 2) to show PA inhibition of insulin signaling.

Using GT1-7 cells over-expressing PKCθ, the role of PKCθ on insulin signaling was further investigated. Insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation level in the wild type GT1-7 cells and in PKCθ over-expressed GT1-7 cells was compared in the presence and absence of a low concentration of palmitic acid (5μM) (figure 4-9). Palmitic acid (5μM) did not reduce insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation significantly in wild type cells, whereas in PKCθ over-expressed GT1-7 cells, palmitic acid induced a greater and statistically significant depression of insulin stimulated Akt$^{\text{Ser473}}$ phosphorylation. The palmitic acid attenuation of insulin signaling was significantly higher in PKCθ over-expressed GT1-7 cells compared to non-transduced control GT1-7 cells.
Effect of PKCθ knock down on insulin signaling
in GT1-7 cell in response to palmitic acid

Since PKCθ over-expression enhanced the palmitic acid inhibition of insulin stimulated Akt^{Ser473} phosphorylation, the effect of PKCθ knock down on insulin signaling in GT1-7 cells was also investigated. As shown in figure 4-10, scrambled siRNA transfected GT1-7 cells responded to palmitic acid (in a dose-dependent manner) to significantly reduce insulin stimulated Akt^{Ser473} phosphorylation. In contrast, neither dose of palmitic acid (5μM and 25μM) depressed insulin stimulated Akt^{Ser473} phosphorylation in PKCθ siRNA transfected GT1-7 cells. Palmitic acid mediated insulin resistance was not detected in PKCθ siRNA transfected GT1-7 cells. To confirm the PKCθ knock down in LV-PKCθ transfected GT1-7 cells, the mRNA and protein expression of PKCθ was assayed in transfected GT1-7 cells. As shown in figure 4-11, both PKCθ mRNA expression (figure 4-11 A and B) and protein expression (figure 4-11 C and D) were reduced in PKCθ siRNA transfected GT1-7 cells compared to scrambled siRNA transfected GT1-7 cells.

5. Discussion

Recently, our laboratory data demonstrated that insulin has an anorectic effect in the CeA and that this effect is rapidly lost after rats are placed on a HFD [35]. Studies in the previous chapters suggest that both HF feeding and an elevation of saturated fatty acid level inhibited insulin-signaling in the amygdala consistent with the loss of insulin’s anorectic effect. Although little attention has been focused on the mechanisms of insulin resistance in the brain, recently it has been reported that a high saturated fat (HSF) diet activates PKCθ and that this might be a possible mediator to inhibit insulin signaling in
the brain. This is supported by the data presented in this chapter showing that both a HFD and elevated saturated fatty acid levels induce PKCθ expression and activation in the amygdala and that this might be a possible mechanism responsible for the alteration of insulin signaling in the amygdala.

A high saturated fat (HSF) diet and elevation of saturated fatty acid level induce the accumulation of lipid metabolites such as long chain fatty acyl-CoA and diacylglycerol (DAG), and these metabolites may be responsible for the induction of insulin resistance in both peripheral tissues [9] and the hypothalamus [37]. Increased DAG levels have been associated with translocation of PKCθ into plasma membrane and its activation. Membrane bound PKCθ has been proposed to prevent insulin signaling by increasing serine phosphorylation of insulin receptors and IRS [5, 8, 13].

Benoit and colleagues [6] reported that PKCθ is expressed on NPY/AgRP neurons in the arcuate nucleus of hypothalamus and that icv infusion of palmitic acid for 3 days, but not oleic acid, induced translocation and activation of PKCθ into the membrane fraction and increased levels of pMARCKS. According to our laboratory data, the level of PKCθ substrate, pMARCKS, was increased in the amygdala of rats fed 3 days of HFD [33]. Furthermore, immunohistochemical analysis demonstrated that 10 days of HFD feeding increased PKCθ expression on both amygdala and hypothalamus. Consistent with the immunohistochemical data, Western blot data demonstrated that PKCθ protein expression levels in the amygdala and hypothalamus of HFD fed rats were significantly increased compared to these in low fat diet fed rats. To confirm that HFD induces translocation of PKCθ to membrane in both the amygdala and hypothalamus, protein samples were separated to cytosolic and membrane fractions using
ultracentrifugation. Both cytosolic and membrane fractions had significantly higher PKCθ expression in both amygdala and hypothalamus of HFD fed rats compared to LFD fed rats. According to the study of Benoit at al., icv palmitic acid infusion significantly decreased cytosolic PKCθ levels but increased membrane PKCθ levels [6]. However, in our data, hypothalamic PKCθ concentration was increased on both cytosolic and membrane fractions. The difference in our data from that of Benoit’s study might be due to the very different experiment design; the physiological effect of fatty acid infusion directly into local brain area is very different to the effect of fatty acid delivery from feeding a high fat diet. Fatty acid infusion directly into a specific region of brain may affect only the local response to fatty acids, whereas, delivery of fatty acids from a high fat diet feeding may reflect not only the effects of the diet but also any induced metabolic or endocrine changes associated with the diet. [33].

The effects of PA infusion into the CeA on PKCθ expression was investigated in this chapter. CeA palmitic acid infusion significantly increased PKCθ protein expression in the amygdala and this increased amount was localized to the membrane fraction compared to only vehicle infused animals. Our data suggests that saturated fatty acid appear to induce the membrane translocation and activation of PKCθ in central nucleus of amygdala similar to its effect on hypothalamic PKCθ activation [6]. Thus PKCθ expression has a similar effect on amygdala insulin signaling as it has on hypothalamic insulin signaling.

However, our data confirm that the effects of HFD are somewhat different from PA infusions. Although both lead to activation of PKCθ, HFDs appear to have a greater effect as cytosolic PKCθ levels were also increased. This may reflect the response to a
larger stimulus, the response of the whole amygdala to HFD rather than the localized CeA response to PA infusion or it could suggest that signals other than PA affect the response to dietary fat.

Palmitic acid infused into the central nucleus of the amygdala locally had no effect on the expression of PKCθ in the hypothalamus. The different PKCθ expression patterns in hypothalamus between HFD and palmitic acid infusion experiment might be due to the different physiological effect of direct infusion of fatty acid compared to its effect on high fat diet feeding as described previously.

PKCθ is expressed in several neuronal populations of the arcuate nucleus, particularly in the NPY and AgRP coexpressing neurons. HFD and elevated saturated fatty acid induced activation of PKCθ on NPY/AgRP neurons might alter the response to insulin by preventing insulin signaling transduction and this, in turn, inhibits downstream signaling transduction to regulated food intake and energy homeostasis. Although the amygdala increases PKCθ levels in response to a HFD and to elevated saturated fatty acid levels, it is not clear which populations of neurons express PKCθ in the amygdala. Since NPY expressing neurons are widely expressed in the limbic system [38] such as central nucleus of amygdala [39, 40], it is possible that PKCθ is expressed in NPY neurons and mediates insulin signaling in response to HFD and elevated saturated fatty acids as in the hypothalamus.

Recently our laboratory investigated the effect of PKCθ over-expression in the amygdala using bilateral injections of LV- PKCθ construct into the central nucleus of amygdala. Bilateral LV-PKCθ injected rat expressed increased body weight and food intake. PKCθ expressing rats lost insulin’s anorectic effect compared to the control rats
indicating the presence of insulin resistance when PKCθ is expressed in the CeA [33]. Western blot analysis showed that the amygdala of PKCθ over-expressed rats had significantly higher PKCθ expression in the amygdala and this was localized on the membrane fractions (figure 4-5). PKCθ over-expressing rats had significantly decreased CeA insulin stimulated Akt signaling in the amygdala (figure 4-6). These data further support the possibility that PKCθ has a similar role in vivo in the amygdala as in hypothalamus and also suggest that HFD or saturated fatty acid induced changes in PKCθ activation in the amygdala may regulate peripheral metabolism.

In chapter 2, the effects of palmitic acid to impair insulin signaling in GT1-7 hypothalamic neuronal cells was reported. To investigate whether palmitic acid induced expression and activation of PKCθ is associated with the alteration of insulin signaling, PKCθ protein expression in GT1-7 cells was analyzed. Palmitic acid application significantly increased PKCθ expression in GT1-7 cells compared to control samples. This data are consistent with the in vivo effects of icv palmitic acid infusion reported in this chapter and with the previous data presented by Benoit and colleagues [6].

Additional support for the effect of PKCθ to inhibit insulin signaling was provided by the experiments reported in this chapter. PKCθ over-expression in GT1-7 cells enhanced the PA inhibition of insulin stimulated phosphorylation of Akt<sup>Ser473</sup> to a significantly higher levels compared to non PKCθ over-expressed GT1-7 cells. Furthermore, knock down of PKCθ in GT1-7 cells to reduce both PKCθ gene and protein expression abolished the palmitic acid inhibitory effect on insulin induced Akt phosphorylation compared to GT1-7 cell transduced with scramble siRNA. Taken
together, these data suggest that PKCθ mediates the inhibitory effect of saturated fatty acid on insulin signaling pathways in neuronal cells.

In summary, the hypothalamus, particularly the arcuate-PVN axis, has been the focus for several years for the control of food intake and peripheral metabolism. Our recent study has shown that insulin also has an anorectic effect in the CeA and that this is rapidly lost after rats are placed on a HFD [35]. The present chapter demonstrated that either HFD or saturated fatty acid infusion also induced the activation of PKCθ expression in the amygdala as well as in hypothalamus. Similar to the effect of PKCθ expression in the hypothalamus, HFD induced loss of insulin’s anorectic effect in the CeA might be due to increased PKCθ expression in metabolic sensing neurons. Activation of PKCθ in these metabolic neurons might impair insulin actions and its downstream signaling for the regulation of food intake and energy homeostasis. However, the neuronal phenotype of cells expressing PKCθ in the amygdala is not known at this time, although, NPY/AgRP coexpressing neurons might be possible candidates since PKCθ was highly expressed on arcuate nucleus in the hypothalamus. Further studies are required to identify the localization of PKCθ in the amygdala.

6. References


% change from Low fat diet control rats

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<th>Signal</th>
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<th>Hypothalamus</th>
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<td>-53</td>
</tr>
<tr>
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<td>-12</td>
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</tr>
<tr>
<td>Akt1S1</td>
<td>-28</td>
<td>+12</td>
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Table 4-1: Effect of 3 days high fat diet on level of phosphorylated proteins in the amygdala and hypothalamus of rats. Values represent Mean increase of duplicate assays. MARCKS myristoylated alanine-rich C kinase substrate; Dok2 Docking protein 2; FKHR1 Forkhead [drosophila] homolog [Rhabdomyosarcoma] like 1; HSP 27 Heat shock protein 27; Akt1S1 proline-rich AKT1 substrate 1. Data from Park et al [33].
Figure 4-1: Effect of 3 days high fat diet (HFD) on PKCθ protein and gene expression in rat’s amygdala. A: Representative Western blot analysis for total, cytosolic and membrane fractions of amygdala of rats fed either low fat diet (LFD) or a HFD for 3 days. B and C: Values in the bar graphs represent Means ± SEMs for 5 rats in each group. *p < 0.05, ***p < 0.001 compared to LFD of each fractions.
Figure 4-2: Effect of 3 days HFD on PKCθ expression in rat’s hypothalamus. A: Representative Western blot analysis for total, cytosolic and membrane fractions of hypothalamus of rats fed either LFD or a HFD for 3 days. B: Values in the bar graphs represent Means ± SEMs for 5 rats in each group. *p < 0.05, ***p < 0.001 compared to LFD of each fractions.
Figure 4-3: Effect of palmitic acid infusion into the CeA for 3 days on PKCθ expression in amygdala. A: Representative Western blot analysis for total, cytosolic and membrane fractions of amygdala of rats infused either vehicle or a palmitic acid for 3 days. B: Values in the bar graphs represent Means ± SEMs for 5 rats in each group. * $p < 0.05$, *** $p < 0.001$ compared to vehicle of each fractions.
Figure 4-4: Effect of palmitic acid infusion into the CeA for 3 days on PKCθ expression in hypothalamus. A: Representative Western blot analysis for total, cytosolic and membrane fractions of hypothalamus of rats infused either vehicle or a palmitic acid for 3 days. B: Values in the bar graphs represent Means ± SEMs for 5 rats in each group. There were no significant differences between any groups.
Figure 4-5: Increased expression of PKCθ in the amygdala of rats injected with a lentiviral-PKCθ construct into the CeA. A: Representative Western blot analysis of PKCθ in total, cytosolic and membrane fractions of amygdala of rats injected with either LV-GFP or LV-PKCθ. B: Bar graph demonstrates the quantitative measure of PKCθ expression. Data are shown with Mean ± SEM for six rats in each group. *p < 0.05 compared to LV-GFP samples in each fraction.
Figure 4-6: Effect of CeA PKCθ expression on insulin stimulated Akt signaling in rat’s amygdala. Representative Western blot analysis of phosphorylation of Akt in amygdala of rats injected with either LV-GFP or LV-PKCθ in response to CeA insulin. Bar graph demonstrates the quantitative measure of Akt phosphorylation level. Data are shown with Mean ± SEM for six rats in each group. **p < 0.005 compared to saline injected groups
Figure 4-7: Effect of CeA PKCθ expression on insulin stimulated Akt signaling in rat’s hypothalamus. Representative Western blot analysis of phosphorylation of Akt in hypothalamus of rats injected with either CeA LV-GFP or LV-PKCθ in response to CeA insulin. Bar graph demonstrates the quantitative measure of Akt phosphorylation level. Data are shown with Mean ± SEM for six rats in each group. *p < 0.05 compared to saline injected groups.
Figure 4-8: Effect of palmitic acid (PA) on PKCθ protein expression in GT1-7 neuronal cells. Representative Western blot of PKCθ and β-actin expression (loading control) and corresponding bar graph demonstrating the quantitative measure of PKCθ protein expression level and β-Actin as a loading control. Data are shown in Mean ± SEM (N = 2). *p < 0.05, **p < 0.01 compared to control samples.
Figure 4-9: Effect of palmitic acid (PA) on insulin stimulated Akt2\textsuperscript{Ser473} phosphorylation in control GT1-7 and the PKCθ transfected GT1-7 neuronal cells. (A), (B) representative Western blot (top band indicates pAkt2) and corresponding bar graph (C) demonstrating the quantitative measure of Akt2\textsuperscript{Ser473} phosphorylation level. Data are shown as Mean ± SEM (N = 2) * p < 0.05 compared to only insulin 50nM treated sample in control GT1-7 cells, † p < 0.05 compared to only insulin 50nM treated sample in PKCθ transfected GT1-7 cells.
Figure 4-10: Effect of palmitic acid (PA) on insulin stimulated Akt2<sup>Ser473</sup> phosphorylation in GT1-7 neuronal cells transfected with either scrambled- or PKC0-siRNA. (A), (B) representative Western blot analyses (top band indicates pAkt2) and corresponding bar graphs (C), (D) demonstrating the quantitative measure of Akt2<sup>Ser473</sup> phosphorylation level. Data are shown as Mean ± SEM (N = 2). *p < 0.05, **p < 0.01, ***p < 0.001 compared to only insulin 50nM treated samples.
Figure 4-11: mRNA and protein expression of PKCθ in GT1-7 cells transfected with either scrambled- or PKCθ siRNA. (A): Representative gel image of semi-quantitative PCR data and (B): Corresponding bar graph demonstrating the quantitative measure of Prkcq mRNA expression from one experiment (no statistics available). (C): Representative Western blot analysis and (D): Corresponding bar graph demonstrating the quantitative measure of PKCθ expression with β-Actin as a loading control. Data are shown as Mean ± SEM (N = 2). \( ^+ p < 0.05 \) compared to no siRNA transfected sample, \( ^* p < 0.05 \) compared to scrambled siRNA transfected sample.
CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

The central nervous system controls food intake and energy expenditure to maintain suitable body weight over time [1]. Particularly, the arcuate nucleus of hypothalamus has been well described as one of the brain areas important in the regulation of energy homeostasis. The current model of energy homeostasis proposes that adiposity signals, insulin and leptin, which circulate proportional to the level of fat storage and energy balance, regulate neuronal circuits in the central nervous system, particularly in the arcuate nucleus PVN region of the hypothalamus, to activate anabolic pathways and inhibit catabolic pathways to reduce food intake and increase energy expenditure (figure 1-3) [1, 2]. The hypothalamus contains metabolic sensing neurons including orexigenic NYP/AgRP and anorexigenic POMC/CART neurons which contain receptors for both insulin and leptin and produce neuropeptides that control activities of downstream neuronal populations to regulate energy homeostasis [1, 2].

The prevalence of obesity, which is considered as a disease has been increasing uncontrollably over the last two decades. Obesity is a state of dis-regulated energy homeostasis characterized by hypothalamic resistance to adiposity signals (insulin and leptin). While many factors are involved in the development of obesity, dietary fat has been proposed as one of the main causal factors that results in disrupted energy homeostasis by inducing both leptin and insulin resistance in the central nervous system.

There has been relatively little investigation on insulin’s effects in the brain, since brain was considered to be insulin independent for a long time. Presently, brain insulin is now recognized to have important functions in the regulation of feeding behavior, energy
expenditure and peripheral metabolism to maintain energy homeostasis. Although the underlying mechanisms are not fully understood, numerous studies suggested that the increased level of free fatty acids that results from eating a high fat diet might be the most critical factor for the development of insulin resistance in both peripheral tissues and the brain. Particularly, an elevated intracellular saturated fatty acid level has been suggested as the most potent lipid factor to induce insulin resistance compared to mono-, and poly-unsaturated fatty acids. In addition, independent of excess nutrient intake, a diet consisting of high levels of saturated fat is sufficient to induce hypothalamic resistance to the negative feedback adiposity signals and cause the level of adiposity to increase.

Numerous investigations have been focused on saturated fatty acid induced insulin resistance in insulin sensitive peripheral tissues and from these several possible mechanisms have been suggested, including mitochondrial dysfunction, ER stress, inflammation, ceramides and activation of PKC-θ. Recently, these proposed mechanisms for peripheral insulin resistance have been implicated for the brain as well. Saturated fatty acid induced hypothalamic insulin resistance is correlated with PKCθ activation in the hypothalamus [3]. Also, both an elevated inflammatory response and ER stress in the central nervous system have been reported in response to high fat feeding and intracerebroventricularly administered saturated fatty acids [4]. These data, from other investigators, suggest that saturated fatty acids induce insulin resistance in the brain through similar mechanisms as it does in the peripheral tissues.

In chapter 2, I discussed the following hypotheses: 1) neuronal cells are also insulin sensitive and insulin activates similar insulin signaling pathways as it does in classic insulin sensitive peripheral cells, and 2) among different kinds of fatty acids, only
saturated fatty acids, specifically palmitic acid, impair insulin signaling pathways in neuronal cells. I presented data to substantiate these hypotheses. *In vitro* experiments using mouse hypothalamic cell lines (GT1-7) demonstrated that insulin activates the PI3K signaling pathway (Akt phosphorylation) and enhanced glucose uptake rate in hypothalamic neuronal cells in a similar to insulin sensitive peripheral cells such as muscle cells and adipocytes. This suggests that insulin also plays a role in the regulation of metabolism in the central nervous system as it does in peripheral tissues. I also examined the effect of fatty acid administration on insulin signaling in GT1-7 neuronal cells. Only the saturated fatty acid, palmitic acid, but not mono- or poly-unsaturated fatty acid significantly impaired insulin stimulated Akt signaling and glucose uptake in GT1-7 neuronal cells suggesting that elevated saturated fatty acids may be a major factor for the hypothalamic insulin resistance as in peripheral cells.

While the majority of studies on fatty acids were performed in neuronal cell lines, I confirmed the inhibition of insulin signaling by palmitic acid in amygdala primary cells. In addition, insulin stimulated Akt phosphorylation in the amygdala primary cells was significantly increased in response to poly-unsaturated fatty acid (PUFA) administration compared to both control and mono-unsaturated fatty acid administrated samples. Amygdala primary cells express GPR120 and PUFA administration increased intracellular calcium mobilization significantly. Although my data is not enough to explain possible mechanisms, as a whole these data suggest that PUFAs may act as signaling molecules activating GPR120 and G_{αq}-PLC pathways, which in turn, possibly potentiate insulin signaling via crosstalk between GPCR signaling and insulin signaling pathways. Since many studies have demonstrated the positive effects of poly-unsaturated
fatty acids (e.g. DHA) on insulin sensitivity, it would be helpful to investigate this further to understand more fully the molecular mechanisms through which PUFAs potentiated insulin signaling. However, according to the calcium imaging data, palmitic acid also significantly increased calcium mobilization in both amygdala and hypothalamic neuronal cells. This suggests the possibility of palmitic acid induced GPCR activation just like the effect of linolenic acid. However, in contrast to linolenic acid effect, palmitic acid inhibited insulin stimulated Akt signaling in GT1-7 and amygdala primary neurons significantly. While both fatty acids rapidly increased fatty acid induced calcium mobilization in neuronal cells, the effect on Akt signaling was contrasting. One suggestive explanation might be that palmitic acid was only applied for a very short time period (3~10 minutes) in calcium imaging experiment whereas palmitic acid was applied for longer time period (12 hours) for analysis of Akt signaling. It is possible that the calcium signaling might be quite different between short term and long term palmitic acid application.

Recently, our lab discovered that insulin has an anorectic effect when it is applied into central nucleus of amygdala (CeA) and this response was similar to its effect when it is intracerebroventricularly (icv) administered into the hypothalamus [4, 5]. Our lab also demonstrated that rats that were fed high fat diet lost the anorectic effect of CeA insulin and became insulin resistant [6]. The amygdala expresses insulin mRNA and insulin receptors [7]. These data suggest that amygdala, a brain region that controls emotions, mainly fear and anxiety, also plays important roles in controlling food intake and energy expenditure similar to the hypothalamus [8, 9]. The data obtained in these cell culture experiments provides confirmation that saturated fatty acids will indeed cause insulin
Further investigations have examined the effect of high fat diet and elevated saturated fatty acids on alteration of insulin signaling in vivo in the amygdala and hypothalamus using Sprague-Dawley (SD) rats. In the first animal experiment described in chapter 3, insulin injection into the central nucleus of the amygdala (CeA) significantly increased Akt phosphorylation both in amygdala and hypothalamus or rats fed 3 days of LFD, whereas 3 days of HFD significantly blunted CeA insulin stimulated Akt phosphorylation in both amygdala and hypothalamus. To determine if the deleterious effects of high fat diets on insulin signaling is a specific response to direct actions of saturated fatty acid within the CNS, the second animal experiment investigated the effect of CeA palmitic acid infusion on insulin signaling in both amygdala and hypothalamus. Three days of palmitic acid infusion into the CeA also significantly inhibited CeA insulin stimulated amygdala Akt phosphorylation but not hypothalamic Akt phosphorylation compared to 3 days vehicle infusion. These data confirm that: 1) insulin also activates signaling pathways in the amygdala similar to both the hypothalamus and peripheral tissues, 2) both short term HFD and saturated fatty acid infusions into the amygdala impair amygdala insulin signaling. This is analogous to other studies that focused on HFD and saturated fatty acid effects on hypothalamus and peripheral tissues. [4, 6, 10, 11] The data also suggests that there are insulin responsive neuronal connections between the amygdala and hypothalamus since CeA insulin injection activated not only amygdala Akt phosphorylation, but it also activated hypothalamic Akt phosphorylation. The data also illustrates that HFD systemically affects both amygdala and hypothalamic insulin resistance in amygdala neurons to substantiate the responses observed in the feeding behavioral studies.
signaling simultaneously, whereas local CeA palmitic acid infusion only alters signaling in the amygdala area locally but not the hypothalamus since CeA insulin stimulated Akt phosphorylation in the hypothalamus was impaired only by HFD but not with CeA palmitic acid infusions. Finally, the data strongly suggest that there might be an alternative insulin signaling pathway in the amygdala that activates mTOR signaling. Even though CeA insulin stimulated Akt phosphorylation in the amygdala was blunted with local CeA palmitic acid infusion, hypothalamic Akt phosphorylation was still responsive to CeA insulin injection. Thus insulin in the CeA must activate neuronal connections to the hypothalamus through an alternative pathway to Akt signaling.

One possible alternative pathway that may underlie differential response of hypothalamic insulin signaling to amygdala palmitic acid infusion might be insulin stimulated mTOR signaling in the amygdala. As described in chapter 3, hypothalamic mTOR signaling has been reported to play an important role to regulate energy homeostasis in response to nutrient as well as hormonal signals such as leptin and insulin [12]. Since the mTOR expression and activity has been detected in various regions of the brain including the hippocampus, thalamus and cortex [13], amygdala mTOR signaling may also have a similar function in the regulation of food intake and/or energy balance. However, mTOR is normally regarded as a downstream effector of Akt signaling. Our data suggests that there must be a non-Akt signaling pathway to activate mTOR. This was investigated in the third experiment in chapter 3 in which the effect of both HFD and amygdala palmitic acid on mTOR signaling in both hypothalamus and amygdala was studied using tissue samples from previous animal and cell culture experiments. These studies confirmed that: 1) insulin significantly increased amygdala mTOR signaling, 2)
insulin stimulated amygdala mTOR signaling was independent of both HFD and palmitic acid effects, 3) hypothalamic mTOR signaling was not affected by insulin stimulated amygdala mTOR signaling and suggest that insulin activation of mTOR signaling in the amygdala may activate a pathway that regulates Akt signaling in the hypothalamus. This suggestion would explain the observed responses of hypothalamic Akt signaling to CeA insulin when insulin stimulation of the signaling in the CeA was inhibited by palmitic acid infusions. Applying the Akt inhibitor, triciribine, into GT1-7 neuronal cells, I also confirmed in chapter 2 that insulin stimulated mTOR signaling in neuronal cells is independent of Akt signaling.

In chapters 2 and 3, the experiments demonstrated that high fat diet impaired insulin stimulated Akt signaling in both hypothalamus and amygdala and suggest that this effect could be mediated through an increase in FA levels. In Chapter 4, I investigated a possible signaling mechanism that could explain how HFD and elevated saturated fatty acid levels impair insulin signaling in the amygdala and hypothalamus. According to a recent study, both HFD and saturated fatty acid can activate protein kinase C theta (PKCθ) in the hypothalamic area and this has been suggested as a critical factor to impair insulin signaling through the activation of serine/threonine kinases such as insulin receptor substrates (IRS). Furthermore, recent data from our lab using immunohistochemical approaches have shown that HFD increased PKCθ levels in both hypothalamus and amygdala areas. This suggests that HFD has a similar role in the activation of PKCθ in the amygdala and its effect on the insulin signaling as in the hypothalamus. I hypothesized that the high fat diet elevation of saturated fatty acid impairs insulin signaling in both amygdala and hypothalamus through the activation of PKCθ. Using
Western blot analysis, I demonstrated that HFD significantly increased PKCθ protein expression in both amygdala and hypothalamus, and that CeA palmitic acid infusion also significantly increased PKCθ protein expression in the amygdala. The increased levels reflected increased membrane localization of the enzyme which is associated with activation. In chapter 3, both HFD and CeA palmitic acid infusion impaired insulin stimulated Akt phosphorylation in the amygdala. I further investigated the effect of PKCθ activation on insulin signaling using GT1-7 mouse hypothalamic cell lines and confirmed the role of PKCθ in fatty acid induced insulin resistance. Insulin stimulated Akt phosphorylation was not impaired by palmitic acid when PKCθ siRNA transfection induced PKCθ knock down in GT1-7 cells in comparison to both control siRNA transfected GT1-7 and wild type GT1-7 cell. Conversely, PKCθ overexpression potentiated the palmitic acid inhibition of Akt signaling in response to insulin.

In conclusion, the data presented in this thesis provide a mechanistic explanation for the observations from our lab that the insulin anorectic effect in the amygdala was lost within 3 days of feeding a high fat diet. It suggests that the anorectic response is mediated through Akt signaling rather than mTOR signaling. This dissertation describes: 1) mechanisms of insulin signaling in both amygdala and hypothalamus and 2) potential mechanisms involved in the development of saturated fatty acid induced insulin resistance in amygdala and hypothalamus. Figure 5-1 summarizes the effects of HFD and elevated intracellular saturated fatty acids on cell signal transduction in metabolic sensing neurons such as NPY/AgRP and POMC in the amygdala and its consequences in a whole animal. HFD induced intracellular saturated fatty acid elevation increases membrane associated DAG concentration which then recruits cytosolic PKCθ into the
membrane region where it is activated by binding with DAG. Serine/threonine kinase activity of PKCθ has been reported to inhibit tyrosine phosphorylation of both insulin receptors and insulin receptor substrates. This inhibits downstream insulin signaling molecules such as PI3K and Akt and blocks further downstream insulin signaling transduction. Based on our observations, amygdala Akt signaling appears to activate hypothalamic Akt signaling through neuronal connections in response to CeA insulin stimulation. However, 3 days of HFD feeding inhibited amygdala Akt signaling as well as hypothalamic Akt signaling. Hypothalamic Akt signaling particularly in arcuate nucleus in the hypothalamus is important in the regulation and the secretion of several neuro-peptides including POMC and AgRP/NPY. Activation of insulin stimulated Akt signaling inhibits the NPY/AgRP anabolic pathway and stimulates the POMC catabolic pathways. These neuro-peptides regulate the activity of secondary neurons located on PVN and LHA/PFA regions which are important areas in the regulation of energy homeostasis and peripheral metabolisms. We also found that amygdala insulin activates amygdala mTOR signaling and it appears to activate hypothalamic Akt signaling as well as amygdala Akt signaling. Also, CeA insulin stimulated amygdala mTOR signaling was independent to both Akt signaling and elevated saturated fatty acids.

However this dissertation also presents a number of very novel observations that deserve further study 1) how is mTOR signaling regulated independent of Akt signaling, 2) what is the insulin-sensitive neuronal pathway from the amygdala to the hypothalamus and which neuronal cell types in the hypothalamus are activated and/or inhibited by amygdala insulin and 3) what is the physiological role of insulin signaling in the amygdala in the control of food intake and peripheral metabolism. Finally, if we could
identify which neuronal cell types in the amygdala are responsive to insulin this would enable us to better target these systems for future studies and to evaluate their potential for future therapeutic treatment of metabolic disorders such as obesity, insulin resistance and type 2 diabetes.

References


Figure 5-1: Model shows the mechanism and effect of both high fat diet and elevated intracellular saturated fatty acid on insulin signaling of the amygdala neurons as well as hypothalamus and its consequence on regulation of energy homeostasis and peripheral metabolism in a whole animal. SFA saturated fatty acid, DAG Diacylglycerol, SLCFACoA saturated long chain fatty acyl Coenzyme A.