The Role of Neuropeptide Y Y1R in Skeletal Muscle Lipid Metabolism

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Abstract

The Hulver laboratory has recently found that the neuropeptide Y Y1 receptor (NPY Y1R) mRNA expression is elevated in skeletal muscle of obese humans (Hulver, unpublished). The goal of this research is to study the role of the NPY Y1R in skeletal muscle lipid metabolism.

Rat L6, mouse C2C12, and human primary myotubes were incubated in ^{14}C palmitate labeled fatty acid oxidation medium containing 80ng/mL, 250ng/mL, and 500ng/mL of NPY and for a three hour period. Experiments were repeated with the addition of 17mg/mL diprotin A to each NPY treatment. Fatty acid oxidation (FAO) and the percentage of lipids stored within the myotubes as diacylglyceride (DAG) and triaclyglyceride (TAG) were measured. Analyses were repeated in rat L6 and mouse C2C12 following a three hour incubation in ^{14}C palmitate labeled fatty acid oxidation medium containing 1µg/mL, 10µg/mL, and 50µg/mL of the NPY Y1R ligand, [Leu31, Pro34] neuropeptide Y (Bachem, Torrance, CA).

Incubation of human primary myotubes in NPY treatments with the addition of diprotin A significantly increased TAG accumulation ($p < 0.05$). Mouse C2C12 mytoube incubation in 500ng/mL NPY with diprotin A increased FAO (p 0.05). All other NPY and NPY Y1R ligand treatments in had no significant effect on FAO or the accumulation of TAG and DAG.

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List of Important Definitions:

1. **NPY- Neuropeptide Y**
2. **NPY Y1R- Neuropeptide Y Y1 Receptor**
3. **DAG- Diacylglycerol**
4. **TAG- Triacylglycerol**
5. **PKC- Protein Kinase C**
6. **FAO- Fatty Acid Oxidation**
7. **CPT1- Carnitine palmitoyl transferase-1**
8. **GLUT4- Glucose Transporter 4**
9. **BMI- Body Mass Index**

Chapter 1: Introduction

Introduction

Obesity is a national issue of growing concern. Currently, 33 percent of American adults have a body mass index greater than 30 kg/m^2 (Ogden et al., 2007). The rising prevalence of both overweight and obesity results in increased national mortality rates and health care costs (Ogden et al., 2007). Increased body mass index is associated with increased risk of cardiovascular disease, some forms of cancer, hypertension, and type II diabetes mellitus (Ogden et al., 2007; Groop, 2000, Pedrazzini et al., 2003).

Of the co-morbidities associated with the metabolic syndrome, insulin resistance is most commonly observed (Eckel et al., 2005). Insulin resistance in models of obesity is believed to be mediated, in part, by abnormal insulin signaling in skeletal muscle, which has been attributed to excess intracellular lipid accumulation. Increased intramyocellular triacylglycerol (TAG) accumulation with obesity is indicative of dysregulated fatty acid metabolism in skeletal muscle (Goodpaster et al., 2000; Hulver et al., 2003; Kelley et al., 1999; Loon, 2004). Skeletal muscle of obese humans is characterized by reduced fatty acid oxidation and increased intracellular lipid accumulation. To date, however; the mechanism(s) responsible these abnormalities are not clear.

The Hulver laboratory has recently observed that a receptor for Neuropeptide Y, the Y1 receptor (NPYY1R), is abundantly expressed in skeletal muscle of obese humans relative to non-obese humans (Hulver, unpublished). Additionally, expression levels of NPYY1R in skeletal muscle are positively related to BMI (r^2=0.56) and increased intramyocellular TAG (r^2= 0.74) (Hulver, unpublished). Neuropeptide Y is a highly conserved peptide that is a member of the gastric peptide family that includes peptide YY and pancreatic peptide. It has a significant role in a variety of biological functions, including locomotion, body temperature regulation, cardiovascular functions, and hormone secretion (Pedrazzini et al., 2003). The peptide is best known for its potent orexigenic effect when injected into the brain (Pedrazzini et al., 2003; Arora & Anubhuti, 2006; Kanatani et al., 2000; Serradeli-Le Gal et al., 2000).

In addition to regulating feeding behavior, there is also evidence that NPY regulates lipid metabolism. The NPY Y1 receptor is highly expressed in adipose tissue.

When activated, lipolysis is inhibited and leptin production increased, leading to increased brown and white fat pad weight (Serradeli-Le Gal et al., 2000).

The role of NPY in the regulation of fatty acid metabolism in skeletal muscle is unknown. Based on preliminary evidence in human skeletal muscle, it is hypothesized that agonism of the NPY Y1R alters fatty acid metabolism in skeletal muscle in a manner that favors increased lipid accumulation. The goal of this study is to study the role of NPY Y1R in skeletal muscle lipid oxidation and accumulation.

Statement of the Problem

There is a well defined correlation between excessive accumulation of skeletal muscle DAG and the development of insulin resistance (Bell et al., 2000; Hulver & Dohm, 2004). Studies have shown that obese skeletal muscle has a reduced capacity to oxidize lipids resulting in higher accumulation of DAG in obese skeletal muscle (Houmard, 2008; Hulver et al., 2005). However; the cellular mechanisms that regulate fatty acid oxidation and the production of DAG are vaguely understood.

Using microchip array analysis, the Hulver laboratory has previously established that mRNA expression of NPY mRNA is significantly higher in skeletal muscle from obese as compared to lean subjects and associated with reduced fatty acid oxidation (Hulver, unpublished). The purpose of this study is to determine if the agonism of NPY Y1R in mammalian skeletal muscle cells resulted in reductions in fatty acid oxidation and increased intracellular lipid accumulation

Significance of the Study

Presently, one in ten Americans over the age of 20 are classified as diabetic (Centers for Disease Control and Prevention). The prevalence of this disease is projected to rise to epidemic status. One in three Americans born in the year 2000 will develop type II diabetes in their lifetimes (Lazar et al., 2005).

While it is widely understood that this disease is linked to obesity and skeletal muscle lipid accumulation, the mechanisms resulting in skeletal muscle lipid accumulation are vaguely understood. Neuropeptide Y as a mediator of increased DAG accumulation seen in obese, insulin resistance skeletal muscle is a novel hypothesis. A

better understanding of NPY's role in lipid accumulation can potentially lead to new pharmaceutical treatments for type II diabetes through restoration of normal skeletal muscle lipid metabolism.

Specific Aims

1. To determine the influence of activation of the NPY Y1R on mammalian skeletal muscle fatty acid oxidation.

2. To determine the influence of activation of the NPY Y1R will increase mammalian skeletal muscle lipid accumulation.

3. To determine if the presence of diprotin A will enhance the effects NPY treatment.

Main Research Hypotheses

1. Mammalian skeletal muscle cells incubated in NPY and NPY Y1R ligand will have lower FAO as compared to control treated cells.

2. Mammalian skeletal muscle cells in NPY and PY Y1R ligand will have higher accumulation of DAG and TAG as compared to control treated cells.

Basic Assumptions

1. The in vivo, physiological characteristics of skeletal muscle are retained in skeletal muscle cell cultures.

2. The skeletal muscle cell lines used in these studies possessed normal metabolic function.

3. The NPY used in these studies has the identical physiological effect as naturally produced NPY in vivo

Limitations

1. Human skeletal muscle myotubes were extracted exclusively from the vastus lateralis muscle.

2. Human skeletal muscle myotubes were derived exclusively from one individual.

3. Rodent skeletal muscle myotubes were derived exclusively from one tissue.

Chapter 2: Review of the Literature

The Metabolic Syndrome

The first documentation of the metabolic syndrome occurred in the 1920's. A Swedish physician, Eskil Kylin, noted the clustering of hypertension, hyperglycemia and gout (Eckel et al., 2005). The understanding of the relationship between obesity and its associated diseases was gradual. It was not until twenty years later, in 1947, that type II diabetes and central obesity were included in the list of symptoms classified under the metabolic syndrome, which was then labeled as Syndrome X (Reaven, 1998).

In 1999 the World Health Organization created an official standard for classifying the disease. Today, the metabolic syndrome is defined by the occurrence of two or more of the following symptoms in an individual; body mass index (BMI) greater than 30, dyslipidemia, hypertension, microalbuinuria, and insulin resistance (Eckel et al., 2005).

As the understanding of the cause and risks associated with the metabolic syndrome has grown, so has the prevalence of the disease. Over 17 percent of American teenagers can be classified as overweight (Ogden et al., 2007). In adults, the prevalence of obesity (BMI) in excess of 30kg/m^2, has increased to ~33 percent (Ogden et al., 2007).

The prevalence of obesity represents a growing burden on the nation's health care system. Obese adults have a higher prevalence of cardiovascular disease, which is the number one cause of death in the US, and type II diabetes (Ogden et al., 2007). The increasing prevalence of obesity also correlates to the increasing prevalence of type II diabetes seen in American youth (Eckel et al., 2005). As the understanding of the cellular and metabolic processes involved in the development of obesity improves so will pharmacological treatments for obesity and its associated diseases.

Lipotoxicity

Subcutaneous white adipose tissue, which is located in a layer beneath the skin, is one of two major lipid deposits in the body. Lipids located in this tissue are used to maintain homeostasis and function as energy reserves (Unger et al., 1999). The second major lipid deposit is visceral adipose tissue, which is located in the peritoneal cavity between internal organs. Excessive plasma free fatty acids and reduced β-oxidation, both

of which are characteristic of obese skeletal muscle, results in excessive accumulation of lipids in both of these regions (Unger et al., 1999).

Adipose cells have the capacity to adjust to chronic positive energy intake by increasing cell size and number (Slawik et al., 2006). However; the plasticity of these cells is limited. In visceral tissue, hyperplasia of adipose cells leads to the development of central obesity. Extremely obese individuals in addition to expansive visceral fat stores also deposit lipids within peripheral organs where they are stored as triacylglycerols (TAG) (Slawik et al., 2006; Unger et al., 1999).

Skeletal muscle, liver, and other peripheral tissue cells require long chain fatty acids for maintaining cell membrane structure, fluidity, and intracellular signaling (Slawik et al., 2006; Unger et al., 1999). Due to the role of lipids in the functioning of these cells, they normally contain small reserves lipids that are converted to TAG for storage purposes. As adipose cells undergo lipid induced hyperplasia; TAG accumulation in peripheral tissues increases. Due to the small size of these deposits, they are quickly saturated (Unger et al., 1999). Lipids in excess of TAG deposits enter non-oxidative pathways. By-products of the non-oxidative reactions create intermediates which cause organ-specific toxic reactions (Slawik et al., 2006).

In pancreatic cells, TAG accumulation is associated with insulin resistance through the disruption of β-cell functioning (Koyama et al., 1997; Lee et al., 1994). In a study conducted by Koyama et al., the group compared fasting, insulin-stimulated secretion in hyperlipidemic, hyoplipidemic, and control rats. Triacylglycerol content in the rat's pancreatic β-cell islets correlates with β-cell insulin production as reflected by fasting insulin and blood glucose levels. Insulin production by hypolipidemic rats was 13% of the amount produced by control rats and 4% of the amount produced by obese rats (Koyama et al., 1997). Lee et al showed that basal insulin secretion increased by several fold when insulin sensitive mouse β-cells were incubated in palmitate for 24 hours (Lee et al., 1994). The results of these studies suggest that beta-cells in the presence of high concentrations of lipids have a diminished capacity to respond to insulin which increases fasting plasma glucose and insulin production.

Reduced insulin sensitivity is also exhibited in skeletal muscle cells that are chronically exposed to high levels of free fatty acids. In the skeletal muscle, TAG are

stored as lipid droplets inside of muscle fibers. The droplets are referred to as intramyocellular TAG (Oscai et al., 1990). While moderate amounts of intramyocellular TAG are necessary for easily accessible substrates for β-oxidation, excessive TAG accumulation in obese skeletal muscle directly correlates with insulin resistance (Phillips et al., 1996; Sebastean et al., 2007).

A host of studies have documented the correlation between intramyocellular TAG and insulin resistance in obese skeletal muscle (Goodpaster et al., 2000; Hulver et al., 2003; Malenfant et al., 2001; Phillips et al., 1996; Ravussin, 2002; Ritov et al., 2005; Slawik et al., 2006). A simultaneous loss of insulin sensitivity and increase in lipid accumulation was documented in rat L6, mouse C2C12, and human muscle cells cultured in high fat media (Sebastian et al., 2007). Two studies reported reduced insulin activation of glycogen synthase, a key enzyme in glycogenesis, in obese tissue containing elevated intramyocellular TAG (Goodpaster et al., 2000; Malefant et al., 2001). Therefore, it is not surprising that skeletal muscle samples from diabetic subjects have higher lipid content than samples from lean subjects (Goodpaster et al., 2000).

In addition to insulin resistance, intramyocellular TAG accumulation correlates with reduced lipid oxidation (Hulver et al., 2003; Malenfant et al., 2001; Phillips et al., 1996; Ravussin, 2002; Ritov et al., 2005; Slawik et al., 2006). Subjects with intramyocellular triglyceride accumulation have 43% decrease in lipid oxidation as compared to lean, insulin sensitive subjects (Hulver et al., 2003).

Despite these correlations, TAG are not directly responsible for the development of insulin resistance or decreased lipid oxidation (Dube et al., 2008; Muhammad et al., 2008). Skeletal muscle from highly trained, insulin sensitive, elite athletes has intramyocellular TAG quantities comparable to those seen in morbidly obese individuals. Unlike obese subjects, muscle samples from athletes had no abnormalities in insulin signaling or oxidative pathways (Dube et al., 2008).

While vaguely understood; it is known that TAG are markers for elevated levels of products of non-oxidative lipolysis; diacylglycerols (DAG), long chain acyl-CoA and ceramides (Bell et al., 2000). These molecules directly disrupt cellular insulin signaling pathways (Bell et al., 2000; Oscai et al., 1990; Slawik et al., 2006). Consequently; high

levels of these intermediates are seen in insulin resistant mammals fed high fat diets (Bell et al., 2000).

The production of ceramides contributes to the pathogenesis of insulin resistance by inhibiting the activity of akt/ protein kinase B (Hulver & Dohm, 2004). This enzyme is necessary for insulin stimulated glucose uptake and glycogen synthesis. The inhibition of akt/ protein kinase B also results in reduced activation of glucose transporter 4 (GLUT4), a transmembrane protein responsible for glucose absorption into the cell (Hulver & Dohm, 2004).

Diacylglycerols disrupt insulin signaling pathways by reducing translocation of GLUT4 to skeletal muscle cell membrane and by activating of conventional and novel protein kinase C including PKCζ and PKCθ (Bell et al., 2000; Hulver & Dohm, 2004). In C2C12 mouse muscle cells, activation of PKC-θ results in translocation of the protein from the cytoplasm to the cell membrane. This disrupts the activity of insulin receptor-1, a key transcription factor involved in insulin signaling, by phosphorlyation of the enzyme at serine and tyrosine residues (Bell et al., 2000).

Due to the severity of damage that lipid intermediates cause, the peripheral tissues including the pancreas and skeletal muscle have protective measures against excessive TAG accumulation. In response to elevated circulating free fatty acids expression of oxidative enzymes are upregulated (Houmard, 2008). Included in upregulated oxidative enzymes are cytochrome c oxidase, an enzyme required for electron transport chain oxidation. Expression of citrate synthase, an enzyme required for the first step of the Krebs cycle, and peroxisome proliferators-activated receptor γ coactivator α (PGC1α), an important regulator of mitochondrial biogenesis, are increased in response to elevated free fatty acid concentrations (Heilbronn et al., 2007; Houmard, 2008). Upregulated expression of these proteins is thought to be an adaptation for the purpose of partitioning fatty acids towards oxidative pathways. When plasma free fatty acids exceed 50 times the normal concentration, protective measures become dysfunctional and activity of the oxidative enzymes described above are suppressed (Unger et al., 1999).

Carnitine palmitoyl transferase-1 (CPT1) is an oxidative enzyme that has an extremely important role in lipid oxidation. Carnitine palmitoyl transferase-1 is used to transfer long chain fatty acids into the inner membrane of the mitochondria where β-

oxidation occurs. Obese transgenic rats that over-express mutant form of liver CPT1 have increased fatty acid oxidation and significantly reduced the activation of PCKθ as compared to wild type obese animals (Sebastian et al., 2007). The incorporation of palmitate into cellular lipids such as TAG, DAG, and phospholipids is also reduced in transgenic rats (Houmard, 2008; Sebastian et al., 2007). Conversely, pharmacological inhibition of CPT1 activity leads to intramuscular lipid accumulation and insulin resistance in previously insulin sensitive rats (Sebastian et al., 2007). Simoneau et al reported that the activity of CPT1 is significantly depressed in extremely obese skeletal muscle (Simoneau et al., 2007). This indicates defects in long chain fatty acid transfer into the mitochondria. To further support this claim, the same group reported a disproportionate ratio of CTP1 to fatty acid binding protein, a peptide used in TAG formation. Lower percentages of CPT1 as compared to fatty acid binding protein are indicative of reduced fatty acid oxidation and increased partitioning of lipids to storage as TAG (Houmard, 2008; Simoneau et al., 1999)

Metabolic Flexibility:

Increased lipid accumulation in obese skeletal muscle is the result of both increased dietary fat consumption and decreased fat oxidation. In normal metabolism, skeletal muscle cells accommodate increases in both carbohydrate and fat consumption at the cellular level (Galgani et al., 2008). Fuel sensors activate or inhibit specific metabolic pathways depending on substrate availability (Galgani et al., 2008). Carbohydrate intake results in increased glycolysis and suppressed lipid oxidation. Conversely, glycolysis is suppressed and β-oxidation increased with fat consumption (Kelly et al., 1999).

In addition to changes in diet composition, substrate metabolism shifts in response to energy intake. When carbohydrate consumption drastically decreases, such as in conditions of fasting; fat oxidation increases to spare glucose for brain usage (Kelly et al., 1999). As a result of this, during periods of prolonged sleeping, skeletal muscle shifts to fat oxidation as its primary source of ATP generation (Galgani, 2008). These phenomenon are known as metabolic flexibility.

Obese skeletal muscle is metabolically inflexible. During fasting, obese skeletal muscle remains predominately glycolytic (Galgani et al., 2008; Kelley et al., 1999). In a

study conducted by Kelley et al., leg muscle samples from lean subjects RQ varied from .83 in fasting conditions to .99 in the presence of insulin (Kelley et al., 1999). Samples from obese subjects maintained a constant average RQ of ~0.9 throughout periods of starvation and exposure to insulin (Kelley et al., 1999). In the same study, it was shown that in resting conditions oxidation is used to generate a third of energy production in obese subjects while it generates two-thirds of energy production in lean subjects (Kelley et al., 1999). When exposed to elevated levels of plasma free fatty acids, obese skeletal muscle partitions lipids towards esterification while retaining glycolysis as the primary source of ATP generation (Kelley et al., 1999).

Suppressed Fatty Acid Oxidation in Obese Individuals:

Respiratory quotient (RQ) is the ratio of carbon dioxide production to oxygen consumption and reflects macronutrient oxidation. A RQ of ~0.9 denotes that glycolytic pathways are the primary source of ATP generation while a RQ of ~0.7 denotes that beta oxidation of fatty acids is the predominant energy producing pathway (Marra et al., 2004; Zurlo et al., 1994). Studies using RQ as a measure of whole body fat oxidation have shown that suppressed β-oxidation is indicative of propensity towards weight gain (Houmard, 2008). Higher amounts of body fat and larger BMI were documented in non-diabetic Pima Indians that displayed high whole body RQ (Zurlo et al., 1994). Non-obese women with an average RQ of greater than .92, which comprised the 90th percentile of distribution, increased body mass by +1.5 kg/ year at the end of a 6 year period. The other 38 participants who displayed lower whole body RQs increased body mass by an average of +0.1kg/ year (Marra et al., 2004).

Calorie restriction and surgical interventions alone are insufficient to correct elevated RQ seen in extremely obese individuals. Several studies have reported that morbidly obese individuals that undergo gastric bypass surgery resulting in stable weight loss for a over a year, retain suppressed fat oxidation despite lower BMI (Kelley et al., 1999; Guesbeck et al., 2001; Houmard, 2008). There was no reported no change in resting whole body RQ before and after weight loss in obese individuals (Kelley et al., 1999). Women that were previously morbidly obese displayed suppressed RQ during physical activity as compared to weight-matched controls (Guesbeck et al., 2001).

Individuals that sustained weight loss solely by calorie restriction also retain suppressed RQ. In a study conducted by Astrup , previously overweight subjects displayed impaired metabolic flexibility when put on a high fat diet as compared to controls. Reduced fatty acid oxidation led to positive energy balance (Astrup, 1993; Houmard, 2008).

Suppressed fat oxidation is partially the result of disproportionate muscle fiber percentages characteristic of obese skeletal muscle. Skeletal muscle fibers are divided into three categories. Type I fibers, also known as red muscle fibers, are dense in mitochondria and rely predominately on lipids for ATP production. These fibers also have more releasable lipoprotein lipase activity and plasma membrane fatty-acid binding protein content (Malefant et al., 2001). Type II fibers, also known as white muscle fibers, are predominately glycolytic. They have high concentrations of insulin-regulated glucose transport and greater insulin sensitivity than type I fibers (Malenfant et al., 2001). Type II fibers are further subdivided into type IIa, fast twitch oxidative, and type IIb, fast twitch glycolytic fibers. The respiratory capacity of type IIa fibers is three to five times greater than that of type IIb fibers (Hickey et al., 1995).

In a study conducted by Hickey et al., subject BMI was negatively correlated to type I fiber composition of skeletal muscle samples (Hickey et al., 1995). Skeletal muscle from lean subjects consisted of ~50 percent type I fibers, 40 percent type IIa fibers, and ten percent type IIb fibers. In obese subjects, average BMI of $47kg/m^2$, type I fibers consisted of only 40 percent of skeletal muscle, type IIa fibers consisted of 45 percent of muscle fibers, and type IIb fibers comprised 15 percent of muscle fibers (Hickey et al., 1995).

The same study found that insulin stimulated glucose transport was correlated to type I fiber content. In obese, diabetic skeletal muscle samples, percentages of type I fibers were further reduced to around 32 percent. Obese, insulin sensitive tissue was composed of ~47% type IIa fibers, and ~17% of type IIb fibers (Hickey et al., 1995). Reduced amounts of oxidative fibers contribute to the six-fold increase in intramyocellular lipid content seen in diabetic subjects as compared to weight matched controls (Phillips et al., 1996).

In addition to lower concentrations of oxidative fibers, muscle fibers in obese individuals have increased lipid content (Goodpaster et al., 2000; Malefant et al., 2001).

Type I, IIa, and IIb skeletal muscle fibers have from obese subjects approximately double the triglyceride content of lean fibers. The majority of lipid aggregates in lean tissue were located near the sarcolemma of the cell making them more accessible for β-oxidation whereas obese skeletal muscle had elevated percentages of aggregates that were located centrally (Malenfant et al., 2001).

Another attribute of obese skeletal muscle contributing to reduced β-oxidation is lower concentrations of skeletal muscle mitochondria as compared to lean individuals (Holloway et al., 2009). Obese skeletal muscle has reduced mitochondrial DNA and mitochondrial electron transport chain capacity as compared to lean skeletal muscle (Ravussin, 2002). In the subsarcolemmal fraction of mitochondria, lipids are used in the generation of ATP for ion exchange, substrate transport, cell signaling, and protein synthesis. Obese skeletal muscle has 3 to 4 fold reductions of mitochondria in this region while diabetic skeletal muscle has 7 fold reductions (Ravussin, 2002). Reduced mitochondrial concentrations are thought to be the result of suppressed mitochondrial biogenesis (Holloway et al., 2009).

Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide that was originally isolated from pig brain (Tatemoto et al., 1982). The molecule has a tyrosine residue at both its amino and carboxyl terminal as well as five other tyrosine residues. This observation led to the name Neuropeptide Y, Y standing for tyrosine (Tatemoto et al., 1982). The carboxyl-terminal of the peptide is responsible for its biological activity while the amino-terminal is involved in receptor affinity (De la Fuente et al., 1993).

NPY is a member of the gastric peptide family that includes peptide YY (PYY) and pancreatic polypeptide (PP). NPY, PYY and PP share 70-50 percent homology (Pedrazzini et al., 2003). The three proteins are also similar in that they control gastrointestinal actions. While the 3 peptides are morphologically very similar, NPY differs from PYY and PP in that it has a much broader function in the body.

NPY has a significant role in the regulation of a number of biological functions such as; control of learning and memory, locomotion, body temperature regulation, sexual behavior, emotional behavior, neuronal excitability, cardiovascular functions, and

hormone secretion (Kamiji et al., 2007). Both the fact that it is widely distributed and that the peptide has been highly conserved throughout evolution in a wide variety of species indicate that NPY is extremely significant to the functioning of the body (Kamini et al., 2007; Tatemo et al., 1982).

Active NPY is synthesized from pre-pro-neuropeptide Y, a 97 residue precursor amino acid (Higuchi et al., 1988; Pedrazzini et al., 2003). Pre-pro-NPY is cleaved at a single dibasic site, Lys-Arg, which results in the formation of active NPY (Brakch et al., 1997). There are four known enzymes that convert pre-pro-NPY into active NPY. The enzymes are prohormone convertase, carboxypeptidase, signal peptidase, and peptidyglycone α-amidating mono-oxygenase. NPY is further cleaved by both amino peptidase P and dipeptidyl I peptidase IV (DPPIV) (Pedrazzini et al., 2003). Dipeptidyl I peptidase IV removes the tyrosine-proline dipeptide from the amino terminal of the protein, this produces $NPY_{3\text{-}36}$. Amino peptidase P converts active NPY into $NPY_{2\text{-}36}$ through hydrolysis of the peptide bond between the first and second amino acid residues. These enzymes are found in the lungs, neurons, endothelial cells, and on smooth muscle (Pedrazzini et al., 2003).

DPPIV both in and ex vivo has the ability convert $NPY_{1\text{-}36}$ from a vasoconstrictive Y1 agonist to a nonvasoconstrictive Y2/Y5 agonist (Kuo et al., 2007; Lee et al., 2003). In vivo inhibition of DPPIV by diprotin A in the hindlimb of rats has been shown to stimulate a Y1-receptor-dependant response that was other-wise unresponsive to NPY infusion (Jackson et al., 2005).

Effect NPY on Feeding Behavior and Metabolism

Two of the many biological functions of NPY are the regulation of feeding behavior and metabolism (Kamini et al., 2007). These processes are regulated through activation of peripheral, central, and cerebral NPY receptors.

The hypothalamus of the brain is a region that integrates neuronal, metabolic, and endocrine signals (Pedrazzini et al., 2003). NPY is one of the most prevalent peptides in this region, indicating that it is extremely important to hypothalamus regulated feeding behavior (Arora and Anubhuti, 2006; Allen et al., 1983). The abundance of NPY in the hypothalamus is in part due to the peptide being synthesized by neurons of the arcuate

nucleus (Beck et al., 1993). The neurons project into the paraventricular nucleus, where NPY regulates feeding behavior (Beck et al, 1993).

Intracerebroventricular (i.c.v.) administration of NPY into mouse and rat hypothalamus, increases consumption leading to weight gain (Billingtion et al., 1991; Egawa et al., 1991). NPY i.c.v. injections into the hypothalamus also stimulate feeding in satiated mice (Vettor et al., 1994).

Zucker rats, whose obese phenotype is associated with over-eating, have higher circulating hypothalamic NPY levels than controls (Beck et al., 1993). In wild type rats, pharmaceutical administration of NPY into the hypothalamus leads to metabolic abnormalities similar to those seen Zucker obese rats (Baran et al., 2002). In addition to increased consumption, NPY i.c.v. mice display characteristics of obese mice such as; elevated body weight, increased plasma leptin levels, increased plasma insulin levels, and increased fat accumulation (Baran et al., 2002).

NPY and Adipose Tissue

NPY-induced weight gain is not solely due to increased food intake. The peptide reduces thermogenesis and lipolysis in both brown and white adipose tissue independent of consumption (Arora and Anubhuti, 2006; Allen et al., 1983; Billington et al., 1991; Egawa et al., 1991). NPY infusion decreased heat production and the expression of a gene involved in the regulation of thermogenesis, uncoupling protein 1 (Egawa et al., 1991). In mice fed ad libitum, this resulted in increased brown fat pad weight (Egawa et al., 1991).

Due to NPY receptors being located throughout the body, agonism of NPY receptors in peripheral tissues results in decreased lipolysis similar to that seen with i.c.v. NPY injections (Kuo et al., 2007a, Serradeli-Le et al., 2000). In human adipocytes, agonism of NPY Y1 receptor has been shown to inhibit adipose cell lipolysis and increases the production of leptin (Serradeli-Le et al., 2000). When combined with a high fat diet, cold and aggressor stressors associated with adipogenesis resulted in up-regulation of NPY and Y2 receptors in rodent abdominal adipose tissue (Kuo et al., 2007a). In vitro, NPY addition to mouse adipocyte cultures resulted in adipogenesis and proliferation of human and mouse adipocytes (Kuo et al., 2007a). Through reduced

thermogenesis and adipose cell lipolysis, agonism of NPY receptors partitions lipids towards storage rather than oxidation (Billington et al., 1991).

Inhibition of thermogenesis is mediated through the sympathetic nervous system (Dryden et al., 1996). In normal conditions, the presence of insulin signals through the SNS to increase the metabolic activity of adipose tissue. The presence of NPY suppresses this effect (Dryden et al., 1996). In white adipose tissue, NPY infusion increase metabolic activity concomitant with skeletal muscle insulin resistance (Vettor et al., 1994). Intracerebroventricular administration of NPY resulted in increased glucose transporter 4 (GLUT4), a protein involved in glucose uptake, protein content in white adipose tissue. Around 7 days after the termination of treatments, GLUT4 levels are returned to normal (Vettor et al., 1991).

NPY inhibition of insulin activated pathways is not exclusive to adipose tissue. In the liver, the peptide has been shown to suppress glycogenolysis but not gluconeogenesis (Nishizawa et al., 2008). It also stimulated de novo lipogenesis (Zarjevski et al., 1993).

NPY and PKC Activation

NPY action is also tightly coupled with PKC activation in macrophages (Wong et al., 1998). NPY and PYY stimulated in mouse peritoneal macrophages; adherence, chemotaxis, ingestion of cells and inert particles, and production of superoxide anion were mediated through a mechanism that also activates PKC (De la Fuente et al., 1993). Chemotactic peptides may increase PKC activity through increased DAG accumulation that occurs in macrophage infiltrated tissues (De la Fuente et al., 1993).

NPY also stimulates PKC activation in smooth muscle. NPY induces calcium mobilization through phospholipase C and PKC dependant pathways (De la Fuente, 1993; Pellieux et al., 2000). Both NPY and PYY stimulation of the Y1 receptor increases membranes fraction activity and decreases PKC activity in the cytosolic fraction (De la Fuente, 1993). This rise is mediated through the Y1 receptor pathway (Shigeri et al., 1995). A 15 second activation of the NPY Y1 receptor using PYY resulted in a 20% increase in membrane associated PKC activity (Selbie et al., 1995). This suggests that PKC is a downstream target of the NPY Y1R signaling pathway.

Summary

Skeletal muscle from obese individuals has reduced mitochondrial content, lower percentages of oxidative fibers, and is metabolically inflexible as compared to lean skeletal muscle (Galgani et al., 2008; Hickey et al., 1995; Houmard, 2008; Kelley et al., 1999; Simoneau et al., 1999). These dysfunctions are associated with reduced β-oxidation and intramuscular lipid accumulation (Slawik et al., 2006; Unger et al., 1999).

Neuropeptide Y is a peptide hormone that stimulates liopogenic pathways in adipose tissue (Dryden et al., 1996). It also increases PKC translocation to the cellular membrane (Selbie et al., 1995).

The peptide’s central and peripheral effects have been heavily studied. However, little is known about the effects of NPY receptor agonism in skeletal muscle. The goal of this study is to examine the role of the NPY Y1 receptor in skeletal muscle lipid metabolism.

Chapter 3: Methods

Skeletal Muscle Cell Lines

Three cell lines were used in NPY experiments; human primary cells, mouse muscle C2C12 cells, and rat L6 cells.

The human primary cell line was extracted from male vastus lateralis muscle, BMI of 20.28kg/m^2. Cell culturing procedures as previously described were used (Hulver et al., 2005). Human growth medium consisting of 500mL Dulbecco's Modified Eagle Media (DMEM), 50mL fetal bovine serum (Invitrogen, Carlsbad, CA), 50mL of bovine serum albumin (Invitrogen), 0.5mL genatmicin sulfate (Lonza, Walkersville, MD), 0.5mL dexamethosine (Lonza), 5mL bovine serum albumin (Lonza), 5mL fetuin bovine serum (Lonza), and 0.5mL human recombinant epidermal growth factor (Lonza) were added to human primary cells incubated in 25cm^2 collage coated flask. After 24 hours of incubation, growth medium was replaced to remove dimethyl sulfoxide (DMSO) present in medium of frozen cultures.

Cells were incubated in growth medium until they reached 80-90% confluency. When confluent, ten milliliters of trypsin (Invitrogen) was added to the cultures and incubated for 2-4 minutes to detach cells from the flask's surface. At the end of the incubation period, growth media was added to the flask to inactivate trypsin. The contents of the flask were transferred to a 50mL conical tube and centrifuged at 1,000 rpm for 3 minutes. The trypsin and growth medium mix was replaced with new growth medium. One milliliter of cell culture was added to each well of twelve well collagen coated plates (Hulver et al., 2005). In plates cells were grown to 80-90% confluency. When cultures became confluent growth medium was replaced with differentiation medium consisting of 500mL Dulbecco's Modified Eagle Media (DMEM), 10mL horse serum (Invitrogen,), 0.5mL genatmcin sulfate (Lonza, Walkersville, MD), 0.5mL dexamethosine (Lonza), 5mL bovine serum albumin (Lonza), 5mL fetuin bovine serum (Lonza), and 0.5mL human recombinant epidermal growth factor (Lonza). Differentiation medium was changed every two days. On day eight of differentiation fatty acid metabolism experiments took place.

Rat and mouse cells were grown in Dulbecco's Modified Eagle Media (DMEM), 10% fetal bovine serum (Invitrogen, Carlsbad, CA), and 2.5% penicillin/ streptomycin (Invitrogen). At 80% confluency, differentiation media was added to the cells; DMEM, 5% horse serum (Invitrogen), and 2.5% penicillin/ streptomycin. Cells were grown in $75cm^2$ flasks and transferred to 12 well plates for experiments using the same procedures as human primary cultures. Differentiation media was changed every two days. Fatty acid metabolism experiments took place on day four of differentiation for rat L6 and mouse C2C12 myotubes. Experiments took place on day eight of differentiation for human primary myotubes.

NPY Treatments

To determine the effects of NPY on skeletal muscle metabolism three concentrations of a NPY Y1 receptor ligand, [Leu31, Pro34] neuropeptide Y (Bachem, Torrance, CA) were added to medium used for fatty acid oxidation, 50µL/mL, 10µL/mL, and 1µL/mL.

In previous experiments, cultures were pre-incubated in NPYY1 ligand for 2 and 6 hour time periods. Ligand pre-treatment had no significant effect on fatty acid oxidation (Hulver, unpublished). In the current studies, the NPY Y1 ligand was added directly to fatty acid oxidation medium to determine effects on skeletal muscle metabolism.

In a separate experiment three concentrations of NPY (Bachem), 80 pg/mL, 250pg/mL, and 500pg/mL, was added to fatty acid oxidation medium. To prevent NPY cleavage by DPPIV present in differentiation medium, 17µL/mL of the DPPIV inhibitor diprotin A (Sigma Chemicals, St. Louis, MO) was added to all NPY treatments. NPY and diprotin A was added directly to fatty acid oxidation medium.

Fatty Acid Metabolism

Fatty acid oxidation medium contained 0.75 µCi of [1-^{14}C] palmitate (New England Nuclear, Boston, MA), .005% palmitic acid, .033% bovine serum albumin (Sigma Chemicals) and 81% differentiation media (Sigma Chemicals), .025% carnitine (Sigma Chemicals), and .125% hepes (should be capitalized and this should be spelled out, at least first time) (Sigma Chemicals). Before addition of differentiation media,

carnitine, and HEPES, both cold and ^{14}C-palmitate were pre-incubated for 30 minutes at 37^0C.

Fatty acid oxidation medium, 0.5 mL, was added to 12-well plates sealed with parafilm, and incubated for 3 hours at 37^0C. After the incubation period the cells were harvested as previously described (Muoio et al., 2002). Palmitate oxidation was measured using previously described procedures (Hulver et al., 2003). Phospholipids, diacylglycerol, and triacylglycerol production were measured by thin layer chromatography as previously described (Hulver et al, 2003).

Statistical Analysis

NPY Y1R ligand and NPY concentrations in cell culture experiments were repeated twice in order to assess statistically significant differences between control and experimental groups. One-way analysis of variance test was used to assess significance between control and experimental concentration means. Post-hoc comparisons were made using Tukey-Kramer HSD. Significance was accepted at $P < 0.05$.

Chapter 4: Results

Rat L6 Myotubes

Incubation of rat L6 myotubes in 80 ng/mL, 250 ng/mL, or 500 ng/mL NPY did not have a statistically significant effect on fatty acid oxidation ($p<0.05$). The addition of 17mg/mL of diprotin A to the NPY treatments also failed to produce an effect on fatty acid oxidation. When incubated in 1 pg/mL, 10 pg/mL, or 50 pg/mL NPY Y1R ligand, no significant change in fatty acid oxidation was observed (Figure 1).

Incubation of rat L6 myotubes in 80 ng/mL, 250 ng/mL, or 500 ng/mL of NPY did not have a statistically significant effect on the percentage of lipids accumulated within the skeletal muscle cell as TAG or DAG ($p<0.05$). The addition of diprotin A to the NPY incubation treatments had no significant effect on the percentage of lipids accumulated within the cell as TAG or DAG. As with the previous two treatment groups, the addition of 1 pg/mL, 10 pg/mL, or 50 pg/mL of NPY Y1R ligand to rat L6 myotubes produced no change in the percentage of ^{14}C labeled lipids accumulated within the cell as DAG or TAG (Figure 2).

Fatty Acid Oxidation
Rat L6 Myotubes

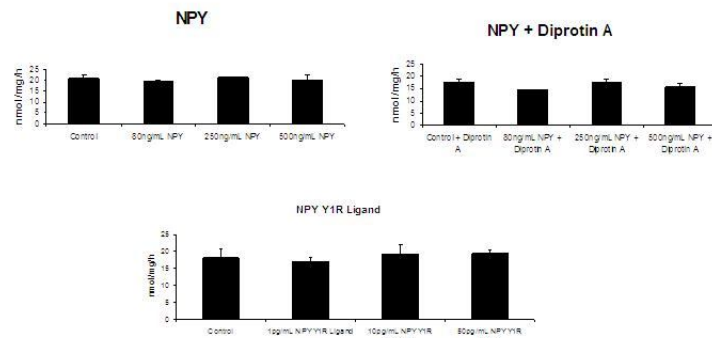

Figure 1

% of Lipid Stored as TAG and DAG
Rat L6 Myotubes

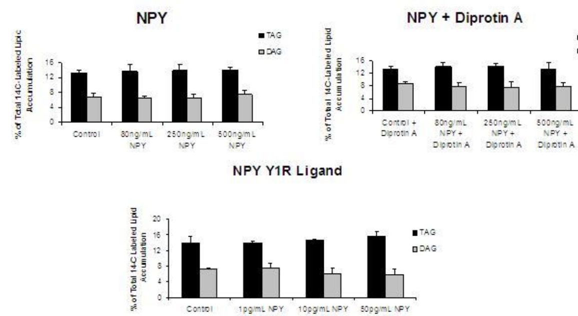

Figure 2

Human Primary Myotubes

Incubation of the human primary myotubes in 80 ng/mL, 250 ng/mL, or 500 ng/mL of NPY or NPY with the addition of 17mg/mL diprotin A produced no effect on fatty acid oxidation. Due to complications with human primary skeletal muscle myotubes cultures; the cells were no incubated in NPY Y1R ligand (figure 3).

Fatty Acid Oxidation
Human Primary Myotubes

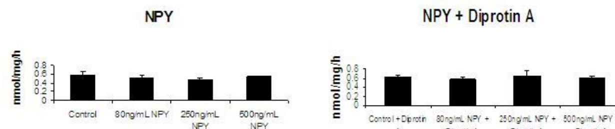

Figure 3

Incubation of human primary myotubes in NPY also produced no significant effect on the percentage of lipids accumulated with in the cell as TAG or DAG. The addition of 17mg/mL to NPY treatments significantly increased the percentage of ^{14}C labeled lipids incorporated into the cell that were stored as TAG ($p < 0.05$). This change was observed in the accumulation of DAG (figure 4).

% of Lipids Stored as DAG and TAG
Human Primary Myotubes

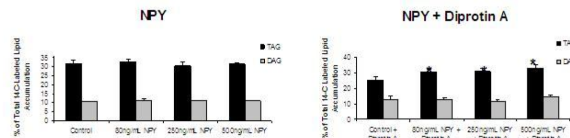

Figure 4

Mouse C2C12 Myotubes

Incubation of mouse C2C12 myotubes in 80 ng/mL, 250 ng/mL, or 500 ng/mL NPY had no significant change in fatty acid oxidation. The addition of 17mg/mL diprotin A to 80 ng/mL and 250 ng/mL NPY treatments resulted in no significant change in fatty acid oxidation. However; the addition of 17mg/mL diprotin A to 500 ng/mL NPY

significantly increased fatty acid oxidation. No change in fatty acid oxidation was observed following incubation of mouse C2C12 myotubes in 1 pg/mL, 10 pg/mL, or 50 pg/mL NPY Y1R ligand (figure 5).

Fatty Acid Oxidaiton

Mouse C2C12 Myotubes

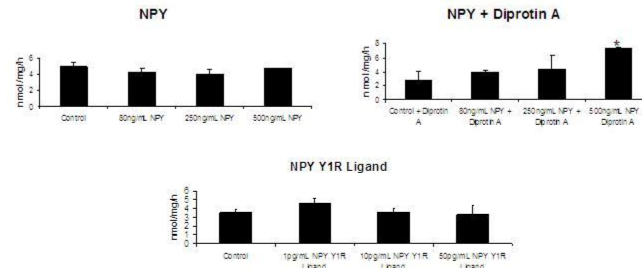

Figure 5

Incubation of mouse C2C12 myotubes in 80 ng/mL, 250 ng/mL, or 500 ng/mL NPY had resulted in no significant change in the percentage of ^{14}C labeled lipids accumulated within the cell as TAG or DAG.

The addition of 17mg/mL diprotin A to NPY treatments also produced no effect on fatty acid oxidation. There was no change in fatty acid oxidation observed following incubation of C2C12 cells in 1 pg/mL, 10 pg/mL, or 50 pg/mL NPY Y1R ligand (figure 6).

% of Lipids Stored as DAG and TAG

Mouse C2C12 Myotubes

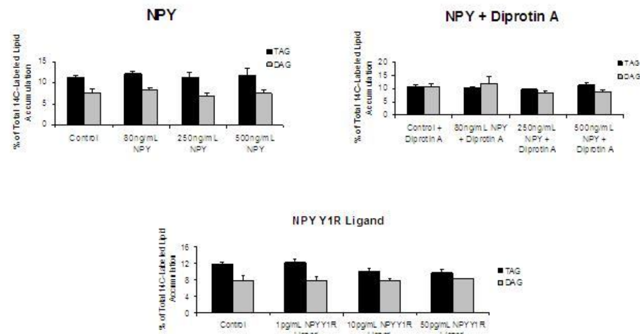

Figure 6

Chapter 5: Discussion

Introduction

There is a large and diverse body of literature which supports the significance of NPY's role in metabolism. Neuropeptide Y is the most abundant peptide in the hypothalamus where it functions as an appetite stimulant and a mediator of energy homeostasis (Pedrazzini et al., 2003). The peptide regulates homeostasis by increasing adipogenesis in both brown and white adipose tissue, decreasing theromgenesis, and increasing the secretion of leptin (Arora & Anubhuti, 2006; Baran et al., 2002; Pedrazzini et al., 2003). These activities contribute to the obese phenotype of *ob/ob* and *db/db* mice and *fa/fa* rats which have elevated hypothalamic levels of the peptide (Zarjevski et al., 1993).

Synthesis of NPY occurs in the hypothalamus. However; NPY receptors are located throughout the body. This allows the protein to regulate adipogenesis and leptin secretion in adipose tissue (Kuo et al., 2007). A number of studies have demonstrated that NPY is an important mediator in adipose tissue metabolism (Baran et al., 2002; Billington et al., 1991; Kuo et al., 2007). However; the role of NPY in the regulation of skeletal muscle lipid metabolism has not previously been observed. Therefore the goal of this research is to study the role of NPY in the regulation of skeletal muscle lipid oxidation and the accumulation of TAG and DAG.

Differentiated non-obese human, rat L6, and mouse C2C12 cells were incubated in ^{14}C palmitate labeled FAO medium containing 80 ng/mL, 250 ng/mL, and 500 ng/mL NPY and 80 ng/mL, 250 ng/mL, and 500 ng/mL NPY containing 17mg/mL diprotin A. In a separate experiment differentiated non-obese human, mouse C2C12, and rat L6 skeletal muscle cells were incubated in 1 µg/mL, 10 µg/mL, and 50 µg/mL NPY Y1R ligand. Fatty acid oxidation in all experiments was determined by the sum of the production of ^{14}C labeled CO_2 and acid soluble metabolite production as measured by scintillation counting. The production of DAG and TAG was measured using thin layer chromatography.

Major Findings

Studies in the Hulver laboratory are among the first to assess the role of NPY is skeletal muscle lipid oxidation and DAG accumulation. The major findings of this study

are (1) that incubation of rat L6, mouse C2C12, and lean human skeletal muscle cells in the presence of 80 ng/mL, 250 ng/mL, and 500 ng/mL NPY produced a no statistically significant change in fatty acid oxidation or the percentage of ^{14}C labeled palmitate stored within the cell as DAG or TAG; (2) incubation of lean human and rat skeletal muscle cells in FAO medium containing NPY treatments and 17mg/mL diprotin A produced no statistically significant change in FAO; (3) incubation of mouse C2C12 skeletal muscle cells in FAO medium containing 500 ng/mL NPY and 17mg/mL diprotin A significantly increased FAO; (4) incubation of lean human cells in 80 ng/mL, 250 ng/mL, or 500 ng/mL NPY with the addition of diprotin A significantly increased the percentage of ^{14}C labeled palmitate incorporated into the cell as TAG.

Cell Incubation in NPY Y1R Ligand

Previous studies have established that [Leu31, Pro34] neuropeptide Y, the Y1R agonist used in ligand experiments, has a high affinity for the NPY Y1R (Fuhlendorff et al., 1990). Despite the addition of 1 pg/mL, 10 pg/mL, and 50 pg/mL of NPY Y1R ligand to rat L6 and mouse C2C12 myotubes to FAO incubation medium; there was no significant effect on lipid metabolism. These findings are supported studies which reported no change in body mass or fat pad weight in NPY Y1R -/- mice (Pedrazzini et al., 1998). It has also been observed that antagonism of the NPY Y5R had no significant effect on whole body weight loss in humans (Erondu et al., 2006).

Preliminary studies displayed a positive correlation between BMI and skeletal muscle NPY Y1R mRNA expression (r^2=0.56, p<0.0008) (Hulver, unpublished). There was also a positive correlation between NPY Y1R mRNA and skeletal muscle fatty acid partitioning ratio (r^2=0.74, p<0.0001) (Hulver, unpublished). The correlation seen between BMI and NPY Y1R was measured exclusively in human skeletal muscle. Had NPY Y1R mRNA expression been measured in lean and obese rat and mouse skeletal muscle, this correlation may not have been observed.

Findings from the current NPY studies indicate that the relationship between elevated BMI and fatty acid partitioning ratio to NPY Y1R mRNA content is not cause and effect. Despite its correlation to obesity, the evidence from this study suggests that NPY Y1R agonism is not a mediator of skeletal muscle metabolic dysfunction as seen in

obese individuals. Skeletal muscle NPY Y1R protein content in was not measured in samples from lean or obese individuals. Increased NPY Y1R mRNA may not be indicative of elevated NPY Y1R protein levels in obese as compared to lean skeletal muscle.

Neuropeptide Y

The inability of NPY to affect FAO on mammalian myotubes is contradictory to results from previous studies which documented an increase in skeletal muscle glucose utilization following a two hour infusion of NPY into the hypothalamus of mice (Vettor et al., 1998). Infusion of NPY into the hypothalamus of rodents increases plasma insulin levels (Baran et al., 2002). Increased glucose utilization seen following NPY administration may be the result of elevated levels of insulin rather than an effect of reduced fatty acid oxidation as previously hypothesized.

The inability of NPY treatments to change TAG and DAG accumulation are also inconsistent with previous studies which documented an increase in tibalis triglyceride content and plasma triglyceride levels following infusion of NPY into the hypothalamus of mice (Vettor et al., 1994).

Metabolic changes following NPY administration were seen in vivo following central administration of the peptide (Baran et al., 2002; Vettor et al., 1994; Vettor et al., 1998). The inability of NPY treatments to change FAO may be a result of the experiments being performed in cell culture rather than in vivo. NPY also goes through several modifications before it is converted to $NPY_{1\text{-}36}$, the ligand which has the highest affinity for the NPY Y1R (Pedrazzini et al., 2003). The addition of NPY directly to skeletal muscle myotubes rather than centrally may have resulted in incomplete modification of the molecule, lowering its affinity for the NPY Y1R.

Addition of Diprotin A

While it was hypothesized that NPY would decrease FAO and increase TAG and DAG accumulation; these effects were expected to be minimal due to cleavage of the $NPY_{1\text{-}36}$ molecule by DPPIV and aminopeptidase P present in horse serum added to FAO incubation medium. The addition of 17mg/mL diprotin A to NPY treatments was

hypothesized to reduce FAO and increase TAG and DAG accumulation within rat L6, mouse C2C12, and human primary myotubes. As hypothesized, the addition of 17mg/mL diprotin A to NPY treatments increased TAG accumulation in human primary myocytes. However; this effect was not observed in any other species

Diprotin A inhibits the activity of DPPIV; aminopeptidase P is an enzyme which also cleaves NPY_{1-36}, the form of the molecule which has the highest affinity for the Y1R (Pedrazzini et al., 2005).

In studies measuring the in vivo response of skeletal muscle to NPY infusion inhibitors of both DPPIV and aminopeptidase P were added to the NPY infusion (Jackson et al., 2005). While the addition of diprotin A inhibited the activity of DPPIV; there was nothing added to the cell culture medium to inhibit the activity of aminopeptidase P. Cleavage of the NPY_{1-36} molecule by aminopeptidase P results in the production of NPY_{2-36}, an agonist for NPY Y2R and Y5R (Pedrazzini et al., 2003). The addition of 17mg/mL diprotin A to NPY treatments may have been ineffective in changing FAO due to cleavage of the NPY_{1-36} molecule by aminopeptidase P present in FAO incubation medium.

Summary

In the present study, agonism of NPY Y1R appears to have little to no effect on the regulation of mammalian skeletal muscle lipid metabolism. The results from this study suggest that the relationship seen between elevated NPY Y1R mRNA and increased FAO partitioning ratio are not cause and effect.

However; incubation of human primary cells in NPY with the addition of diprotin A significantly increased TAG accumulation. The use of NPY to regulate skeletal muscle lipid metabolism may be exclusive to human skeletal muscle. More studies are needed to determine if this effect was mediated through the NPY Y1R.

Future Directions

This investigation suggested that NPY Y1R does not play a major role in skeletal muscle lipid metabolism; but this conclusion may be due to methodological limitations. Future studies may include measures of skeletal muscle calcium release to determine if

the NPY Y1R is being activated by both NPY and NPY Y1R ligand administered to mammalian skeletal muscle cell lines.

Administration of NPY into the hypothalamus of rodents may produce results not seen with NPY incubation in skeletal muscle cell cultures. As in vivo assessment of the role of NPY Y1R in skeletal muscle lipid metabolism may be used in future studies.

It was observed that NPY Y1R mRNA expression was positively correlated to BMI in human skeletal muscle (Hulver, unpublished). Future studies may involve an assessment of NPY Y1R protein content in mice with a wide range of body mass to determine if a correlation between NPY Y1R and body mass is present in rodent skeletal muscle.

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Appendix A: Table Appendix

Table 1: Rat L6 Myotubes

Treatment	FAO
Control	20.90 ± 1.40
80ng/mL NPY	19.5653 ± 0.
250ng/mL NPY	21.38 ± 0.25
500ng/mL NPY	20.28 ± 2.02
Control + Diprotin A	17.92 ± 0.89
80ng/mL NPY + Diprotin A	14.45 ± 0.08
250ng/mL NPY + Diprotin A	17.30 ± 1.70
500ng/mL NPY + Diprotin A	15.89 ± 1.35

Table 2: Rat L6 Myotubes

(percentage of 14C palmitate incorporated into myotubes as lipids)

Treatment	TAG	DAG
Control	13.20 ± 0.83	6.99 ± 0.79
80ng/mL NPY	13.72 ± 1.75	6.48 ± 0.65
250ng/mL NPY	14.04 ± 1.39	6.45 ± 1.09
500ng/mL NPY	14.08 ± 0.59	7.44 ± 1.22
Control + Diprotin A	13.26 ± 0.87	8.61 ± 0.61
80ng/mL NPY + Diprotin A	14.15 ±1.37	7.69 ± 1.14
250ng/mL NPY + Diprotin A	14.18 ± 0.94	7.66 ± 1.55
500ng/mL NPY + Diprotin A	13.50 ± 2.09	7.79 ± 0.99

Table 3: Rat L6 Myotubes

Treatment	FAO
Control	18.19 ± 2.62
1ng/mL NPY Y1R Ligand	16.93 ± 1.27
10ng/mL NPY Y1R Ligand	19.25 ± 2.66
50ng/mL NPY Y1R Ligand	19.47 ± 1.15

Table 4 Rat L6 Myotubes

(percentage of 14C palmitate incorporated into myotubes as lipids)

Treatment	TAG	DAG
Control	13.82 ± 1.70	7.26 ± 0.24
1µg/mL NPY	13.89 ± 0.35	7.55 ± 1.20
10µg/mL NPY	14.64 ± 0.23	6.00 ± 1.59
50µg/mL NPY	15.61 ± 1.34	5.81 ± 1.36

Table 5: Human Primary Myotubes

Treatment	FAO
Control	0.59 ± 0.05
80ng/mL NPY	0.52 ± 0.06
250ng/mL NPY	0.48 ± 0.03
500ng/mL NPY	0.55 ± 0.02
Control + Diprotin A	0.62 ± 0.05
80ng/mL NPY + Diprotin A	0.58 ± 0.06
250ng/mL NPY + Diprotin A	0.65 ± 0.11
500ng/mL NPY + Diprotin A	0.61 ± 0.04

Table 6: Human Primary Myotubes

(percentage of 14C palmitate incorporated into myotubes as lipids)

Treatment	TAG	DAG
Control	31.65 ± 1.84	10.74 ± 0.04
80ng/mL NPY	32.75 ± 1.34	11.21 ± 0.79
250ng/mL NPY	30.14 ± 2.18	11.26 ± 0.15
500ng/mL NPY	31.34 ± 0.68	10.98 ± 0.42
Control + Diprotin A	25.12 ± 2.34	12.69 ± 2.49
80ng/mL NPY + Diprotin A	30.55 ± 1.63	12.85 ± 1.05
250ng/mL NPY + Diprotin A	30.91 ± 1.48	11.80 ± 0.84
500ng/mL NPY + Diprotin A	32.82 ± 2.55	14.77 ± 0.92

Table 7: Mouse C2C12 Myotubes

Treatment	FAO
Control	4.96 ± 0.49
80ng/mL NPY	4.25 ± 0.44
250ng/mL NPY	4.02 ± 0.62
500ng/mL NPY	4.75 ± 0.07
Control + Diprotin A	2.83 ± 1.22
80ng/mL NPY + Diprotin A	3.92 ± 0.28
250ng/mL NPY + Diprotin A	4.39 ± 1.91
500ng/mL NPY + Diprotin A	8.9 ± 0.15

Table 8: Mouse C2C12 Myotubes

(percentage of 14C palmitate incorporated into myotubes as lipids)

Treatment	TAG	DAG
Control	11.20 ± 0.62	7.64 ± 0.83
80ng/mL NPY	12.06 ± 0.67	8.27 ± 0.40
250ng/mL NPY	11.28 ± 1.21	6.90 ± 0.62
500ng/mL NPY	11.71 ± 1.82	7.45 ± 0.88
Control + Diprotin A	10.51 ± 0.84	10.59 ± 1.32
80ng/mL NPY + Diprotin A	10.16 ± 0.30	11.85 ± 2.57
250ng/mL NPY + Diprotin A	9.71 ± 0.18	8.27 ± 0.91
500ng/mL NPY + Diprotin A	11.38 ± 1.10	8.66 ± 0.78

Table 9: Mouse C2C12 Myotubes

Treatment	FAO
Control	3.83 ± 0.19
1μg/mL NPY	5.16 ± 1.27
10μg/mL NPY	3.49 ± 0.54
50μg/mL NPY	3.22 ± 0.18

Table 10: Mouse C2C12 Myotubes

(percentage of 14C palmitate incorporated into myotubes as lipids)

Treatment	TAG	DAG
Control	11.62 ± 0.63	7.92 ± 1.13
1μg/mL NPY	12.08 ± 0.88	7.67 ± 1.03
10μg/mL NPY	9.99 ±0.86	7.73 ± 0.54
50μg/mL NPY	9.66 ± 0.98	8.22 ± 0.11

Appendix B: Raw Data

Raw Data

Lean Human

Treatment	PL	DAG	TAG	Total
Control	114.10	21.1	60.7	195.9
Control	152.00	29.3	92.3	273.6
Control	123.60	31.4	67.2	222.2
80µL NPY	116.20	25.3	67.1	208.6
80µL NPY	123.80	23.5	69.4	216.7
80µL NPY	118.10	22.8	72.8	213.7
250µL NPY	116.50	17	53.3	186.8
250µL NPY	114.40	21.4	56.2	192
250µL NPY	121.70	24.7	70.9	217.3
500µL NPY	125.60	24.7	84	234.3
500µL NPY	134.40	26.5	71.8	232.7
500µL NPY	94.50	18.2	52.6	165.3
Control + dpA	117.40	20.8	52.2	190.4
Control + dpA	78.50	18.8	32.8	130.1
Control + dpA	78.60	26.4	30.9	135.9
80µL NPY + dpA	101.50	23	57.8	182.3
80µL NPY + dpA	86.20	18.1	47.4	151.7
80µL NPY + dpA	90.10	22	45.1	157.2
250µL NPY + dpA	97.50	20.3	56.7	174.5
250µL NPY + dpA	104.20	19.4	51.9	175.5
250µL NPY + dpA	60.60	13.6	32.8	107
500µL NPY + dpA	102.00	26.9	61.6	190.5
500µL NPY + dpA	109.70	31.3	62	203
500µL NPY + dpA	107.70	26.7	74.2	208.6

Rat

Treatment	PL	DAG	TAG	Total
Control	167.80	14	26.9	208.7
Control	186.40	14.7	28.9	230
Control	163.20	16.5	29.6	209.3
80µL NPY	147.70	13.4	26.6	187.7
80µL NPY	144.40	10.7	27.8	182.9
80µL NPY	157.40	12.4	22.7	192.5
250µL NPY	145.60	13.6	29	188.2
250µL NPY	153.90	10.9	27	191.8
250µL NPY	166.30	45.3	30.6	242.2
500µL NPY	162.80	18.1	29.7	210.6
500µL NPY	180.50	17.3	30.8	228.6
500µL NPY	155.60	12.1	28.8	196.5
Control + dpA	143.00	17.2	24.7	184.9
Control + dpA	146.80	15.1	22.8	184.7
Control + dpA	181.20	19.5	32.9	233.6

80µL NPY + dpA	160.30	17	26.8	204.1
80µL NPY + dpA	173.70	14.2	35	222.9
80µL NPY + dpA	159.30	17.1	27.8	204.2
250µL NPY + dpA	150.90	15.2	27.9	194
250µL NPY + dpA	175.70	13.4	33.4	222.5
250µL NPY + dpA	180.80	21.2	30.6	232.6
500µL NPY + dpA	191.40	16.9	28.8	237.1
500µL NPY + dpA	156.30	14.9	32.4	203.6
500µL NPY + dpA	205.80	23.4	32.6	261.8
Control	171.30	37.1	28.3	236.7
Control	188.90	17.9	34.2	241
Control	177.50	16.2	35	228.7
1µg/mL NPY	183.90	18.4	32.5	234.8
1µg/mL NPY	175.30	19.5	32.4	227.2
1µg/mL NPY	162.10	12.6	27.4	202.1
10µg/mL NPY	193.90	33	39.3	266.2
10µg/mL NPY	207.30	18.9	39.2	265.4
10µg/mL NPY	202.20	12.2	36	250.4
50µg/mL NPY	190.40	10.3	41.2	241.9
50µg/mL NPY	197.90	17.1	36.1	251.1
50µg/mL NPY	182.10	14.8	35.9	232.8
Mouse				
Control	279.70	29.7	36.4	345.8
Control	298.40	26.8	41.5	366.7
Control	237.20	20.5	34.3	292
80µL NPY	262.90	28.8	38.5	330.2
80µL NPY	271.60	27.1	39.5	338.2
80µL NPY	257.10	26.2	41.7	325
250µL NPY	302.10	27.7	37.7	367.5
250µL NPY	291.60	24.8	45.7	362.1
250µL NPY	312.00	23.8	41.4	377.2
500µL NPY	287.60	23.2	35.8	346.6
500µL NPY	130.80	12	22.8	165.6
500µL NPY	198.00	20.7	27.1	245.8
Control + dpA	267.80	37.1	36.4	341.3
Control + dpA	308.10	34.7	36.4	379.2
Control + dpA	273.40	41.7	40	355.1
80µL NPY + dpA	285.20	49.3	38.7	373.2
80µL NPY + dpA	308.30	50.9	19.4	378.6
80µL NPY + dpA	297.70	32.6	36.5	366.8
250µL NPY + dpA	240.20	21.3	13.3	274.8
250µL NPY + dpA	267.30	30.7	31.6	329.6
250µL NPY + dpA	294.10	27.6	35.1	356.8
500µL NPY + dpA	314.20	32.6	39.1	385.9
500µL NPY + dpA	290.10	35.3	45.2	370.6
500µL NPY + dpA	330.80	33.1	48.8	412.7
Control	229.20	23.4	34.5	287.1
Control	252.30	28.1	34.3	314.7
Control	227.20	18.7	33.4	279.3

1µg/mL NPY	231.40	18.2	31.4	281
1µg/mL NPY	263.80	27.5	43.3	334.6
1µg/mL NPY	222.10	23.2	33.8	279.1
10µg/mL NPY	288.10	24.7	33.7	346.5
10µg/mL NPY	322.20	30.6	36.1	388.9
10µg/mL NPY	263.00	26.6	35.6	325.2
50µg/mL NPY	332.00	32.8	34.9	399.7
50µg/mL NPY	326.40	33.6	43.1	403.1
50µg/mL NPY	303.60	29.9	35.2	368.7

Mouse	
Control	5.16
Control	4.41
Control	5.32
80ng/mL of NPY	4.76
80ng/mL of NPY	4.07
80ng/mL of NPY	3.94
250ng/mL of NPY	4.36
250ng/mL of NPY	3.31
250ng/mL of NPY	4.39
500ng/mL of NPY	4.70
500ng/mL of NPY	1.57
500ng/mL of NPY	4.80
Control + Diprotin A	4.21
Control + Diprotin A	2.40
Control + Diprotin A	1.87
80ng/mL of NPY + Diprotin A	3.68
80ng/mL of NPY + Diprotin A	3.87
80ng/mL of NPY + Diprotin A	4.22
250ng/mL of NPY + Diprotin A	2.61
250ng/mL of NPY + Diprotin A	4.15
250ng/mL of NPY + Diprotin A	6.40
500ng/mL of NPY + Diprotin A	4.28
500ng/mL of NPY + Diprotin A	9.01
500ng/mL of NPY + Diprotin A	8.80
Control	3.94
Control	3.94
Control	3.61
1ng/mL NPY ligand	4.63
1ng/mL NPY ligand	4.25
1ng/mL NPY ligand	6.61
10ng/mL NPY ligand	3.71
10ng/mL NPY ligand	3.89
10ng/mL NPY ligand	2.88
50ng/mL NPY ligand	3.39
50ng/mL NPY ligand	3.04
50ng/mL NPY ligand	3.25

Human	
Control	0.52
Control	0.63
Control	0.60
80ng/mL of NPY	0.52
80ng/mL of NPY	0.57
80ng/mL of NPY	0.45
250ng/mL of NPY	0.45
250ng/mL of NPY	0.49
250ng/mL of NPY	0.52
500ng/mL of NPY	0.53
500ng/mL of NPY	0.55
500ng/mL of NPY	0.56
Control + Diprotin A	0.57
Control + Diprotin A	0.67
Control + Diprotin A	0.62
80ng/mL of NPY + Diprotin A	0.53
80ng/mL of NPY + Diprotin A	0.57
80ng/mL of NPY + Diprotin A	0.64
250ng/mL of NPY + Diprotin A	0.53
250ng/mL of NPY + Diprotin A	0.69
250ng/mL of NPY + Diprotin A	0.73
500ng/mL of NPY + Diprotin A	0.59
500ng/mL of NPY + Diprotin A	0.58
500ng/mL of NPY + Diprotin A	0.66

rat	
Control	20.79
Control	19.56
Control	22.36
80ng/mL of NPY	19.19
80ng/mL of NPY	19.13
80ng/mL of NPY	20.28
250ng/mL of NPY	16.62
250ng/mL of NPY	21.55
250ng/mL of NPY	21.20
500ng/mL of NPY	23.20
500ng/mL of NPY	17.33
500ng/mL of NPY	20.34
Control + Diprotin A	19.59
Control + Diprotin A	15.84
Control + Diprotin A	18.34
80ng/mL of NPY + Diprotin A	14.40
80ng/mL of NPY + Diprotin A	14.50
80ng/mL of NPY + Diprotin A	16.79
250ng/mL of NPY + Diprotin A	19.31
250ng/mL of NPY + Diprotin A	15.09
250ng/mL of NPY + Diprotin A	17.50
500ng/mL of NPY + Diprotin A	15.99
500ng/mL of NPY + Diprotin A	17.63

500ng/mL of NPY + Diprotin A	14.08
Control	15.41
Control	18.52
Control	20.63
1ng/mL NPY ligand	15.69
1ng/mL NPY ligand	16.90
1ng/mL NPY ligand	18.22
10ng/mL NPY ligand	16.28
10ng/mL NPY ligand	20.07
10ng/mL NPY ligand	21.41
50ng/mL NPY ligand	20.64
50ng/mL NPY ligand	18.34
50ng/mL NPY ligand	19.45