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NaKtide Targeted to Adipocytes Ameliorates Western Diet Induced Obesity

Rebecca D. Pratt

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**NAKTIDE TARGETED TO ADIPOCYTES AMELIORATES WESTERN DIET
INDUCED OBESITY**

A dissertation submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Doctorate

In
Biomedical Research
by

Rebecca D. Pratt

Approved by

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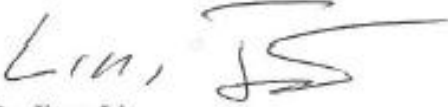
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APPROVAL OF THESIS

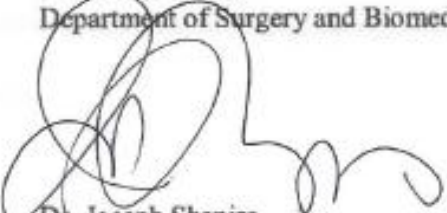
We, the faculty supervising the work of Rebecca D. Pratt, affirm that the dissertation, *NAKTIDE TARGETED TO ADIPOCYTES AMELIORATES WESTERN DIET INDUCED OBESITY*, meets the high academic standards for original scholarship and creative work established by the Biomedical Research Program and Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.


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ABSTRACT

Obesity has become a worldwide epidemic and is a major risk factor for metabolic syndrome. It is believed that cellular oxidant stress plays a key role in both the development and maintenance of obesity as well as its associated comorbidities such as diabetes, cardiovascular disease, and nonalcoholic steatohepatitis (NASH). We have previously reported that systemic administration of pNaKtide, which targets the Na/K-ATPase oxidant amplification loop was able to decrease oxidative stress and adiposity in mice fed a high fat and fructose supplemented western diet (WD). As adipocytes are believed to play an active role in the development of obesity and its related comorbidities, we examined whether lentiviral-mediated adipocyte-specific expression of NaKtide, the portion of pNaKtide derived from the $\alpha 1$ Na/K-ATPase N domain without the TAT leaders sequence used to make pNaKtide cell permeant, could have a similar ameliorative effect. To test this hypothesis, studies were first performed in isolated murine preadipocytes, 3T3-L1 cells; it was found that specific activation of Na/K-ATPase signaling (with the cardiotonic steroid ouabain which is specific to the Na/K-ATPase) significantly increased adipogenesis in 3T3-L1 cells in the presence of adipogenic medium, and pNaKtide administration attenuated oxidative stress and lipid accumulation. When administered through a lentiviral construct, NaKtide showed a similar decrease in oxidative stress. Complementary *in vivo* studies were performed as well. C57BL6 mice fed a WD activated adipocyte Na/K-ATPase signaling and increased adiposity, systemic oxidative stress and insulin resistance as well as induced development of NASH. Also of note, WD increased the plasma levels of leptin, IL-6 and TNF α along with decreased locomotor activity, expression of the D2 receptor and tyrosine hydroxylase in defined brain regions as well as markers of neurodegeneration including neuronal apoptosis. Selective adipocyte expression of NaKtide in these mice fed a WD attenuated all of these

changes including the brain biochemical alterations and behavioral adaptations. These data suggest that adipocyte derived cytokines play an essential role in the development of obesity induced by a WD and that targeting the adipocyte Na/K-ATPase oxidant amplification loop (NKAL) may serve as an effective therapeutic strategy.

CHAPTER 1

INTRODUCTION

Na/K-ATPase: History and Signaling Function

The Na/K-ATPase is an enzyme that is found in the plasma membrane of all animal cells and holds several important cellular responsibilities (Skou, 1957, 1986). It is responsible for not only the movement of sodium (Na^+) and potassium (K^+) across their ion gradients at the expense of adenosine triphosphate (ATP), but in more recent years has been implicated as a signaling transducer that interacts with multiple protein kinase cascades and is a factor in many disease states [reviewed in (Aperia, 2007; Z. Xie & Cai, 2003)].

Before its discovery, the existence of the Na/K-ATPase had been speculated for years. Many important findings occurred that finally led to its discovery in 1957(Skou, 1957). Carl Schmidt demonstrated the existence of a Na^+/K^+ concentration gradient across the cell membrane and Rudolph Heidenhain proposed a “microscopic steamship” in the membrane that was capable of maintaining this gradient(Heidenhain, 1894; Zaleski, 1894) . Ernest Overton demonstrated that muscle cells had active transport mechanisms that allowed cells to move Na^+ and K^+ across the cell membrane, via energy consumption (Overton, 1899, 1902). Heppel and Steinbach confirmed these findings in muscle cells using radioactive isotopes, and this was further confirmed by US scientists who found that cardiac glycosides (such as ouabain) were specific inhibitors of active transport in red blood cells (RBCs) (Heppel, 1940; Steinbach, 1940). ATP dependent potassium uptake further supported and linked the transport system to membrane bound ATPase sensitive to cardiac glycosides or cardiotonic steroids (CTS) (Skou, 1986).

In 1957, while doing anesthesia research in crab leg nerves, Jens Christian Skou found that the Na/K-ATPase was at its most active when combined with the correct combination of Na^+ , K^+ , and magnesium (Mg^+) (Skou, 1957; Skou & Esmann, 1983, 1992). During the same time, Robert Post (in the US) found that the Na/K-ATPase is responsible for moving 3 Na^+ and 2 K^+ ions across the cell membrane in RBCs; the first discovery of the Na/K-ATPase was due to Skou, but was confirmed by Post (Post, 1969).

Around the same time as these discoveries, renal physiologists and cell biologists had developed a kidney Na/K-ATPase purification protocol. From this, we understand that the Na/K-ATPase exists in a dynamic state of conformation equilibrium; this is vital for the ability to convert ATP hydrolysis to binding and movement of ions across the plasma membrane (Blanco, DeTomaso, Koster, Xie, & Mercer, 1994; Blanco, Koster, Sanchez, & Mercer, 1995). Digitalis specific inhibition of the Na/K-ATPase was recognized in the late 50's; in cardiac myocytes inhibition of Na/K-ATPase activity by cardiotonic steroids increases intracellular Na^+ concentration, which leads to accumulation of intracellular calcium (Ca^{2+}) via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Blanco et al., 1994; Blanco et al., 1995).

Molecular cloning of sheep kidneys in 1985 by G.E. Shull, Schwartz, and Lingrel led to further information about the complexity of the Na/K-ATPase; their work indicated the possibility of more than one Na/K-ATPase isoform (G. E. G. Shull, J.; Lingrel, J.B, 1986; G. E. S. Shull, A.; Lingrel, J.B, 1985). Sweadner further discovered there are four different isoforms of the Na/K-ATPase in rat tissues (Sweadner, 1989). These isoforms are expressed in a tissue-specific manner, but the tertiary structure of each isoform is relatively the same: $\alpha 1$ is expressed in virtually all tissues (especially endothelium), $\alpha 2/\alpha 3$ are expressed in cardiac myocytes, skeletal muscle, and neurons, and $\alpha 4$ is primarily found in the testis and is involved with sperm

motility (Blanco, Melton, Sanchez, & Mercer, 1999; Sweadner, 1989; Z. Xie & Cai, 2003).

Finally, in 1987 Marks and Seeds confirmed the previous discovery regarding Na/K-ATPase isoforms (Marks, 1978).

Between 1987 and 1990, the crystal structure of the Na/K-ATPase was determined. It contains 10 transmembrane helices, with both N and C domains in the cytoplasm, and 2 non-covalently linked α and β subunits (Laursen, Yatime, Nissen, & Fedosova, 2013; Morth et al., 2007; Nyblom et al., 2013; Z. Xie, 2003). The α subunit consists of the actuator domain, the second cytosolic domain, the phosphorylation domain and the nucleotide binding domain, and the β subunit is essential for the formation of a competent pump [reviewed in (Blanco, 2005; Z. Xie, 2003; Z. Xie & Askari, 2002)].

During the early 21st century it was determined that the Na/K-ATPase belongs to a class of ion pumps known as the P-Type ATPases. This family of ATPases is also known as the E1/E2 ATPases and are known for their role in carrying out many fundamental biological and physiological processes; the Na/K-ATPase is a unique P-Type ATPase in its ability to bind CTS, such as ouabain (Jorgensen & Andersen, 1988; Kaplan, 2002; Lutsenko & Kaplan, 1995; Z. Xie & Cai, 2003).

Years of research has shown that through binding interactions between the $\alpha 1$ subunit of the Na/K-ATPase and Src, a signaling cascade is produced that has various effects on both protein and lipid cascades, responsible for cell growth and cellular homeostasis (Z. Li et al., 2009; Z. Li & Xie, 2009; Z. Xie, 2003; Z. Xie & Cai, 2003; Z. Xie et al., 1995; Z. Xie et al., 1999). Specifically, the Na/K-ATPase $\alpha 1$ subunit and Src are bound at multiple locations, seen in Figure 1 [subject reviewed in (Tian et al., 2006; Y. Wang et al., 2014; Z. Xie, 2003; Z. Xie & Askari, 2002)]. Under normal physiological conditions, interaction at both the kinase domain and SH2 domain of Src with the Na/K-ATPase $\alpha 1$ subunit ND1 and CD2 segments keeps the molecule in an E1 or “inactive” state (Jorgensen & Andersen, 1988; Z. Xie, 2003; Z. Xie & Askari, 2002; Z. Xie & Xie, 2005).

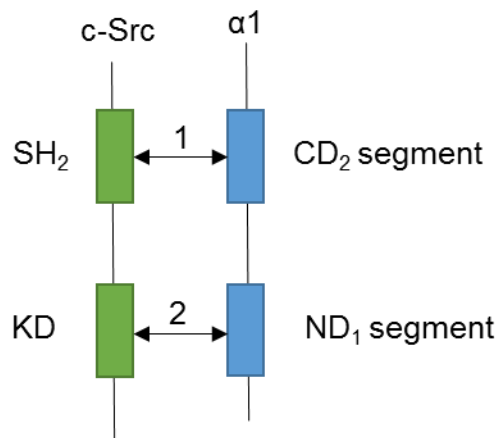


Figure 1. Na/K-ATPase $\alpha 1$ and Src Interacting Domains.

Specific binding at multiple locations on both the $\alpha 1$ subunit and c-Src are responsible for a signaling cascade that has various effects on protein and lipid cascades and impacts cell growth and cellular homeostasis.

In cell culture models, ouabain binding to the Na/K-ATPase at its specific binding site causes two things to occur: increased intracellular Na^+ leads to an inhibition of pumping and Na/K-ATPase associated c-Src is activated (Aizman, Uhlen, Lal, Brismar, & Aperia, 2001; Haas, Askari, & Xie, 2000; J. Liu et al., 2000; Peng, Huang, Xie, Huang, & Askari, 1996). This phosphorylation of Na/K-ATPase-bound Src stimulates protein tyrosine phosphorylation (at

tyrosine residue 418 (Tyr 418) on Src) and results in the downstream signaling cascade; this pushes the pump into an E2 or “active” state (Figure 2). (Haas et al., 2000; Jorgensen & Andersen, 1988; Z. Li & Xie, 2009). Ouabain activates Na/K-ATPase associated Src at Tyr 418, this is kind of a repeat of previous sentence increases Src binding to the Na/K-ATPase, and this activation is the crucial first step in the downstream signaling cascade (Z. Xie, 2003; Z. Xie & Askari, 2002).

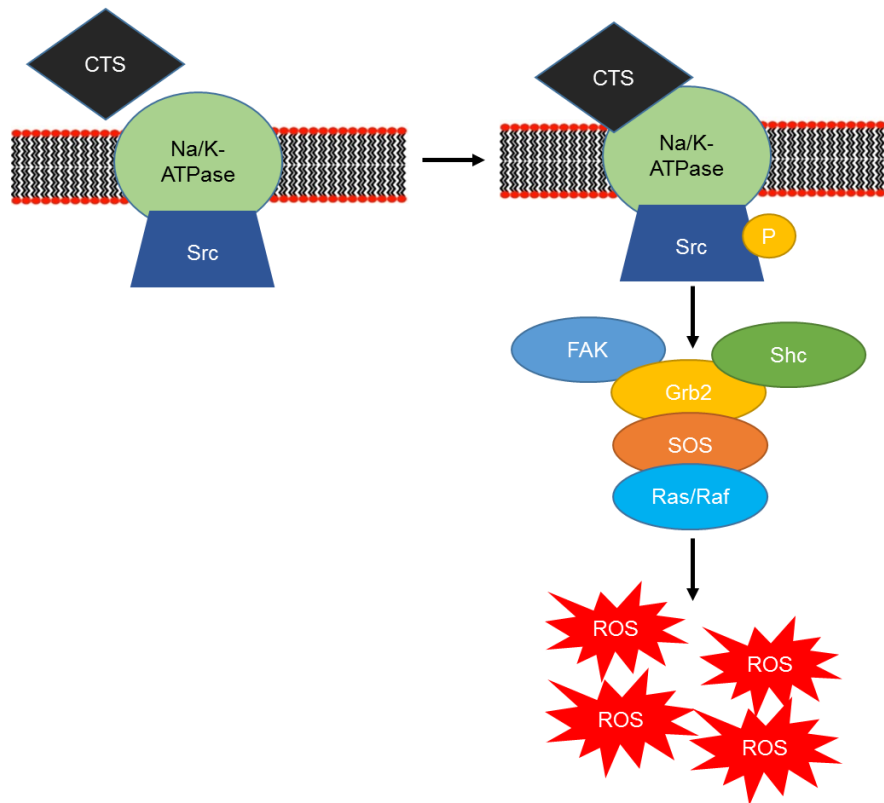


Figure 2. Na/K-ATPase Signaling Cascade.

When stimulated by cardiotonic steroids (CTS) such as ouabain, c-Src bound to the Na/K-ATPase is phosphorylated; this phosphorylation activates the signaling cascade.

The Na/K-ATPase has multiple signaling partners and its downstream signaling/protein tyrosine effects have been implicated in many signaling pathways and disease states (Z. Xie, 2003; Z. Xie & Askari, 2002; Yan et al., 2016). Xie et al proposed that there are three potential

chemical classes (pathological and physiological) that can serve as Na/K-ATPase ligands: cardiac glycosides, chemical modifiers, and ionic alterations(Z. Xie, 2003).

Cardiac glycosides are a class of organic compounds that increase contraction rate in the heart by increasing calcium alterations via interactions with the Na/K-ATPase (Oweis et al., 2006; Pierre & Xie, 2006; Z. Xie & Xie, 2005). Cardiac glycosides can be exogenous, like the commonly used heart failure medication digoxin, but they have also been found to be endogenous. Ouabain is known best for its role in modulating blood pressure and its role in hypertension; it is regulated by hormones such as adrenocorticotrophic hormone and angiotensin II (Ang II) (Blaustein & Hamlyn, 2010; Dostanic et al., 2005; Z. Li & Xie, 2009; Z. Xie, 2003). As a specific ligand, ouabain exerts very significant effects on the Na/K-ATPase, such as inhibiting ion-pumping activity as well as activating Src associated with the Na/K-ATPase (Z. Li & Xie, 2009; Z. Xie, 2003). Na/K-ATPase associated c-Src, once active, then converts and amplifies the ligand binding signal to cause significant increases in protein tyrosine phosphorylation and increases the activity of downstream kinase activity(Z. Xie, 2003). It is known that in low doses, ouabain induces substantial endocytosis of the Na/K-ATPase in a Src dependent manner; the endocytosis and subsequent recycling of the pump is a necessary process that is mediated by Src and caveolin-1(J. Liu et al., 2004; J. Liu et al., 2005; Z. Xie, 2003).

Chemical modifiers that irreversibly or reversibly alter Na/K-ATPase can also act as ligands(Z. Xie, 2003). The most prominent of these examples is reactive oxygen species (ROS). ROS are chemically reactive, and contain oxygen. There are several examples including peroxides, superoxide, and hydroxyl radicals, among more (J. Liu et al., 2000; Lushchak, 2014; Sodhi et al., 2015; Z. Xie & Cai, 2003; Z. Xie et al., 1999; Yan et al., 2013; Yan et al., 2016). ROS are a natural byproduct of normal oxygen metabolism, and have roles in cell signaling and

homeostasis. During times of “stress” (low oxygen levels, starvation, inflammation, etc.) ROS levels can increase dramatically and lead to cellular damage (Furukawa et al., 2004; Jakus, 2000; Sodhi et al., 2017; Srikanthan, Feyh, Visweshwar, Shapiro, & Sodhi, 2016; Srikanthan, Shapiro, & Sodhi, 2016; Vincent & Taylor, 2006). Mitochondria are potent ROS producers, and give off ROS during the production of ATP in the electron transport chain (Halliwell, 1989; Harman, 1992; Yan et al., 2013). The free radical oxygen produced during the electron transport chain is normally converted to water, but in a small number of electrons passing through the chain, the free radical is released. In disease states such as obesity, an increased inflammatory profile is implicated with increased ROS (Fernandez-Sanchez et al., 2011; Furukawa et al., 2004; Lafontan, 2014; Le Lay, Simard, Martinez, & Andriantsitohaina, 2014). When an increased level of ROS is present, it can damage RNA/DNA, increase lipid peroxidation, and oxidize amino acids in proteins (Halliwell, 1989; Jakus, 2000). Ouabain binding can increase mitochondrial ROS production (J. Liu et al., 2004). This increase in ROS production can lead to abnormal signaling, desensitization of the Na/K-ATPase to stop signals, and throws the Na/K-ATPase into what is known as an oxidant amplification loop; this loop has been implicated in several disease states over the last few years (J. Liu et al., 2016; Sodhi et al., 2015; Sodhi et al., 2018; Sodhi et al., 2017).

To further understand the interaction between $\alpha 1$ and c-Src, the domain between the two was mapped. This mapping led to the discovery of two separate binding domains that are important for c-Src targeting and the signaling cascade, seen above in Figure 1 (Z. Li et al., 2009). Specifically, the SH₂ and KD (kinase domain) of c-Src and the CD₂ and ND₁ segment of $\alpha 1$ are necessary for proper signaling and binding. The SH₂ domain is crucial for the targeting of c-Src, but the real signaling power comes from the interaction between the KD of c-Src and ND₁

segment of $\alpha 1$ (Z. Li et al., 2009; Z. Xie, 2003). When bound, the $\alpha 1/c$ -Src complex is inactive, and takes on an E1 conformation; when stimulated by ouabain or ROS, the bond between KD and ND₁ is broken, pushing the receptor complex into an E2 state and allowing the signaling cascade to begin (Blanco, 2005; Tian et al., 2006; Z. Xie et al., 1995). Tyr 418 on c-Src is phosphorylated and therefore activated. This allows for phosphorylation on other proteins such as MAPK, ERK1/2, IP3, as well as other proteins in various pathways that have yet to be elucidated (Aperia, 2007; Z. Xie, 2003).

Mapping of these domains led to the identification of a 20 amino acid sequence that could inhibit c-Src activation, called NaKtide. This peptide was derived from the ND₁ segment, and therefore mimics binding to the KD, which prevents c-Src phosphorylation, inhibiting the signaling cascade (Z. Li et al., 2009).

NaKtide ranges from serine residue 415 (Ser 415) to glutamine residue 434 (Gln 434) and acts on Na/K-ATPase c-Src complexes outside of the cell membrane (it is not cell permeable) (Z. Li et al., 2009). In order to further explore this relationship, a cell-permeable version of NaKtide was created, pNaKtide. pNaKtide functions similarly to NaKtide, by targeting the receptor complexes close to the plasma membrane inside the cell; its cell permeability comes from a 13 amino acid twin-arginine translocation (TAT) leader sequence, which makes the peptide positive (Z. Li et al., 2009). Both NaKtide and pNaKtide act on c-Src but the major difference lies in it is cell membrane permeable or not. (Z. Li et al., 2009).

For close to a decade pNaKtide has been used as a specific antagonist of Na/K-ATPase signaling. It seems to be an ideal candidate for treatment of obesity, inflammation, nonalcoholic steatohepatitis (NASH), uremic cardiomyopathy, and seems to reverse the aging process (Bartlett,

Miller, Thiesfeldt, Lakhani, Khanal, et al., 2018; J. Liu et al., 2016; Sodhi et al., 2015; Sodhi et al., 2018; Sodhi et al., 2017). Recently, pNaKtide has been shown to decrease c-Src activation, carbonylation and damage from ROS, as well as decreasing adiposity and other biomarkers (J. Liu et al., 2016; Sodhi et al., 2017). Previous data has shown a potentially (and slightly) toxic effect for NaKtide, which was ameliorated with development of pNaKtide (Z. Li et al., 2009).

Adipocyte Biology

Obesity is currently named as the most important noncommunicable disease. In 1990, the prevalence of obesity was no more than 15% across the United States; as of 2010, the incidence is no less than 20%, with some states creeping towards the 30% or higher mark (Courtesy of the CDC). It is a multifactorial disease, and in more recent years has been characterized as a low grade chronic inflammatory disease that is linked to metabolic disorders including Type 2 diabetes and insulin resistance, nonalcoholic steatohepatitis (NASH), cardiovascular disease, and has more recently been implicated in neurodegenerative disorders (Figure 3) (Kothari et al., 2017; Sodhi et al., 2015; Sodhi et al., 2017; Srikanthan, Shapiro, et al., 2016; E. Stolarczyk, 2017). Adipose tissue was previously thought to only be an energy reservoir, but research has shown that it plays a larger role in whole body homeostasis; adipose tissue is a secretory endocrine organ that is responsible for the secretion of between 50 and 100 cytokines, hormones, and proteins that impact the functionality of cells and tissues everywhere in the body (Gomez-Hernandez, Beneit, Diaz-Castroverde, & Escribano, 2016; Greenberg & Obin, 2006).

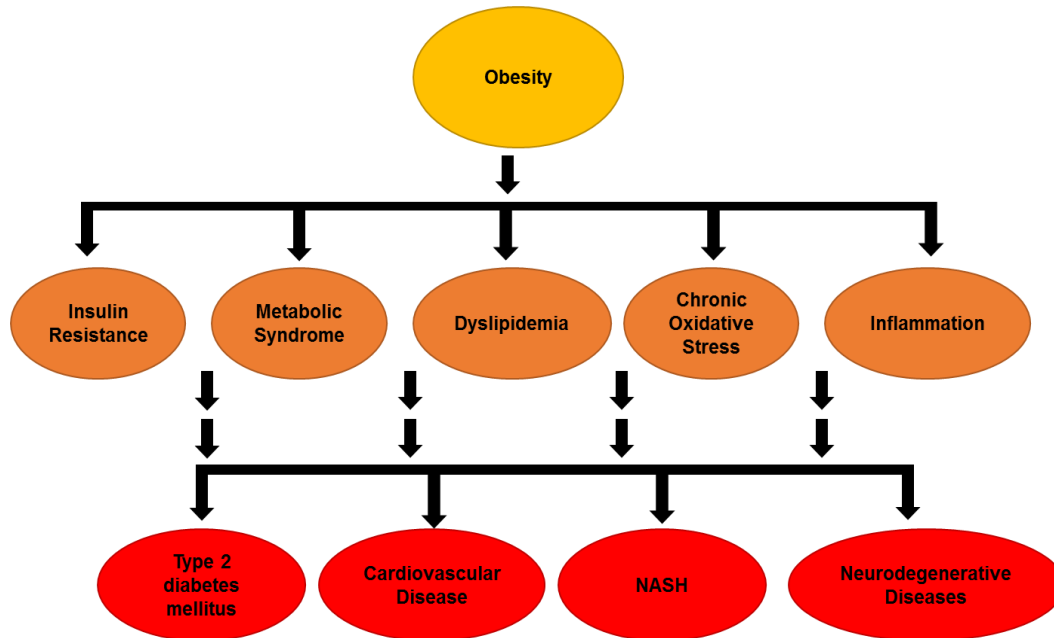


Figure 3. Obesity and Related Comorbidities.

Obesity has been classified as a low grade chronic inflammatory disease that is linked to metabolic disorders including Type 2 diabetes and insulin resistance, nonalcoholic steatohepatitis (NASH), cardiovascular disease, and has more recently been implicated in neurodegenerative disorders

White adipose tissue is classically spherical in shape, ranging in size from 25 μM to 200 μM . The nucleus is flat and the cytoplasm is thin. The majority of the cell's size (~90%) is taken up by a single large lipid droplet; there are limited mitochondria and small amounts of smooth and rough endoplasmic reticulum. The adipocytes are held together by poorly vascularized and innervated connective tissue. Obesity triggers accumulation of lipids in adipocytes, triggering cellular stress, and activating apoptotic and inflammatory pathways (Greenberg & Obin, 2006; Lumeng, Bodzin, & Saltiel, 2007). Adipocytes are one part of what makes up adipose tissue. White adipose tissue is made up of adipocytes, macrophages, leukocytes, fibroblasts, cell progenitors, and endothelial cells; this diverse mix gives evidence to the vast array of hormones, cytokines, and proteins that are secreted by white adipose tissue (Gil, Olza, Gil-Campos, Gomez-Llorente, & Aguilera, 2011; Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016). Adipose

tissue macrophages (ATMs) accumulate in adipose tissue with adiposity. ATMs are a prominent source of proinflammatory cytokines that block insulin action in the adipocytes and are augmenters of ROS; in fact, ATMs are responsible for most of the cytokine production from dysfunctional adipose tissue (Greenberg & Obin, 2006; Lumeng et al., 2007).

Macrophage activation has been defined across two polarization states, and in normal conditions, macrophages are likely activated as a continuum between the two states: the classically activated M1 macrophages and the alternatively activated M2 macrophages (Hausman, DiGirolamo, Bartness, Hausman, & Martin, 2001; Lumeng et al., 2007). M1 macrophages are induced by proinflammatory mediators and have enhanced proinflammatory cytokine production (tumor necrosis factor α (TNF α) and interleukin-6 (IL-6)) and generate significant amounts of ROS (Hausman et al., 2001; Lumeng et al., 2007). M2 macrophages, on the other hand, are generated by exposure to interleukin-4 (IL-4) and interleukin-13 (IL-13), and have a higher expression level of anti-inflammatory cytokines compared to pro-inflammatory cytokines (Greenberg & Obin, 2006; Lumeng et al., 2007). Exposure to high fat diet (HFD) feeding causes a phenotypic switch from M2 to M1 macrophages, switching the cell from an anti-inflammatory state to a pro-inflammatory state; this shows that ATMs are a significant contributor to inflammation in obesity (Greenberg & Obin, 2006; Lumeng et al., 2007; Medh, 2002).

The inflammatory response in obesity appears to be triggered by, and reside, in the adipose tissue itself (Wellen & Hotamisligil, 2003). Obesity features both metabolic and inflammatory pathway abnormalities, through dysregulated production of the released factors, or adipokines (Sodhi et al., 2015; Sodhi et al., 2017; Wellen & Hotamisligil, 2003). Hypertriglyceridemia, hyperglycemia, and oxidative stress increases plasma free fatty acids

(FFA) in circulation, which further increases oxidative stress; these events can trigger inflammatory responses in other areas of the body, including the liver (via the portal vein) and the brain (through systemic circulation) (Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016; Sodhi et al., 2015; Sodhi et al., 2017; Wellen & Hotamisligil, 2003). ROS production increases in parallel with fat accumulation. ROS upregulates expression of proinflammatory cytokines and macrophage chemoattractive molecules, which stimulates the production of more ROS (Srikanthan, Shapiro, et al., 2016; Wellen & Hotamisligil, 2003). This leads to a vicious cycle of ROS production that throws the already imbalanced pro/anti-oxidant system into further distress, leading to an increase in systemic oxidative stress and maintenance of the obese phenotype. This increase in ROS also leads to hyperglycemia and hyperlipidemia. Hyperglycemia is caused when ROS activates polyol and hexosamine pathways, which leads to the production of advanced glycation end products and increases in diacylglycerol synthesis (Boyer et al., 2015; Le Lay et al., 2014). Hyperlipidemia results from increased lipid oxidation.

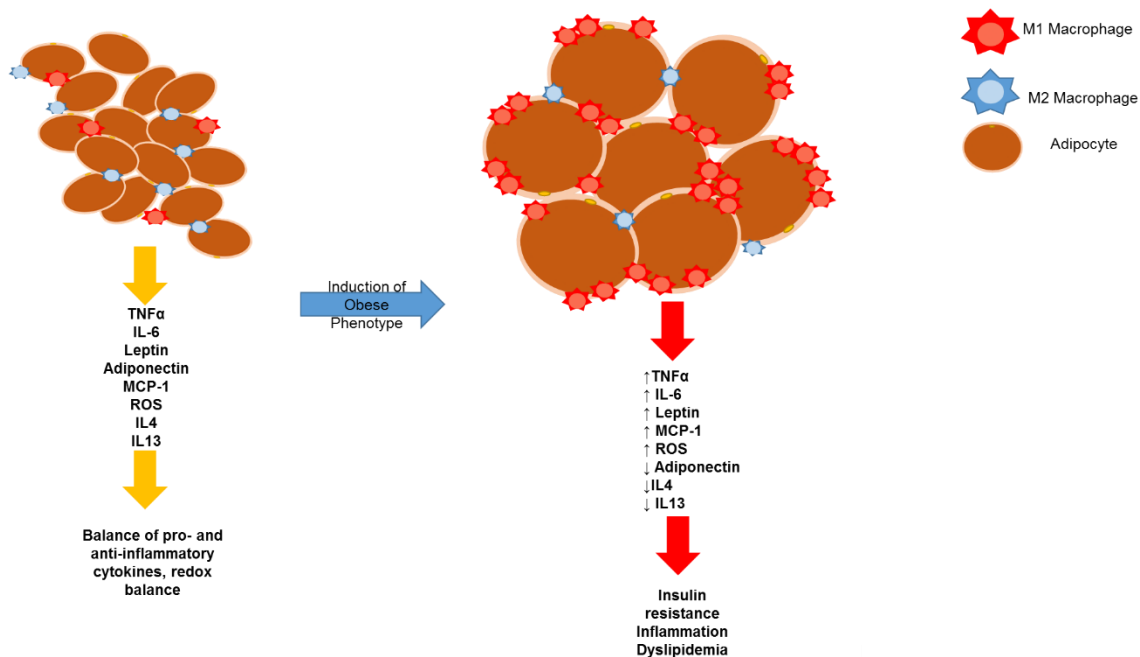


Figure 4. Differences in adipose tissue in lean vs obese scenario.

Adipose tissue contains a milieu of components, including adipocytes, macrophages, leukocytes, fibroblasts, cell progenitors, and endothelial cells; this diverse mix gives evidence to the vast array of hormones, cytokines, and proteins that are secreted by white adipose tissue. The inflammatory response characteristic of obesity appears to be triggered by and reside in the adipose tissue itself.

TNF α and IL-6 are local inflammatory markers generated by obesity. TNF α levels in plasma are positively correlated with the size of adipose depots, and increased TNF α levels are linked to development of insulin resistance (Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016). Under ordinary conditions, TNF α activates lipolysis and inhibits the expression of lipoprotein lipase (LPL) and glucose transporter type 4 (GLUT4) as a mechanism to reduce excessive size of adipose tissue depots. LPL is an important factor in lipid metabolism, and studies have shown that relative levels of LPL activity in adipose tissue determine whether lipids are stored or utilized (Garcia-Arcos et al., 2013; Mead, Irvine, & Ramji, 2002). Increased insulin in the blood, after increased presence of glucose in the blood and activation of glucose receptors, causes an increase of glucose uptake into cells through GLUT4. Abnormal levels of TNF α could

be accountable for the metabolic alterations seen in obesity, including abnormal action of LPL and GLUT4 (Garcia-Arcos et al., 2013). Further, TNF α increases FFA levels, induces insulin resistance, and has an inhibitory effect on insulin action in the liver; this causes an increase in hepatic glucose production (Cawthorn & Sethi, 2008; Greenberg & McDaniel, 2002; Lumeng et al., 2007). Activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) pathway in adipose tissue may also contribute to metabolic abnormalities influenced by TNF α (Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016; Kothari et al., 2017). Increased FFA are known to promote insulin resistance in the muscle by secondary alterations in phosphoproteins (perilipins) found on the surface of the adipocyte (Greenberg & Obin, 2006). TNF α inhibits insulin-stimulated autophosphorylation of the insulin receptor, and phosphorylation of the insulin receptor substrate 1 (IRS1) in vivo; regulation of glucose transporter number and activity, modulation of steps downstream from IRS1, and even inhibiting transcription of IRS1 and GLUT4 may be part of the complex process (Hotamisligil, Arner, Caro, Atkinson, & Spiegelman, 1995; Z. Liu et al., 2012; E. Stolarczyk, 2017). TNF α has been implicated in activation of signaling transduction pathways leading to proapoptotic effects in virtually all tissues, and has even more recently been connected to neurodegenerative disorders and progression of the insulin resistant component of Alzheimer's disease (Cawthorn & Sethi, 2008; Kothari et al., 2017).

IL-6 is another proinflammatory cytokine implicated in the negative prognosis of obesity and other diseases. Its role in adiposity is similar to TNF α ; it sports pro and anti-inflammatory properties and increases insulin resistance by inhibiting LPL (E. Stolarczyk, 2017). This inhibition leads to increases in fat cell size as they store more lipids. IL-6, like TNF α , is secreted primarily by macrophages in adipocytes. TNF α and IL-6 are some of the few cytokines that are

able to cross the blood brain barrier, and some studies have shown that they are capable of making the blood brain barrier permeable to other cytokines, making it a factor in increased inflammation in neurodegenerative disorders (Greenberg & Obin, 2006; Youdim, Lavie, & Riederer, 1994).

Leptin, adiponectin, and Ang II are protein hormones that are associated with the regulation of body weight and vascular function; unlike IL-6 and TNF α , whose functions in obesity are very similar, these three protein hormones have very different roles. Ang II has a positive effect on differentiation of adipose tissue, and regulates adiposity due to its lipogenic actions; it is a pro-oxidant molecule and under abnormal conditions (i.e. increased adiposity in obesity) it stimulates NADPH oxidases and activates MAPK and protein kinase pathways, leading to increases of ROS (Cao, Sodhi, Inoue, et al., 2011; Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016; Srikanthan, Shapiro, et al., 2016). Plasma Ang II has been shown to increase with increasing body weight, and in addition to its role in adiposity, it has been shown to play a critical role in cardiovascular diseases (Cao et al., 2012; Cao, Sodhi, Inoue, et al., 2011).

Both adiponectin and leptin are important hormones with central and peripheral effects on metabolism and energy balance by reducing circulating FFA (Greenberg & Obin, 2006). Adiponectin is only produced by adipocytes, and enhances insulin sensitivity in muscle and liver by increasing FFA oxidation and decreasing glucose production; it is inversely related to adiposity, and as lipids increase and the adipocyte grows, adiponectin release is decreased (therefore decreasing its beneficial effects) (Greenberg & Obin, 2006; E. Stolarczyk, 2017). Adiponectin is also responsible for suppressing M1 macrophage activation and promoting M2 proliferation (E. Stolarczyk, 2017).

Leptin is primarily produced by adipocytes, but some leptin can also be produced by other tissues (it has been argued that these sources of leptin are more local mediators than systemic, and the major source of leptin is adipocytes) (Trayhurn & Bing, 2006). Leptin has a direct action on the hypothalamus to reduce food intake; levels rise significantly in obesity and targeted tissues become resistant to its effects. It also has a role in regulating the immune response, as an inflammatory molecule, and can activate adaptive immune cells (such as B and T cells) in different nutritional states (obese, malnourished, etc.) (E. Stolarczyk, 2017). Leptin exerts central effects on appetite and peripheral effects on energy expenditure; in fact, significantly increased leptin (as seen in obesity) inhibits dopamine receptor 2 signaling and transcription in the brain, leading to decreased dopamine receptors, decreased dopamine and tyrosine hydroxylase, and a decrease in physical activity (Fulton et al., 2006; Ishibashi et al., 2012; Opland, Leininger, & Myers, 2010; Perry et al., 2010). Abnormal levels of leptin also promote inflammation and lipid peroxidation, leading to an increase in oxidative stress in obese states (Srikanthan, Shapiro, et al., 2016). Low adiponectin and high leptin seen in obesity are associated with a significantly increased risk for metabolic and cardiovascular complications associated with obesity, including hypertension, Type 2 diabetes, NASH, and neurodegeneration (Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016; Park et al., 2010; Sodhi et al., 2017).

Traditionally, fat can be broken down into two categories, and there are many differences between the two. White fat has been discussed extensively above, and is what is more typically implicated in obesity and metabolic syndrome. Brown fat is typically found in the supraclavicular and spinal region of infants and rodents, but studies have shown that human adults also have brown fat specific depots (Gomez-Hernandez, Beneit, Diaz-Castroverde, et al.,

2016). Whereas white fat is primarily an energy storing molecule, brown fat can dissipate stored chemical energy as heat (therefore giving it an energy releasing phenotype)(Alvarez et al., 1995; Ballinger & Andrews, 2018). Brown fat is richly innervated and uses the mitochondrial uncoupling protein 1 (UCP1) to uncouple ATP from the electron transport chain, and use the energy to combat hypothermia, obesity, and diabetes; brown fat can be activated by either cold exposure, or β adrenergic signaling (Barbatelli et al., 2010; Bernardis & Bellinger, 1991; Gomez-Hernandez, Benoit, Diaz-Castroverde, et al., 2016).

Brown and white fat come from two different cellular lineages. Brown fat comes from a myogenic factor 5 (Myf5+)/ paired box protein (Pax7+) cellular lineage; muscle tissue also stems from this cellular lineage(Christian & Parker, 2010; Garcia-Arcos et al., 2013; Gomez-Hernandez et al., 2012). The PR-domain contain 16 protein (PRDM16) is an important transcriptional factor for brown adipocytes; in-vitro studies have shown it can be a determining factor in the muscle/brown adipose Myf5+ lineage. If PRDM16 is knocked out, then the cells become muscle (Christian & Parker, 2010; Garcia-Arcos et al., 2013; Gomez-Hernandez et al., 2012). While the cellular lineages may be different, the core elements of the adipogenic cascade are shared by all types of fat. The master regulator of fat cell formation is peroxisome proliferator-activated receptor gamma (PPAR γ) (Srikanthan, Shapiro, et al., 2016). CCAAT Enhancer Binding Protein alpha (CEBP α) is an important transcriptional cofactor and is locked in a differentiation loop with PPAR γ (as one increases so does the other) (Carmona et al., 2005; Kajimura et al., 2008; Kozak, 2011). To a point, this cascade is the same for both white and brown fat. Early B Cell Factor 2 (ebf2) is an important protein that recruits PPAR γ to sites that will be brown fat (Elattar & Satyanarayana, 2015; J. Wang & Tontonoz, 2017).

While the differentiation cascade is similar, the function of brown fat is significantly different from white fat. Brown fat consists of brown adipocytes with a polygonal shape, centered oval nucleus, and a large cytoplasm that contains multiple small lipid droplets (Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016; Gomez-Hernandez, Beneit, Escribano, et al., 2016). It has a large number of mitochondria, is highly vascularized, and is responsible for the secretion of a different set of adipokines (sometimes known as “batokines”)(Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016). Brown adipose tissue doesn’t accumulate macrophages, due to the larger number of mitochondria present; the macrophages that brown adipose acquires have a different expression profile compared to white adipose tissue macrophages (Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016; Greenberg & Obin, 2006; Wellen & Hotamisligil, 2003). Activation of brown adipose tissue has been implicated as a protective effect against obesity and reduces adiposity (Gil et al., 2011; Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016).

Brown adipocytes can sometimes be found inside white adipose; they do not come from the same Myf5 lineage as brown cells, and are known as “beige” adipocytes(Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016). Beige fat is a unique type of fat that is genetically distinct. Beige cells seem to be capable of expressing abundant UCP1. They express the ability to switch between an energy storing and energy releasing phenotype, depending on environmental cues/conditions, and have a high respiratory capacity (Ahima, 2016; Becerril et al., 2013; Chondronikola & Sidossis, 2019). They have characteristics of both white and brown fat; while white fat cannot be completely changed to brown fat, under certain conditions, it can gain a beige phenotype or undergo what is called “beiging” of fat (Cohen & Spiegelman, 2015; Dempersmier & Sul, 2015). When unstimulated, these beige cells have very low basal UCP1

expression, similar to white adipose; but once stimulated, these cells have a thermogenic profile similar to brown fat suggesting that the cells are bifunctional, suited for energy storage in the absence of thermogenic stimuli but fully capable of turning on energy releasing mechanisms when necessary (Harms & Seale, 2013). These cells have an overlapping but distinct gene pattern compared to classical brown fat (Carobbio, Guenantin, Samuelson, Bahri, & Vidal-Puig, 2019; Cohen & Spiegelman, 2015). Another feature of beige fat is that it has a higher proportion of M2 macrophages compared to M1 and an anti-inflammatory cytokine profile, indicating a lean towards a healthier metabolic phenotype(Harms & Seale, 2013).

Beige cells are highly responsive to the polypeptide hormone irisin (Bostrom et al., 2012; M. V. Wu, Bikopoulos, Hung, & Ceddia, 2014). Irisin is released during exercise and secreted by skeletal muscle; it has been shown to induce browning of subcutaneous adipose tissue(Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016). Irisin has little effect on brown fat itself, suggesting that the responsiveness to irisin may be a selective feature of beige cells localized in subcutaneous white adipose tissue(Gil et al., 2011; Gomez-Hernandez, Beneit, Diaz-Castroverde, et al., 2016).

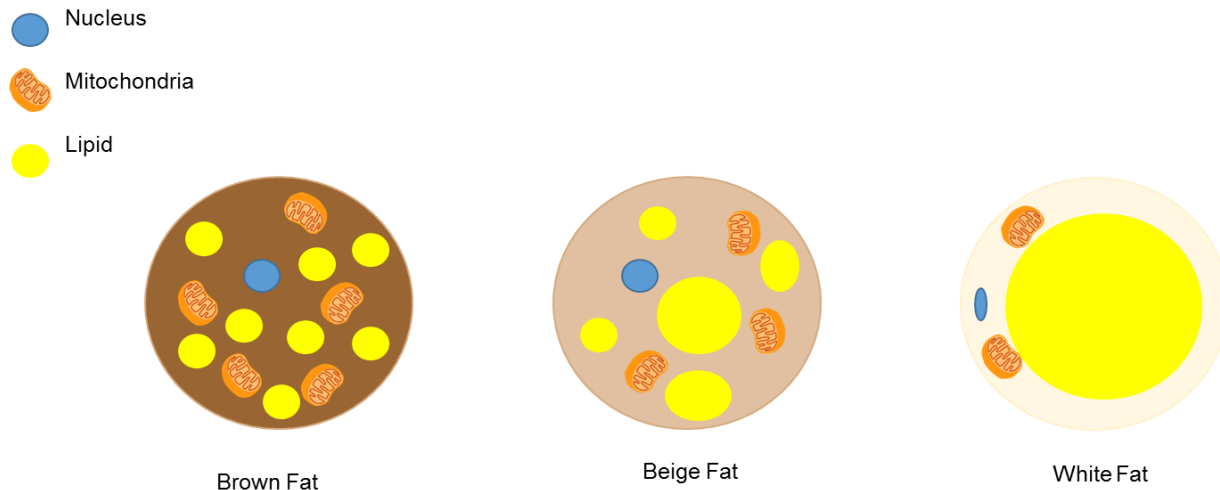


Figure 5. White Adipose vs Brown Adipose vs Beige Adipose.

White fat cells are the most common fat cell; they are used to store fat and are found subcutaneously and surrounding organs in the abdomen. Brown fat converts chemical energy to heat to protect against certain stimuli. Beige fat is a hybrid type of fat that behaves similarly to both types of fat.

Connections between Na/K-ATPase Oxidant Amplification Loop and Obesity: Current Research

ROS has been implicated in both abnormal Na/K-ATPase signaling and the exacerbation of obesity; simply put, the pathological natures of dysregulated Na/K-ATPase signaling and dysfunctional adiposity are linked by ROS.

ROS has been established as one of the chemical classes that can act as a ligand for the Na/K-ATPase; it is a chemical modifier that can either reversibly or irreversibly change the conformation of the Na/K-ATPase (Z. Xie, 2003). Binding of ouabain to the Na/K-ATPase results in activation of the Ras-Raf-MEK-ERK pathway, which subsequently leads to activation of MAPK and an increase in intracellular Ca^{2+} concentrations; this is responsible for the opening

of mitochondrial ATP-sensitive K⁺ channels and increased mitochondrial ROS production (Greenberg & Obin, 2006; Srikanthan, Shapiro, et al., 2016; Z. Xie, 2003).

Increased ROS production from obesity (a disease of chronic inflammation) can oxidize the Na/K-ATPase, which inhibits its activity and promotes susceptibility to degradation by proteosomal and endosomal/lysosomal proteolytic pathways; this oxidized modification is normally reversible, but increased presence of ROS speeds it up, leading to a decrease in functional Na/K-ATPase α 1, and a decrease in the ability of the cell to “rescue” recycled Na/K-ATPase (Blanco, 2005; J. Liu, Kennedy, Yan, & Shapiro, 2012; Srikanthan, Shapiro, et al., 2016; Z. Xie, 2003; Yan et al., 2013).

It has been discussed above that ROS production increases in parallel with fat accumulation in obesity; macrophages infiltrate adipose tissue and increase inflammatory cytokines and augment ROS production, which in turn upregulates expression of proinflammatory cytokines and stimulates more ROS production (Srikanthan, Shapiro, et al., 2016; Z. Xie, 2003). Obesity tends to decrease expression and activity of cytoprotective systems including heme oxygenase 1 (HO-1) and adiponectin while increasing inflammation (Cao et al., 2012; Cao, Sodhi, Inoue, et al., 2011). Hypertriglyceridemia and hyperglycemia, both associated with increased adiposity, have been shown to increase plasma FFA into circulation, which can lead to increased ROS generation and oxidative stress (Sodhi et al., 2017). The imbalance of pro- and anti-oxidants that comes from an obese phenotype results in systemic oxidative stress, which maintains the phenotype and sustains oxidation of the Na/K-ATPase, leaving it in a constant E2 conformation. This prevents the Na/K-ATPase signaling cascade from turning off, and leads to abnormal signaling phenomena, increased c-Src activation, further increased ROS expression, and exacerbation of conditions related to redox imbalance (i.e. obesity, dyslipidemia, diabetes,

and atherosclerosis) (Bartlett, Miller, Thiesfeldt, Lakhani, Khanal, et al., 2018; Sodhi et al., 2017; Srikanthan, Shapiro, et al., 2016; Z. Xie, 2003; Yan et al., 2013). This points to the interaction between ROS, obesity, and the Na/K-ATPase, indicating that the Na/K-ATPase is a feed forward amplification loop for oxidants; it acts as a receptor for ROS, inducing conformational changes in the $\alpha 1$ subunit and an impaired signaling profile that intensifies the development of and pathogenesis of obesity (J. Liu et al., 2016; Sodhi et al., 2015; Sodhi et al., 2018; Sodhi et al., 2017).

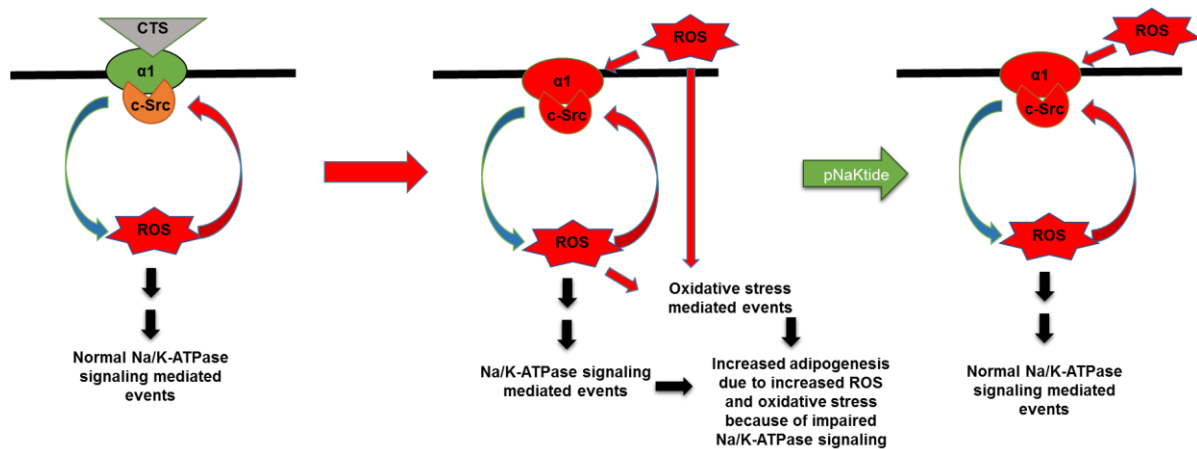


Figure 6. Proposed ROS Mediated Interaction Between Na/K-ATPase and Obesity.

There is a complex interaction between ROS, obesity, and the Na/K-ATPase, which indicates that the Na/K-ATPase is a feed forward amplification loop for oxidants; it acts as a receptor for ROS, inducing conformational changes in the $\alpha 1$ subunit and an impaired signaling profile that intensifies the development of and pathogenesis of obesity.

When administered systemically, pNaKtide was shown to decrease adiposity, improve glucose tolerance, decrease incidence of NASH, and decrease the inflammatory profile of mice given a western diet (WD) (Sodhi et al., 2015). Dysfunctional adipogenesis is a hallmark of obesity and is characterized by altered endocrine function of adipose tissue; adipose tissue regulates energy metabolism via the secretion of adipokines. Dysregulated production of these adipokines has harmful effects including increased obesity and an impaired metabolic profile (Sodhi et al., 2015; Srikanthan, Shapiro, et al., 2016). Given this, we wanted to examine whether

adipocyte-specific expression of NaKtide could have a similar ameliorative effect as when pNaKtide is administered systemically. This tissue specific administration was done via a lentiviral mechanism which allowed NaKtide to permeate the cell. We believe that using a lentiviral vector with NaKtide can both avoid the potentially toxic effect, and also prevent the risk of exocytosis of NaKtide to neighboring tissues.

Lentivirus is a complex retrovirus that has been rendered replication incompetent to prevent random mutations; lentivirus (with a specific gene/drug attached) infects the host cell and integrates its own genome into the host cell's DNA. Each new cell has expression of the drug or gene, but no new virus is formed (Figure 7) (Cao et al., 2012; Cao, Sodhi, Inoue, et al., 2011; Z. J. Liu et al., 2011; Manickam et al., 2018).

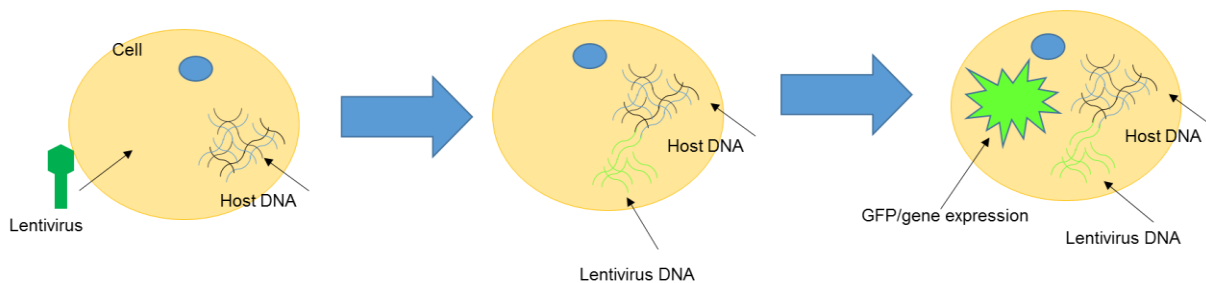


Figure 7. Lentiviral infection of host cell.

Lentivirus (with a specific gene or drug product) infects the host cell. It integrates its genome (and thus the gene or drug) into the host cell's DNA, so that each new cell will have expression of the gene or drug but lentivirus has been rendered replication incompetent. Each new cell will have expression of the drug, but no new virus is being

Over the last decade, lentivirus has emerged as a promising gene therapy. There is little to no risk of infection in humans. It is stable with low mutation rates and, most importantly, it provides the ability to study the effect of a drug or gene product on a specific tissue based on the specificity of the promoter (Cao et al., 2012; Cao, Sodhi, Inoue, et al., 2011; Gao et al., 2018). In the case of this work, an adiponectin promoter was used to drive the lentivirus with NaKtide to

adipose tissue. There are other viral transfection therapies. Adenovirus, influenza virus, and cytomegalovirus are three examples. There are individual risks associated with each of these viruses, but the overall problem is that it is difficult to knock out replication abilities in these viruses and thus humans are extremely susceptible to infection by these viruses(Bian, Bai, Zhao, Zhang, & Liu, 2013; Cao, Sodhi, Inoue, et al., 2011).

Previous data, and the data herein, suggest that adipocyte derived cytokines play an essential role in the development of obesity induced by a WD, and that targeting the adipocyte Na/K-ATPase may serve as an effective therapeutic strategy. Specific expression of NaKtide in adipocytes via lentiviral vectors modulates the adipocyte's redox state and ameliorates systemic oxidative stress, therefore preventing development of pathological adipocyte phenotype.

CHAPTER 2

ROLE OF ADIPOCYTES IN HYPERTENSION

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Introduction

Hypertension is defined as elevated blood pressure—typically a systolic blood pressure of ≥ 140 mmHg or a diastolic pressure ≥ 90 mmHg (or both) in a relaxed state (Abraham, Asija, Drummond, & Peterson, 2007; Adedayo et al., 2014; Canning, Brown, Jamnik, & Kuk, 2014). Currently, 29% (or 70 million) of Americans have been diagnosed with hypertension (Cohen & Spiegelman, 2015; Yang, Bi, & Kuang, 2014). There are several stages of hypertension, as well as salt sensitive and salt resistant types of hypertension (J. Chen et al., 2009). Hypertension may complicate and/or worsen other diseases such as diabetes, cardiovascular disease, chronic kidney disease, and obesity (Adedayo et al., 2014; Albert, Aguglia, Chiarle, Bogetto, & Maina, 2013; T. M. Brown et al., 2008; Herath Bandara & Brown, 2013). Obesity itself has reached pandemic proportions; according to the World Health Organization (WHO), over 500 million adults (10-14% of world population) were obese in 2008, and this number keeps increasing (Adedayo et al., 2014; T. M. Brown et al., 2008; Herath Bandara & Brown, 2013). As of 2014, this number has jumped to 600 million. There is an association between hypertension and obesity, but the mechanism(s) by which obesity predisposes to hypertension in humans has not been clearly established. In this review, we will explore some aspects of this important relationship.

The three known types of adipose tissue

Currently there are three known types of adipose tissue, each with its own specific characteristics: white, brown, and a mixture type, known as beige (or “brite”). The main purpose of adipose, regardless of the type, is to store excess energy that can be released as needed. The way the energy is stored varies between types. White adipose is the type one would think about when thinking about typical obesity. White adipocytes (WAT) are characterized by a spherical shape, a large lipid droplet that takes up 90 % of the volume of the cell, very few mitochondria,

and a flattened peripheral nucleus (Alvarez et al., 1995; Bonet, Serra, Matamala, Garcia-Palmer, & Palou, 1995; Brestoff et al., 2015; Farooqi & O'Rahilly, 2009; Fonseca-Alaniz, Takada, Alonso-Vale, & Lima, 2007; Gil et al., 2011). WAT can release triglycerides during a time of energy crisis in the body. WAT can be found virtually anywhere on the body, but are mainly located in subcutaneous abdomen, viscera, retroperitoneal, inguinal, and gonadal areas (Adams et al., 2008; Alvarez et al., 1995; Burgess et al., 2010; Cinti et al., 1997; Cohen, Ntambi, & Friedman, 2003). White adipocyte cells are known to secrete several kinds of proteins, such as inflammatory factors and the protein leptin (Abraham et al., 2014; Almabrouk, Ewart, Salt, & Kennedy, 2014; Bianco, Kieffer, & Silva, 1992; Cinti et al., 1997; Cohen & Friedman, 2004; Cohen et al., 2003).

Leptin is known as the satiety protein; when released, it inhibits feelings of hunger (Farooqi & O'Rahilly, 2009; Fonseca-Alaniz, Takada, et al., 2007). The antagonist of leptin is ghrelin; this is thought to be one of the hunger hormones (Cinti et al., 1997; Cohen & Friedman, 2004; Cohen et al., 2003; Farooqi & O'Rahilly, 2009). It has clearly been shown by Friedman and colleagues that patients with an inability to produce leptin develop profound hyperphagia and obesity (D. J. Friedman et al., 2009; J. Friedman, 2014; J. M. Friedman, 2011; Sennello et al., 2005). However, in most obese subjects, leptin levels are high (J. Friedman, 2014; J. M. Friedman, 2011). In these subjects, it is thought that as leptin levels are chronically elevated, responses to leptin are diminished (J. M. Friedman, 2011). Despite the high amount of energy already stored, the body ignores satiety signals and thinks it requires more energy to store; this higher level of leptin is consistent with a higher amount of adipocytes which are believed to be the primary source of leptin (J. Friedman, 2014; J. M. Friedman, 2011; Simonds et al., 2014; Y. T. Zhou et al., 1997). This hormone and protein releasing function therefore places white adipose

tissue as an endocrine organ (Fonseca-Alaniz, Takada, et al., 2007; Villacorta & Chang, 2015; M. V. Wu et al., 2014).

In addition to leptin, adipocytes also release other hormones and peptides, including TNF α and IL-6 (Sodhi et al., 2015; Sodhi et al., 2014; Wong, Ward, Fong, Yap, & Brown, 2015; Yoda et al., 2015). These are inflammatory cytokines, and the increased levels are indicative of inflammation in the body (Sodhi et al., 2014; Wong et al., 2015; Yoda et al., 2015). Whether this inflammation leads to increased reactive oxygen species (ROS) production creating oxidative stress or oxidative stress from signaling leads to inflammation is currently unclear.

Brown adipocytes (BAT) are a bit different from white adipocytes; they are polygonal in shape, contain fewer and smaller lipid molecules, have abundant mitochondria, and a central round nuclei (Alvarez et al., 2000; Alvarez et al., 1995; Bonet et al., 1995). Brown adipocytes are mainly found in the subscapular region of rodents and human infants (Alvarez et al., 1995; Martin et al., 1999). Whereas white adipocytes use lipids to store energy, brown adipocytes store energy in the form of fat and break them down to produce heat in a process known as non-shivering thermogenesis (Klingenspor, 2003; Klitsch & Siemen, 1991; Strack, Bradbury, & Dallman, 1995). Thermogenesis is the production of heat in an organism; non-shivering thermogenesis occurs in the brown adipose tissue because of the presence of thermogenin (Herron et al., 1990; Palou, Pico, Bonet, & Oliver, 1998; Puigserver, Vazquez, Bonet, Pico, & Palou, 1996; Rehnmark, Nechad, Herron, Cannon, & Nedergaard, 1990). Thermogenin (also known as uncoupling protein 1) allows the uncoupling of protons moving down their gradient from ATP synthesis; this energy is then dissipated as heat (Herron et al., 1990; Palou et al., 1998). Free fatty acids from the brown adipose tissue remove any proteins that could inhibit thermogenin (Puigserver et al., 1996; Rehnmark et al., 1990). Thermogenin then causes an influx

of H⁺ into the mitochondrial matrix, bypassing the ATP synthase normally used to make ATP (C. Z. Wang, Wei, Guan, & Xue, 2014; S. Wang et al., 2015; Xue et al., 2015; J. Zhou et al., 2015; Zietak & Kozak, 2015). This uncouples oxidative phosphorylation, and the energy normally used to convert ADP to ATP is released as heat (Sacks et al., 2009; Xing, Yang, & Wang, 2015; Yan et al., 2013; J. Zhou et al., 2015). Interestingly, thermogenesis can also be produced by ion pump leakage (Bednar & Soukup, 2003; Kopecky et al., 1990; Yan et al., 2013). It is thought that a leaky ion pump in mitochondria releases H⁺ ions; the intensity of heat is proportional to the amount of H⁺ released during this process (Palou et al., 1998; Rehnmark et al., 1990). The ability of brown adipocytes to turn excess energy into heat is a property the white adipocytes lack (Sacks et al., 2013; Sharp et al., 2012; Vargas et al., 2016; Yang et al., 2014). Circulating factors, such as irisin, FGF-21, and natriuretic peptides play a role in regulating brown adipocytes. It is thought that these factors can encourage proliferation of brown adipocytes, and increase the amount of present beige adipocytes (Sharma et al., 2014; Sharp et al., 2012; Vargas et al., 2016).

Beige adipocytes are a combination of brown and white adipocytes (Yang et al., 2014). Beige adipocytes are born through a browning process; white adipocytes become more like brown adipocytes, the one large lipid droplet becomes many, and uncoupling protein 1 becomes expressed, and thermogenic activity increases (Cohen & Spiegelman, 2015; Sharma et al., 2014; Sharp et al., 2012). All three types of adipocyte cells, along with muscle cells, come from the same precursor cell—a mesenchymal stem cell (Cohen & Spiegelman, 2015; Yang et al., 2014). The expression of different genes at different points during the life cycle of these cells determines their fate (Cohen & Spiegelman, 2015; Yang et al., 2014), see Figure 8. The

potential for obese adults to spontaneously form beige adipocytes from their white adipocytes is unclear, but it brings to mind the possibility that such a phenomenon is possible.

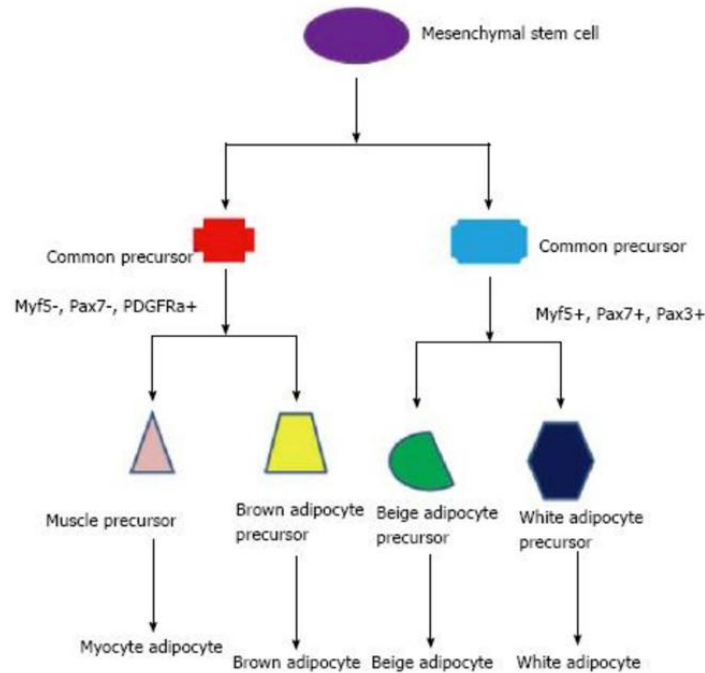


Figure 8. Schematic demonstrating links between three basic type of adipocytes and muscle.

All cells come from the same common cell, mesenchymal stem cell. Presence or absence of certain factors during development, such as Myf5, Pax 3 and 7, PDGFRa and myogenin can affect the final product of the cell. Myf5: Myogenic factor 5; Pax 7: Paired box protein 7; Pax 3: Paired box protein 3; PDGFRa: Platelet-derived growth factor receptor, alpha polypeptide.

The relationship between hypertension and obesity

Obesity can increase the susceptibility to metabolic syndromes, cardiovascular diseases, Type 2 diabetes, cancer, and hypertension. Although some patients with hypertension are not obese, and vice versa, there is a strong correlation across populations (P.T. Shah, Shapiro, Khitan, Santhanam, & Shapiro, 2016). The interactions between obesity, salt sensitivity and hypertension are shown schematically in Figure 9. When blood pressures reach the hypertensive range, there is almost always small vessel disease of the arterioles, or arteriolosclerosis, as well as kidney

damage (Sasaki, Uchida, Niiyama, Yoshida, & Saito, 1998; Sharma et al., 2014; Watanabe et al., 2013). This strongly suggests that there are both vascular and renal components to the disease (J. E. Hall et al., 2012). Hypertension has genetic and environmental factors in addition to those associated with obesity (Shungin et al., 2015; Simonds et al., 2014; Yang et al., 2014; S. Zhang et al., 2016). Salt sensitive hypertension refers to an increase in blood pressure related to an increase in salt (specifically sodium) intake (J. Chen et al., 2009; Gilibert et al., 2008; Mori et al., 1999). Some workers in this field believe that all hypertension reflects either excessive sodium intake or some form of renal salt sensitivity, but this is admittedly still controversial (Fedorova, Shapiro, & Bagrov, 2010; J. E. Hall et al., 2012; J. E. Hall, Guyton, & Brands, 1996; J. X. Xie, Shapiro, & Shapiro, 2014).

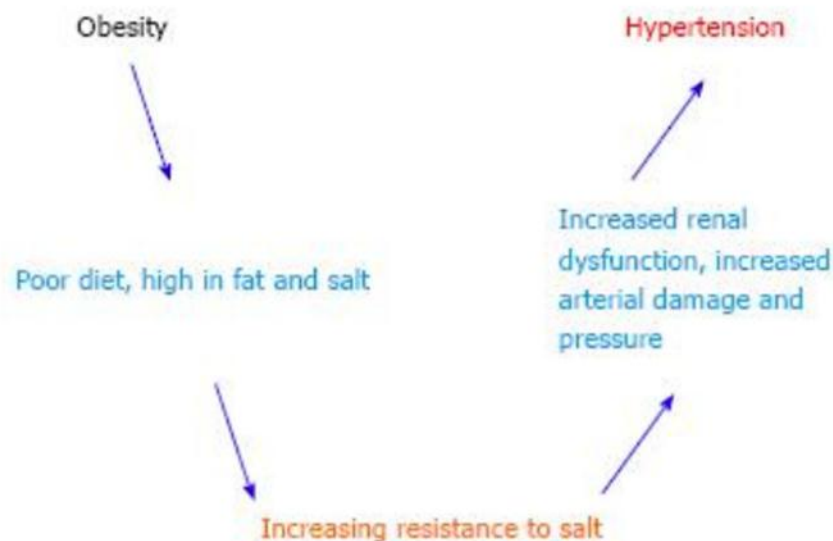


Figure 9. Schematic demonstrating potential relationship between obesity and hypertension.

Present theories linking obesity to hypertension

Obesity appears to be associated with or complicated by “increased sympathetic nervous system (SNS) activity, activation of the renin-angiotensin aldosterone system (RAAS), and physical compression of the kidneys by extra-renal fat and by increased intrarenal extracellular matrix”(J. E. Hall et al., 2010; J. E. Hall, do Carmo, da Silva, Wang, & Hall, 2015; J. E. Hall, Granger, Reckelhoff, & Sandberg, 2008). This physical compression can directly activate the RAAS which, in turn, leads to increased SNS outflow as well as increased circulating concentrations of angiotensin II, a well-known vasoconstrictor and aldosterone, an anti-natriuretic hormone. The net effect is sodium retention and increased blood pressure (J. E. Hall et al., 2012; J. E. Hall et al., 2008; J. E. Hall, Jones, et al., 2003; J. E. Hall, Kuo, et al., 2003) . Leptin levels, as discussed above, appear to be increased in obese patients. This hormone through several biochemical mechanisms, affects appetite as well as SNS outflow, and can cause increases in blood pressure (J. E. Hall et al., 2010; J. E. Hall, Jones, et al., 2003). The duration of obesity also plays a role; the longer one is obese, the more renal damage occurs which further impairs pressure natriuresis, exacerbating hypertension (da Silva, do Carmo, & Hall, 2013; J. E. Hall et al., 2010; J. E. Hall et al., 2015; J. E. Hall et al., 2012; M. E. Hall et al., 2014).

Another potentially contributing factor is Obstructive Sleep Apnea (OSA), which is more than just another co-morbidity of obesity. OSA is much more common in people who are overweight or obese (Abraham et al., 2007; Adedayo et al., 2014). OSA occurs when the airway becomes blocked or constricted and can cause snoring, and lapses in breathing that are common to sleep apnea (Adedayo et al., 2014; Al-Jehani, Hall, & Maleki, 2013; Dharia, Unruh, & Brown, 2015). Untreated sleep apnea can lead to increases in blood pressure, obesity, heart attack risk, and diabetes, among other problems (Adedayo et al., 2014; Albert et al., 2013; Allison, Gertsch,

Mahan, Sheean, & Brown, 2014; Alonso-Galicia, Brands, Zappe, & Hall, 1996; Fonseca-Alaniz, Brito, et al., 2007; J. E. Hall, Kuo, et al., 2003). In addition to increasing the risk for hypertension, OSA can lead to other problems. Hypoxia, or lack of oxygen that occurs when breathing is stopped or obstructed, is also a risk factor for generating ROS and increasing oxidant stress and SNS activity (Abraham et al., 2007; Adedayo et al., 2014; Al-Jehani et al., 2013; Fonseca-Alaniz, Brito, et al., 2007).

Recent work suggests that the adipocyte itself could play an important role in hypertension. Research has shown that high dietary sodium can increase the white adipocyte mass as well as leptin levels in rats (Fonseca-Alaniz, Brito, et al., 2007), and blood pressure was significantly increased as well. The increase in adipocyte mass has a cascading effect; the mass increases, the release of additional adipokines occurs, and these lead to an increase in inflammation. This inflammation causes further exacerbation of disordered metabolism and insulin resistance (Alon & Friedman, 2006; Enzi et al., 2015; Fonseca-Alaniz, Brito, et al., 2007; Gutierrez, Puglisi, & Hasty, 2009). These interactions are shown schematically in Figure 10 and Figure 11.

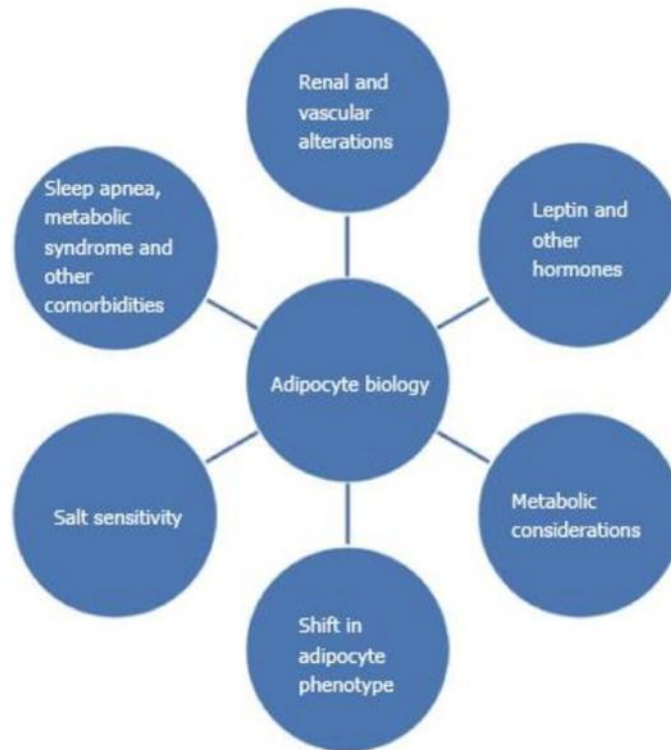


Figure 10. Visual representation of factors contributing to the complex pathophysiology linking adipocyte biology and hypertension

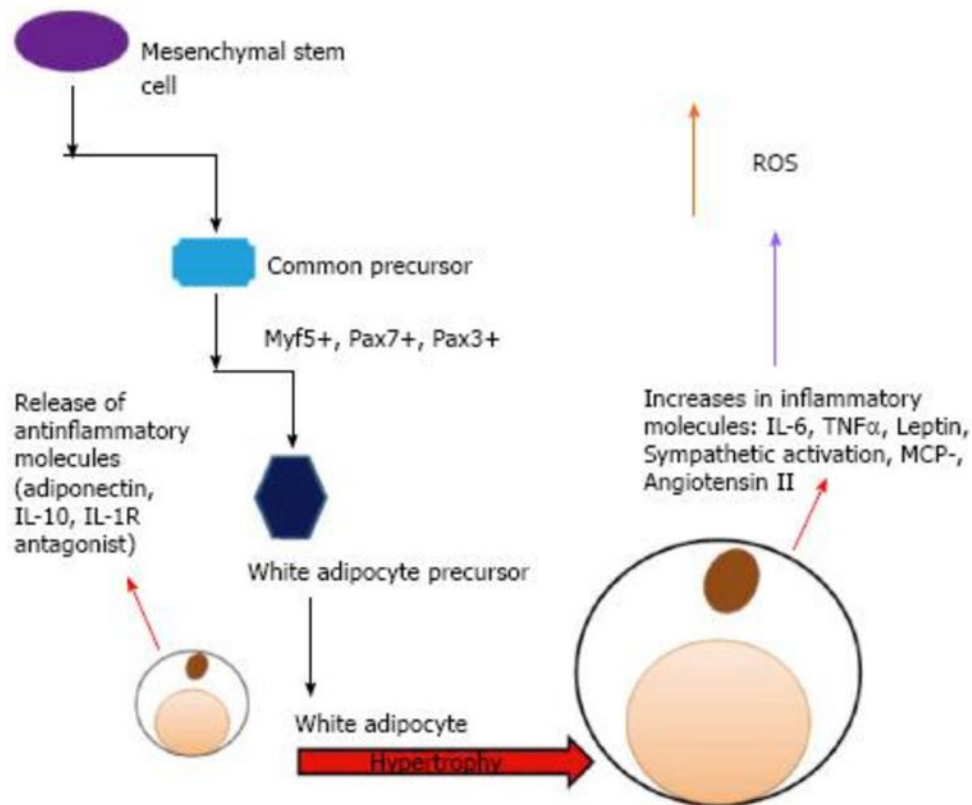


Figure 11. Small adipocytes release anti-inflammatory agents such as adiponectin, interleukin 10, and interleukin 1 receptor antagonist.

Hypertrophy of small adipocytes into large adipocytes changes the biochemical release products, into inflammatory markers [interleukin-6 (IL-6), tumor necrosis factor alpha (TNF α), and monocyte chemoattractant protein 1 (MCP-1)] and other chemicals such as leptin and angiotensin II that contribute to the disease. Increased activation of the sympathetic nervous system also contributes. IL-10: Interleukin-10; IL-1R: Interleukin 1 receptor; Myf5: Myogenic factor 5; Pax 7: Paired box protein 7; Pax 3: Paired box protein 3; ROS: Reactive oxygen species.

Current treatments of obesity and their effects on hypertension

Currently, there are several treatment methods to deal with obesity, and as it is so closely related with hypertension, treatments for the two can often overlap (Blood Pressure Lowering Treatment Trialists et al., 2015; G. Bray, 1998; A. D. Brown, Barton, & Lambert, 2009). The treatments can be broken up into several categories; lifestyle changes (including nutritional

changes and exercise addition), drug therapy, and surgical methods(Alemaný, Remesar, & Fernandez-Lopez, 2003). It has been shown that a reduction in a patient's weight by 5-10% is enough to reduce their risk of cardiovascular complications, including hypertension (Alemany et al., 2003; Atkinson, Blank, Loper, Schumacher, & Lutes, 1995; Barja-Fernandez, Leis, Casanueva, & Seoane, 2014). When looking at the drug treatment route, it is important to consider however that some drugs are not recommended for patients who have pre-existing conditions, such as hypertension or diabetes. Sibutramine, for example, has been associated with small increases in blood pressure and heart rate, and is not recommended for patients suffering from hypertension (Alemany et al., 2003; Ioannides-Demos, Proietto, Tonkin, & McNeil, 2006). Some drugs that are used to treat hypertension can be used as a weight loss agent, such as the drug orlistat (Alemany et al., 2003; Charakida, Tousoulis, & Finer, 2013). These drugs can work on multiple levels; some are known as feeder modulators, and change the way the patient receives signals that the body needs food (Alemany et al., 2003; G. A. Bray, 1999). Some effect the formation of agents such as angiotensin II and nitric oxide synthase (Alemany et al., 2003; Charakida & Finer, 2012; Cheung, 2011). Still others work at the molecular level and effect the afferent signaling that can lead to obesity (Alemany et al., 2003; Charakida & Finer, 2012). Serotonin drugs have been found to be an effective treatment of obesity, but the downside is they can cause an increased risk of primary hypertension because of their effects on vascular smooth muscle(Halford, Harrold, Lawton, & Blundell, 2005). If we look at surgical approaches, the benefits of surgery on hypertension itself and the abnormal hormonal milieu appear to be huge, at least over the first year or so (Ruano et al., 2005).

Redox reactions and their relation to obesity and hypertension

In addition to salt intake and obesity, nitrous oxide synthase and heme oxygenase both play a role in the cause and treatment of hypertension (De Simone et al., 2015; Ishima et al., 2015; X. Zhou et al., 2010). Obesity leads to an imbalance in the circulating level of nitric oxide (NO); this is due to increased oxidative stress and decreased NO production (Ishima et al., 2015; Merial, Bouloumie, Trocheris, Lafontan, & Galitzky, 2000). Decreasing the availability of the NO can predispose an individual to hypertension (De Simone et al., 2015; Ishima et al., 2015; X. Zhou et al., 2010). NO contributes to vasodilation, which is the relaxation of the vasculature (De Simone et al., 2015; Ishima et al., 2015; X. Zhou et al., 2010). If there is less NO present (because of a decrease in NO synthase), vasoconstriction can occur, which can exacerbate the damage of increased pressure from the other factors related to hypertension (De Simone et al., 2015; Ishima et al., 2015; X. Zhou et al., 2010). Human adipose tissue expresses angiotensinogen, angiotensin-converting enzyme (ACE) as well as AT1 (angiotensin type 1), and AT2 (angiotensin type 2) receptors (Hilzendeger et al., 2012; Peterson, Frishman, & Abraham, 2009; Westphal et al., 2008). The role of angiotensin is not well known, but it has some kind of redox purpose; it appears to involve inhibition of endothelial cell migration, proliferation and induction of apoptosis (De Simone et al., 2015; Ishima et al., 2015; X. Zhou et al., 2010). There is a link between NO synthase dysfunction and the ACE enzyme in the obese population (De Simone et al., 2015; Ishima et al., 2015; X. Zhou et al., 2010). Excessive NO formation by the inducible member of the nitric oxide synthase (NOS) family (iNOS or Nos2) has been shown to cause nonspecific tissue damage; it is thought to be involved in the pathogenesis of inflammatory and autoimmune diseases (Marcano et al., 2007; Rodella et al., 2008; Zaitone et al., 2015). By inhibiting this inducible factor, obesity still occurs but the pathologies associated are reduced

(Rodella et al., 2008). A similar study showed that even though mice protected from pathologies associated with INOS inhibition, they are still subjected to increased blood pressure and increased ROS (Y. Chen, Liu, et al., 2015; Klein, Fasshauer, Benito, & Kahn, 2000). iNOS is associated with increased inflammatory responses, which is related to the cascade of responses associated with obesity and hypertension (Cao, Sodhi, Puri, et al., 2011; Sodhi et al., 2012; Sodhi et al., 2014; Wong et al., 2015). It is known that increased NO can induce cellular stress, which can exacerbate the current problems present.

Similarly to NO synthase, heme oxygenase (HO) has a role in amelioration of hypertension (Abraham et al., 2007; Ishima et al., 2015; Peterson et al., 2009; Rodella et al., 2008; Vanella et al., 2013). An increase in HO expression can cause reductions in reactive oxygen species, or ROS. Increases in ROS, also called oxidant stress, are believed to be important in the progression of hypertension and associated cardiovascular diseases. The isoform HO-1 is the inducible form of heme oxygenase, and when induced it can cause a decrease in weight, and therefore a decrease in obesity (Kamble, Litvinov, Aluganti Narasimhulu, Jiang, & Parthasarathy, 2015; Vanella et al., 2010; Vanella et al., 2013). HO does this by changing the phenotype of the adipocyte (Nicolai et al., 2009; Vanella et al., 2013). HO-1 can interact with NO in several ways, one of which is through AngII (Valladares, Roncero, Benito, & Porras, 2001; Westenbrink et al., 2015; X. Zhou et al., 2010). Increased AngII production causes an increase in ROS, which may inhibit the action of NO (Ishima et al., 2015; P. Wang et al., 2014). This can also increase salt reabsorption. When HO-1 expression is increased, the increases in AngII levels are attenuated; this decreases AngII's downstream signaling effects (Valladares et al., 2001; Westenbrink et al., 2015; X. Zhou et al., 2010). Induction of HO-1 can also reduce the renal vasculature resistance

that is increased with AngII level increases (Valladares et al., 2001; Westenbrink et al., 2015; X. Zhou et al., 2010).

Several studies have shown that induction of HO-1 not only decreases weight and obesity, but it can also prevent the development of hypertension, even if its expression is limited to adipocytes (Abraham et al., 2007; Cao et al., 2012). It is not clear if blood pressure is lowered through the indirect effects on the vasculature, kidney, or through the release of other enzymes and factors. If induction of HO-1 is done at any step in the pathway described above, what are the specific effects? If induced during any stage of hypertension, will effects still be seen, or does it need to be induced early in obesity?

Abraham, et. al., look specifically at the role of HO-1 and the effects it can have on various aspects of obesity. One study specifically examines adipocyte dysfunction; induction of HO-1 can reverse adipocyte dysfunction and to an extent reverse effects of damage (Khitan, Harsh, Sodhi, Shapiro, & Abraham, 2014). This lab has also shown significant findings of the role of HO-1 and the attenuating effects it can have with hypertension (Abraham, 2008; Abraham et al., 2007; Abraham & Kappas, 2011). We have also looked from the other perspective, namely ROS generation. We have recently observed that attenuation of ROS generation with pNaKtide (Z. Li et al., 2009; Z. Li et al., 2011), a peptide designed to ameliorate the Na/K-ATPase mediated feed forward amplification of ROS (Yan et al., 2013), prevents phenotypical changes within adipocytes as well as ameliorates diet induced obesity in mice (Sodhi et al., 2015).

Conclusions and future directions

There is clearly a very strong relationship between obesity and hypertension. While a plethora of mechanisms potentially link obesity to hypertension, we are left with the provocative

possibility that adipocyte biology may play an important role in blood pressure regulation, a topic which to date has not been systematically explored.

CHAPTER 3

THE NA/K-ATPASE SIGNALING: FROM SPECIFIC LIGANDS TO GENERAL REACTIVE OXYGEN SPECIES

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Abstract: The signaling function of the Na/K-ATPase has been established for 20 years, and is widely accepted in the field with many excellent reports and reviews that we did not cite. Even though there is debate about the underlying mechanism, the signaling function is unquestioned. This short review looks back at the evolution of Na/K-ATPase signaling, from stimulation by cardiotonic steroids (also known as digitalis-like substances) as specific ligands to stimulation by reactive oxygen species (ROS) in general. The interplay of cardiotonic steroids and ROS in Na/K-ATPase signaling forms a positive-feedback oxidant amplification loop that has implicated in some pathophysiological conditions.

Key word: Na/K-ATPase; ROS; sodium; potassium; signaling; Src. Endocytosis

Introduction

Since J.C. Skou's discovery in 1957 (Skou, 1957), the energy-transducing Na/K-ATPase has been extensively studied for its ion pumping function and later on, its signaling function. The latter signaling function was first demonstrated about two decades ago, and evolved to a much bigger signaling network (and has kept evolving) one could not imagine before.

All starts with the possible role of Na/K-ATPase in cardiac hypertrophy. In a classic view, partial inhibition of Na/K-ATPase ion-exchange activity raises intracellular sodium concentration ($[Na^+]_i$), which in turn, increase intracellular calcium concentration ($[Ca^{2+}]_i$) by coupling with Na^+/Ca^{2+} exchanger (NCX) to execute inotropic effect. This is the basis of the treatment of heart failure with digitalis-like drugs. Furthermore, partial inhibition of Na/K-ATPase not only causes intracellular ionic changes, but also stimulates transcriptional up-regulation of several marker genes including Na/K-ATPase itself. However, further studies were unable to directly link ouabain-mediated gene regulation effects to changes in intracellular $[Na^+]_i$ or $[K^+]_i$ caused by Na/K-ATPase inhibition. In cultured cardiac myocytes, treatment with nontoxic concentrations of ouabain not only partially inhibited Na/K-ATPase activity and increased cardiac contractility, but also stimulated cell growth and protein synthesis through induction of early response proto-oncogenes and activation of transcription factors (L. Huang, Kometiani, & Xie, 1997; L. Huang, Li, & Xie, 1997; Kometiani et al., 1998; Peng et al., 1996; Sugden, 1999; Z. Xie & Askari, 2002; Z. Xie et al., 1999). These discrepancies started the search for mechanism(s) other than ionic changes.

Na/K-ATPase signaling and intracellular ionic concentration

As mentioned above, changes in $[Na^+]_i$, $[K^+]_i$, and $[Ca^{2+}]_i$ were largely attributed to changes in Na/K-ATPase activity that can be regulated by specific ligands cardiotonic steroids.

Interestingly, Na/K-ATPase activity can also be regulated by changes in cellular redox status, induced by either cardiotonic steroids or other factors. In cardiac myocytes, inhibition of Na/K-ATPase ion exchange function leads to decrease of $[K^+]_i$ and increase of $[Na^+]_i$. By coupling to NCX, this increase in $[Na^+]_i$ elevates intracellular $[Ca^{2+}]_i$, which is the leading force of the positive inotropic action induced by digitalis drugs for treatment of heart failure (Barry, Hasin, & Smith, 1985; Reuter et al., 2005). To address the role of these ionic changes, ouabain-induced ROS generation (an essential second messenger) and increase in $[Ca^{2+}]_i$ (a shared secondary messenger) were manipulated to investigate the possible interplay. Ouabain-induced ROS generation was compared in cardiac myocytes cultured in Ca^{2+} -free medium (with 0.1 mM EGTA) and Ca^{2+} -containing medium, respectively. In neonatal cardiac myocytes cultured in Ca^{2+} -free medium, in which ouabain did not change $[Ca^{2+}]_i$, ouabain was still able to stimulate ROS generation as shown in myocytes cultured in Ca^{2+} -containing medium, but unable to stimulate increase of $[Ca^{2+}]_i$ and contractility in neonatal cardiac myocytes (J. Liu et al., 2000). Furthermore, in neonatal cardiac myocytes cultured in Ca^{2+} free medium with high Na^+ (150mM), monensin, a Na^+ -specific ionophore capable of equilibrating Na^+ concentration across cell membrane, failed to increase ROS generation. Interestingly, inhibition of c-Src or Ras, as well as antioxidants can block ouabain-stimulated ROS generation, but not ouabain-induced increases in $[Ca^{2+}]_i$ (Tian, Gong, & Xie, 2001; Tian, Liu, Garlid, Shapiro, & Xie, 2003; Z. Xie et al., 1999). These observations suggested that increases in $[Ca^{2+}]_i$ is necessary in ouabain-induced increases in cardiomyocytes contractility and gene regulatory effects, but is not necessary in ouabain-stimulated ROS generation. Moreover, Ouabain-stimulated Na/K-ATPase signaling also increases generation of ROS which functions as a second messenger. Pretreatment with antioxidants, such as *N*-acetylcysteine (NAC) or vitamin E, neutralized the increases of ROS,

and therefore, prevented ouabain-stimulated activation of NF- κ B and protein synthesis (J. Liu et al., 2000; Z. Xie et al., 1999). Ouabain-induced increases in ROS production involves opening of mitochondrial ATP-sensitive K⁺ channels (mitoK_{ATP}) (J. Liu et al., 2000; Tian et al., 2003).

Notably, ouabain-induced increases in [Ca²⁺]_i is also involved in ouabain-stimulated Na/K-ATPase signaling. In renal epithelial cells, low doses of ouabain (only inhibit Na/K-ATPase activity partially) functioned as an inducer/trigger of regular, low frequency [Ca²⁺]_i oscillations is involved the Na/K-ATPase/ inositol 1,4,5-trisphosphate receptors (IP₃Rs) signaling micro-domain that leads to NF- κ B activation (Aizman et al., 2001; Miyakawa-Naito et al., 2003). This phenomena does not depend on partial inhibition of Na/K-ATPase using low extracellular K⁺ and depolarization of cells, but is achieved by ouabain-stimulated activation of tyrosine kinase c-Src and phospholipase C- γ (PLC- γ), which transmitted the signal to IP₃Rs (Ying Chen et al., 2008; Yuan et al., 2005). Depletion of intracellular endoplasmic reticulum (ER) Ca²⁺ by sarco-endoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor, as well as blockage of store-operated calcium-mediated cytosolic Ca²⁺ influx and inhibition of IP₃Rs-induced Ca²⁺ release abolished ouabain-induced Ca²⁺ oscillations (Miyakawa-Naito et al., 2003). Truncation of 32 amino acids from the α 1 NH₂ terminus results in a functional enzyme but abolishes ouabain-induced Ca²⁺ oscillations, indicating that the cytoplasmic α 1 NH₂ terminus plays a central role in ouabain-induced Ca²⁺ oscillations (Miyakawa-Naito et al., 2003). The data from this study also indicates that increased [Na⁺]_i is not the main cause of ouabain-induced Ca²⁺ oscillations, rather the release of α 1 NH₂ terminus during Na/K-ATPase E1 to E2 conformational change (ouabain binding favors E2 conformation) serves as a mean of α 1/IP₃Rs complex formation. This was further confirmed that the α 1 NH₂ terminus binds directly, through motif LKK, with the IP₃R NH₂ terminus (Songbai Zhang et al., 2006). On the other hand, ouabain

stimulated the formation of a functional Ca^{2+} -signaling complex including the Na/K-ATPase $\alpha 1$ /c-Src/PLC- γ /IP₃R in LLC-PK1 cells, and knock-down of the Na/K-ATPase $\alpha 1$ redistributed IP₃R (Ying Chen et al., 2008). Overexpression of the $\alpha 1$ NH₂ terminus (amino acids 1-160) not only disrupted the interaction of the Na/K-ATPase $\alpha 1$ and IP₃R, but it also functioned as a negative regulator of ATP-induced ER Ca^{2+} release (Ying Chen et al., 2008). The p42/44 MAPK-mediated activation of Ca^{2+} channels partially contributes to ouabain-induced regulation of $[\text{Ca}^{2+}]_i$ (Fitzgerald, 2000; Marban & Tsien, 1982).

Because the effects of ouabain on c-Src are independent of changes in intracellular ion concentrations (Aydemir-Koksoy, Abramowitz, & Allen, 2001; Haas et al., 2000; Haas, Wang, Tian, & Xie, 2002; J. Liu et al., 2000), it seems that ouabain-induced inhibition of Na/K-ATPase enzymatic activity (ion exchange) and ouabain-induced c-Src-dependent signaling are, at least partially, two separated regulatory events under these experimental conditions. In addition to ouabain, changes in intracellular Na^+ or extracellular K^+ affect not only the enzyme conformation, but also change other ion transporter-related activities. For example, lowering of extracellular K^+ activates protein kinases and raises intracellular Ca^{2+} in cardiac myocytes (Haas et al., 2000), but it differs from the effects of ouabain on smooth muscle and kidney epithelial cells (Aizman et al., 2001; Aydemir-Koksoy et al., 2001). Large bulk changes in $[\text{Na}^+]_i$ or $[\text{K}^+]_i$ that are not induced by ouabain-Na/K-ATPase axis also affect other intracellular signal pathways (Kuroki, Minden, Sanchez, & Wattenberg, 1997; S. Li & Wattenberg, 1998; Pressley, 1992). For example, in cultured porcine aortic endothelial cells, ouabain-mediated complete inhibition of Na/K-ATPase causes cell necrosis that is independent of ouabain-mediated ion fluxes and change of $[\text{Na}^+]_i/[\text{K}^+]_i$ ratio, but K^+ -free ($[\text{K}^+]_0=0$) medium-caused inhibition of Na/K-ATPase, which elevates $[\text{Na}^+]_i$, does not induce necrosis but protects against apoptosis (Orlov et

al., 2004). The anti-apoptosis effect is regulated by a $[\text{Na}^+]_i$ -mediated, Ca^{2+} -independent mechanism (Taurin et al., 2002). In cultured cortical neurons, ouabain-induced concentration-dependent neuron death that involved apoptosis and necrosis, which is mediated by intracellular depletion of K^+ and accumulation of Ca^{2+} and Na^+ (Xiao, Wei, Xia, Rothman, & Yu, 2002). These observations suggest that ouabain- Na^+/K^+ -ATPase-mediated signaling differs from solely ionic change ($[\text{Na}^+]_i/[\text{K}^+]_i$)-mediated signaling pathways. The possible interplay and different mechanism(s) between them are still not totally understood.

In smooth muscle cells of rat arteries, both NCX and ouabain-sensitive Na^+/K^+ -ATPase α_2 - and α_3 -isoforms reside closely in plasma membrane regions adjacent to the sarcoplasmic reticulum, a sub-plasmalemmal space called plasmERosome (Golovina, Song, James, Lingrel, & Blaustein, 2003; Juhaszova & Blaustein, 1997a, 1997b; Moore et al., 1993). While low doses of ouabain do not increase bulk cytosolic Na^+ levels, it can stimulate a local transient rise of $[\text{Na}^+]_i$ in the plasmERosome, which can lead to a local transient increase of $[\text{Ca}^{2+}]_i$ via the NCX and increased muscle contractility (Arnon, Hamlyn, & Blaustein, 2000a, 2000b; Lee et al., 2006).

In cells without expression of plasma membrane NCX, alteration of $[\text{Na}^+]_i/[\text{K}^+]_i$ ratio, by high ouabain concentrations or palytoxin through inhibition of Na^+/K^+ -ATPase, is able to activate some protein kinase signaling pathways (Contreras, Shoshani, Flores-Maldonado, Lazaro, & Cerejido, 1999; Kuroki et al., 1997; S. Li & Wattenberg, 1998). This suggests that, in cells lacking NCX expression, changes in $[\text{Na}^+]_i$ or $[\text{K}^+]_i$ or both may also stimulate Ca^{2+} -independent Na^+/K^+ -ATPase signaling functions.

These observations indicate a complicated interplay amongst Na^+/K^+ -ATPase ion-exchange activity, signaling, and ROS in regulation of different cellular events. The Na^+/K^+ -ATPase signaling-ROS axis might play an important role to dissect these regulations since chronic

regulation of ion homeostasis could be a consequence of Na/K-ATPase signaling and ROS regulation.

Na/K-ATPase signaling and ROS: the positive oxidant amplification loop

The interplay amongst Na/K-ATPase signaling, ROS, and oxidative modifications has been a topic for decades. The effect of ROS on the Na/K-ATPase activity has been well-documented (Figtree, Keyvan Karimi, Liu, & Rasmussen, 2012; Figtree et al., 2009; W.-h. Huang, Y. Wang, A. Askari, N. Zolotarjova, & M. Ganjeizadeh, 1994; W. H. Huang, Wang, & Askari, 1992; Petrushanko et al., 2012; THÉVENOD & FRIEDMANN, 1999; Z. Xie et al., 1999; Z. J. Xie, Wang, Askari, Huang, & Klaunig, 1990; Yan et al., 2013; Yan et al., 2016). Different oxidative modification mechanisms and subunits of the Na/K-ATPase showed different outputs.

In rabbit ventricular myocytes, glutathionylation of cysteine residue (Cys-46) of the Na/K-ATPase β 1 subunit inhibits the Na/K-ATPase activity by either stabilizing the enzyme in an E2-prone conformation, a process that could be reversibly regulated by glutaredoxin 1 and FXYD, the proposed γ subunit of the Na/K-ATPase (Bibert et al., 2011; Figtree et al., 2012; Figtree et al., 2009). In rat myocardium, S-glutathionylation of cysteine residues (Cys-454, -458, -459, and -244) of the Na/K-ATPase α 1 subunit also inhibits the Na/K-ATPase activity by blocking the ATP-binding site Na/K-ATPase α 1 subunit when the ATP concentration below 0.5mM. This s-glutathionylation of the α 1 subunit as well as inhibition of the Na/K-ATPase activity can be reversed by deglutathionylation with glutaredoxin or dithiothreitol (Bogdanova, Petrushanko, Hernansanz-Agustin, & Martinez-Ruiz, 2016; Petrushanko et al., 2012). In various chronic inflammatory conditions, circulating cardiotoxic steroids are elevated that are capable of stimulating proinflammatory response in murine and human macrophages. This process involves

ouabain-stimulated activation of NF- κ B through a signaling complex of Na/K-ATPase, CD36, and TLR4, leading to increases in proinflammatory cytokines MCP-1, TNF α , IL-1 β , and IL-6 (Y. Chen et al., 2017; Y. Chen, Kennedy, et al., 2015; Kennedy et al., 2013).

In rat neonatal myocytes, ouabain-stimulated activation of Na/K-ATPase signaling function increases mitochondrial ROS generation that functions as an essential second messenger (J. Liu et al., 2000; Z. Xie et al., 1999). More importantly, increases in ROS can cause conformational changes in Na/K-ATPase like ouabain (Bibert et al., 2011; Figtree et al., 2012; Figtree et al., 2009; W.-h. Huang et al., 1994; Petrushanko et al., 2012; Z. J. Xie et al., 1990) . One question asked was that if ROS is able to stimulate the signaling function of Na/K-ATPase like ouabain, and if ouabain (or ROS) \rightarrow Na/K-ATPase signaling \rightarrow ROS \rightarrow Na/K-ATPase signaling could form a positive amplification loop that could amplify Na/K-ATPase and subsequent signaling events and functional changes. This is of particular interest since it was well accepted that ROS play an important role in the pathogenesis of cardiovascular diseases and chronic kidney diseases, and many others.

In primary cultures of cardiac myocytes, it was demonstrated that partial inhibition of Na/K-ATPase by ouabain increased c-Src- and Ras-dependent, and mitochondrial K_{ATP} channel-related ROS generation, and ouabain-induced cardiac hypertrophic growth involved ROS-dependent signaling pathways (Z. Xie et al., 1999). In Langendorff-perfused rat hearts, pretreatment with ouabain demonstrated a cardioprotective effects against ischemia-reperfusion injury by an improved recovery of contractile function and a reduction of infarct size. This ouabain effect is due to activation of Na/K-ATPase signaling function that involves Src, mitochondrial K_{ATP} channel, and ROS (Pasdois et al., 2007). Exogenous ROS (for example, induced by glucose oxidase), acting as ouabain, also caused ROS-dependent cardiac

hypertrophic growth. Inhibition of c-Src and ERK1/2 abrogated the effects of ROS-induced protein synthesis that was not affected by chelating intracellular Ca^{2+} by BAPTA-AM (L. Liu et al., 2006). Moreover, ouabain-induced increase in $[\text{Ca}^{2+}]_i$ was ROS-independent and involved mainly the inhibition of Na/K-ATPase ion transport function (J. Liu et al., 2000). These observations indicated that ROS act like ouabain, and the Na/K-ATPase could be a target for ROS-initiated signaling.

In porcine LLC-PK1 cells (an immobilized renal proximal tubule cell line), exogenous peroxide (H_2O_2) activated Na/K-ATPase signaling pathways including phosphorylation of c-Src and ERK1/2 (Y. Wang et al., 2014). By using LLC-PK1 cells, it was further demonstrated that low concentration of ouabain also stimulated Na/K-ATPase signaling function which leads to increased ROS generation and protein carbonylation modification of Na/K-ATPase (direct carbonylation of two amino acid residues, Pro222 and Thr224, in the actuator domain of the $\alpha 1$ subunit) (Yan et al., 2013). Pretreatment with antioxidant *N*-acetyl-L-cysteine (NAC) or disruption of the Na/K-ATPase/c-Src signaling complex attenuated ouabain- and glucose oxidase-stimulated Na/K-ATPase/c-Src signaling, protein carbonylation, redistribution of Na/K-ATPase, and inhibition of active transepithelial $^{22}\text{Na}^+$ transport. It indicated that ROS are critical in initiating ouabain-stimulated Na/K-ATPase/c-Src signaling, and carbonylation modification of the $\alpha 1$ subunit is involved in a feed-forward mechanism of regulation of ouabain-mediated Na/K-ATPase signal function and subsequent Na^+ transport. Interestingly, there is an undefined de-carbonylation mechanism of ouabain-stimulated protein carbonylation after removal of ouabain, this could be another new regulatory mechanism of Na/K-ATPase signaling because it was believed that protein carbonylation modification could not be reversed. Furthermore, stable overexpression of rat $\alpha 1$ mutant Pro224/Ala (Pro224 of rat $\alpha 1$ is the same as the Pro222 of pig

$\alpha 1$) prevented ouabain-stimulated signal function of Na/K-ATPase, protein carbonylation, Na/K-ATPase endocytosis, and active transepithelial $^{22}\text{Na}^+$ transport (Yan et al., 2016). Taken together, we proposed that, in LLC-PK1 cells, there is a positive-feedback amplification loop of Na/K-ATPase signaling and ROS generation, in which carbonylation of the Pro222 of the $\alpha 1$ subunit plays a critical role. In this working model, both Na/K-ATPase specific ligands cardiotonic steroids (including ouabain) and ROS increases (induced by other stimuli including exogenous added glucose oxidase) could activate the Na/K-ATPase signaling. The Na/K-ATPase/c-Src complex functions as a “receptor” of ROS signaling. This Na/K-ATPase signaling-ROS axis may explain the role of Na/K-ATPase signaling in the development of different pathophysiological conditions. However, it is not clear (1) if a “decarbonylation” process could regulate the carbonylation modification, and (2) to which point the oxidant amplification loop will be forced to stop.

Na/K-ATPase signaling and pNaKtide: a specific antagonist of c-Src kinase that breaks oxidant amplification loop.

One question for above-mentioned “Na/K-ATPase signaling-mediated oxidant amplification loop” is, could this amplification loop be controlled and targeted for possible therapeutic implication(s)? In the Na/K-ATPase/c-Src signaling complex model, it was demonstrated that the $\alpha 1$ ND1 domain binds to the c-Src tyrosine kinase domain and the $\alpha 1$ CD2 domain binds to the c-Src SH2 domain in the “resting” state (Tian et al., 2006). Upon ouabain stimulation, c-Src is activated (phosphorylation of Tyr418) due to the disruption of the binding between the $\alpha 1$ ND1 domain and the c-Src tyrosine kinase domain. Based on this “working” model, mapping of these domains led to the identification of a peptide named NaKtide (derived from the Ser415-Gln434 of the pig $\alpha 1$ ND1 domain). In order to further explore this

relationship, a cell permeable version of NaKtide, named pNaKtide was created. A 13 amino acid TAT leader sequence makes pNaKtide positive and therefore cell permeable. pNaKtide targets the α 1/Src receptor complex close to the plasma membrane inside the cell (Figtree et al., 2009; Z. Li et al., 2011). Both NaKtide and pNaKtide act as specific antagonists of c-Src phosphorylation (Figure 12), further demonstrated that the binding of the α 1 and c-Src and the conformational change are critical in activation of Na/K-ATPase signaling.

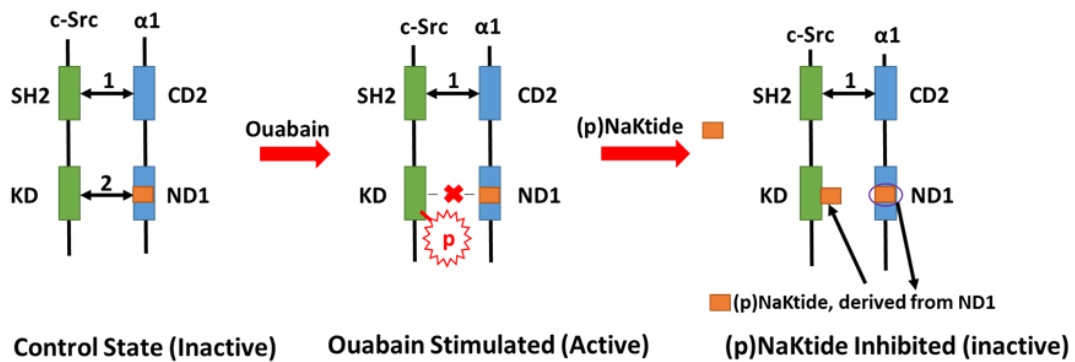


Figure 12. A schematic illustration of action of pNaKtide.

Under control state, c-Src SH2 domain binds to α 1 CD2 segment, and c-Src KD binds to α 1 ND1 that keep c-Src inactive. Upon ouabain binding to the α 1 subunit, the α 1 subunit favors E-2P conformational status, and c-Src KD released from α 1 subunit that leads to phosphorylation of Tyr418 in c-Src KD. NaKtide and pNaKtide are derived from 20 aa (Ser415-Gln434) in α 1 ND1, which can bind to the c-Src KD to competitive binding of α 1 ND1 and KD, thus prevent phosphorylation of Tyr418 in c-Src KD. In the illustration, ouabain is used as a representative of cardiotonic steroids. SH2, c-Src SH2 domain; KD, c-Src kinase domain; CD2, α 1 subunit CD2 segment; ND1, α 1 subunit ND1 segment.

Oxidative stress plays an important role in many pathophysiological conditions. The role of pNaKtide in Na/K-ATPase signaling mediated oxidant amplification loop has been investigated in different cell types and animal models. For example, systemic administration of pNaKtide significantly and effectively attenuates (1) 5/6th partial nephrectomy (PNx) induced uremic cardiomyopathy phenotypes in C57BL/6 mice (J. Liu et al., 2016), (2) a high fat diet

induced adipogenesis, a model of obesity and metabolic syndrome (Sodhi et al., 2015), (3) a Western diet (containing high fat and high fructose) induced obesity, hepatic steatosis, and fibrosis in C57BL/6 mice, as well as steatohepatitis and aortic atherosclerosis in ApoE knockout mice (Sodhi et al., 2017), (4) a Western diet accelerated aging process involving nuclear oxidative stress in C57BL/6 mice (Sodhi et al., 2018), as well as (5) unilateral ureteral obstruction (UUO) mediated interstitial fibrosis in C57BL/6J mice (Cheng, Song, & Wang, 2018). In these animal models, administration of pNaKtide specifically breaks Na/K-ATPase signaling mediated oxidant amplification loop, demonstrated by pNaKtide-induced attenuation of c-Src activation, protein carbonylation, and other regulations. More molecular mechanistic studies are necessary for possible therapeutic usage.

Na/K-ATPase signaling-mediated transporter endocytosis and renal sodium handling

Over the last decade, the role of Na/K-ATPase signaling in renal proximal tubular sodium handling, and the role of oxidative modification of the Na/K-ATPase $\alpha 1$ subunit in Na/K-ATPase signaling were explored both *in vitro* and *in vivo*. The findings may explain some mechanism(s) related to the Na/K-ATPase signaling-ROS amplification loop and subsequent regulation of salt-sensitivity.

It is well-documented that the renal proximal tubule mediates over 60% of the filtered Na^+ reabsorption, mainly through apical Na^+ entry via NHE3 and basolateral Na^+ extrusion through the Na/K-ATPase. Coordinated and coupled regulation of NHE3 and the Na/K-ATPase is critical in maintaining intracellular Na^+ homeostasis and extracellular fluid volume (J. Liu & Xie, 2010; Alicia A. McDonough, 2010; A. A. McDonough, Leong, & Yang, 2003).

In LLC-PK1 cells, like dopamine (Bacic et al., 2003; Chibalin, Katz, Berggren, & Bertorello, 1997; Chibalin et al., 1999; Hu et al., 2001), low concentrations of ouabain stimulate

endocytosis of the $\alpha 1/\beta 1$ subunits, NHE3 (Na^+/H^+ exchanger, isoform 3), and c-Src into early and/or late endosomes, leading to a net decrease in abundance of Na/K-ATPase and NHE3 in cell surface, and thus decreases in transcellular $^{22}\text{Na}^+$ transport (J. Liu, 2005; J. Liu et al., 2004; J. Liu et al., 2005; J. Liu et al., 2002; J. Liu & Shapiro, 2007; J. Liu & Xie, 2010; Periyasamy et al., 2005; Yan et al., 2012). This phenomenon is mainly through a clathrin-dependent endocytic pathway, and requires caveolin-1 and activation of c-Src and PI3K. Furthermore, ouabain-induced endocytosis of Na/K-ATPase and NHE3, thus decreases in transcellular $^{22}\text{Na}^+$ reabsorption, is dependent on ouabain-stimulated signaling function of Na/K-ATPase without significantly affect $[\text{Na}^+]_i$ (H. Cai, Wu, L., Qu, W., Malhotra, D., Xie, Z., Shapiro, JI, and Liu, J., 2008; J. Liu et al., 2004; Oweis et al., 2006). Inhibition of c-Src and PI3K activity prevented ouabain-induced endocytosis of Na/K-ATPase and NHE3. Pretreatment of LLC-PK1 cells with membrane-permeable Ca^{2+} chelator BAPTA-AM attenuated ouabain-induced regulation of NHE3 (H. Cai, Wu, L., Qu, W., Malhotra, D., Xie, Z., Shapiro, JI, and Liu, J., 2008), suggesting ouabain-induced Ca^{2+} signaling might be involved in regulation (Aizman et al., 2001). In male Sprague-Dawley rats fed a high salt (4.0% NaCl) or normal salt (0.4% NaCl) diet for 1 week, a high salt diet redistributes the Na/K-ATPase $\alpha 1$ subunit from plasma membrane fraction to early/late endosomes, accompanying a reduction of proximal tubular Na/K-ATPase ion-exchange activity and enzymatic activity, but increases in urinary excretion of marinobufagenin (MBG) and sodium. These effects were attenuated by administration of anti-MBG antibody prior to salt load (Periyasamy et al., 2005). Moreover, this observation was further confirmed in vivo. By using Dahl salt-sensitive and salt-resistant rats (Jr strains) as models, in vivo studies demonstrated that impairment of renal proximal tubular Na/K-ATPase signaling is a causative of experimental Dahl salt sensitivity (J. Liu et al., 2011). In Dahl salt-resistant but not salt-sensitive

rats, a high salt (2% NaCl, 1 week) diet activated proximal tubular Na/K-ATPase signaling and stimulated coordinated redistribution of the Na/K-ATPase and NHE3, leading to increases total and fractional urinary sodium excretion as well as normal blood pressure. However, the underlying mechanism(s) that the difference of Na/K-ATPase signaling function between Dahl salt-sensitive and salt-resistant rats, as well as the translation of Na/K-ATPase signaling to NHE3 regulation are still unclear.

It is well established that both oxidative stress and high blood pressure are a cause and consequence of each other. Based on the findings of amplification loop of Na/K-ATPase signaling and ROS generation, we tested whether oxidative stress could activate the signaling function of Na/K-ATPase and induce above-mentioned endocytosis process and regulation of renal sodium handling. In our “working” model, increases in ROS generation, either by ouabain or by other stimuli like glucose oxidase, is critical in activation of Na/K-ATPase signaling which mediates transporter trafficking, transcellular Na⁺ transport, and urinary sodium excretion (Yan et al., 2013; Yan et al., 2016). In one hand, pretreatment with anti-oxidant NAC abrogates ouabain-stimulated Na/K-ATPase signaling and transcellular Na⁺ transport, suggesting that a certain level of basal ROS is required for initiation of Na/K-ATPase signaling. On the other hand, without the presence of ouabain, increases in ROS by extracellularly added glucose oxidase is able to activate Na/K-ATPase signaling, indicating that activation of Na/K-ATPase signaling does require its specific ligands and general stimuli like oxidative modification alone are able to activate the Na/K-ATPase signaling (P. T. Shah, Martin, Yan, Shapiro, & Liu, 2016).

However, the effect(s) and consequence(s) of ouabain- and ROS-induced endocytosis of Na/K-ATPase/c-Src/EGFR (J. Liu et al., 2004; J. Liu et al., 2005) are not clear. It has been shown that endocytosis of signaling molecules could be a way to terminate or propagate the

signaling, and could further regulate endocytosis itself (Cavalli, Corti, & Gruenberg, 2001; Di Guglielmo, Baass, Ou, Posner, & Bergeron, 1994; Kuwada et al., 1998; McPherson, Kay, & Hussain, 2001; Roy, Wyse, & Hancock, 2002; Ware, Tice, Parsons, & Lauffenburger, 1997; Wilde et al., 1999; Wiley & Burke, 2001). In this regard, it is possible that ouabain- and ROS-induced endocytosis could be an effective way to terminate Na/K-ATPase signaling mediated oxidant amplification loop by degradation of carbonylated Na/K-ATPase, to maintain certain basal level of ROS and carbonylated protein (J. Liu, Lilly, & Shapiro, 2018).

Perspectives: The “working” models of Na/K-ATPase signaling

There are different proposed “working” models which explain the mechanisms underlying the activation of the Na/K-ATPase signaling function, including (1) the direct interaction of the Na/K-ATPase $\alpha 1$ subunit with c-Src kinase which forms a functional Na/K-ATPase/c-Src signaling receptor complex, a model has been demonstrated both *in vitro* and *in vivo* (Bagrov, Shapiro, & Fedorova, 2009; Z. Li & Xie, 2009; Pierre & Xie, 2006; Tian et al., 2006); (2) c-Src is activated primarily by an ATP-sparing effect (observed in a cell-free system) (Gable, Abdallah, Najjar, Liu, & Askari, 2014; Weigand, Swarts, Fedosova, Russel, & Koenderink, 2012); and (3) c-Src is activated by transiently interaction with a Na/K-ATPase $\alpha 1$ /caveolin-1 complex (also observed in a cell-free system) (Figure 13).

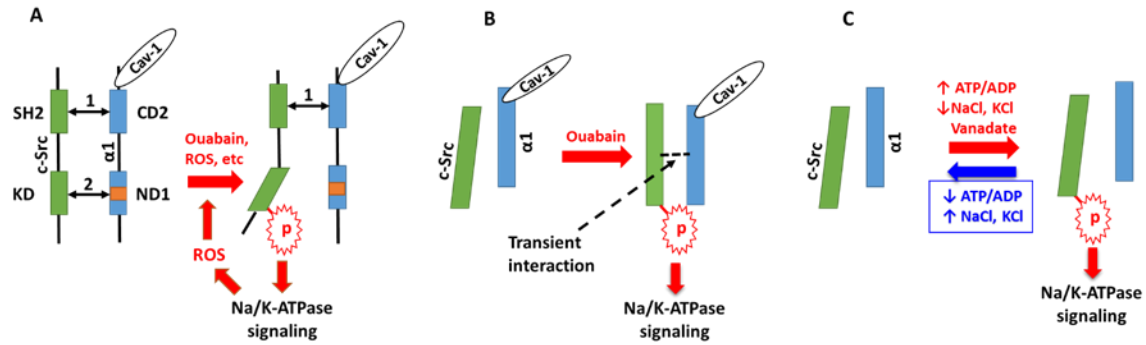


Figure 13. Schematic illustration of different “working” models.

In the illustration, ouabain is used as a representative of cardiotonic steroids. **A**, the model of Na/K-ATPase/c-Src (binding) receptor complex. **B**, the model of c-Src activation by transiently binding to Na/K-ATPase/Cav-1 complex. **C**, the model of c-Src activation regulated by ATP/ADP ratio, ATPase inhibitor vanadate, and low concentration of Na^+ and K^+ . In this model, there is no binding between Na/K-ATPase and c-Src. The role of Cav-1 was not tested. Please refer to the references for details. Cav-1, caveolin-1; ROS, reactive oxygen species.

In these models, there is no doubt that c-Src activation is a proximal step in the Na/K-ATPase signaling. It is not a surprise that different “working” models are proposed based on different experimental systems, and an ideal “working” model is developed based on new developments and new technologies. As mentioned above, ouabain (and other cardiotonic steroids), ROS/reactive nitrogen species (RNS), changes of ionic concentrations (bulky or local), and other stimuli can activate different signaling pathways to excute different functional regulations. Moreover, these different signaling pathways and functional regulation are also cell-dependent. A common charateristic in these “working” models is that they are, at least partially, dependent on the conformation change. Specifically, the E2-P conformational state of the Na/K-ATPase is favored, and stabilized by Na/K-ATPase inhibitors (ouabain, vanadate, oligomycin), energy status (ATP/ADP ratio), and change in $[\text{Na}^+]$ and $[\text{K}^+]$. While the E2-P conformational state of the Na/K-ATPase is favored, a “slower” dynamic E-2P \leftrightarrow E1-P conformational change (in the presence of inhibitors and/or energy status) might be an effective way to maintain and control the

signaling strength and function. Nevertheless, these hypotheses need to be experimentally demonstrated.

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CHAPTER 4

RELATIONSHIP BETWEEN THE NA/K-ATPASE OXIDANT AMPLIFICATION LOOP AND ADIPOGENESIS IN 3T3-L1 CELLS

Introduction

Obesity has reached epidemic proportions worldwide; the prevalence of obesity has increased by 28% in adults and 47% in children from 1980 to 2013 and these numbers are still increasing (Smith & Smith, 2016). Obesity is a chronic multifactorial disease that has been associated with metabolic syndrome, diabetes mellitus, cardiovascular disease, chronic kidney disease, hypertension, insulin resistance, and even dementia (Andre, Dinel, Ferreira, Laye, & Castanon, 2014; Greenberg & McDaniel, 2002; J. E. Hall, Jones, et al., 2003; Kothari et al., 2017; Lafontan, 2014) . The typical “Western diet”, containing excessive fat and fructose, may predispose one to both obesity and these complications (Ohtomo et al., 2017; Sodhi et al., 2017).

It has been demonstrated that chronic oxidative stress contributes to the development of obesity (Le Lay et al., 2014; Srikanthan, Shapiro, et al., 2016; Vincent & Taylor, 2006). In many models of obesity, there is an increase in reactive oxygen species (ROS) and oxidative stress; it is thought that increased ROS lead to obesity, and obesity increases ROS (Furukawa et al., 2004; Srikanthan, Shapiro, et al., 2016). This increase in ROS related to obesity can be linked to dysfunctional Na/K-ATPase signaling (Rebecca D. Pratt et al., 2019; Srikanthan, Shapiro, et al., 2016).

Other than functioning as an “ion-pumping” pump, the Na/K-ATPase also functions as a receptor, signal transducer, and scaffold through multiple protein–protein interactions [reviewed in (J. Liu et al., 2018; R.D. Pratt, Brickman, Cottrill, Shapiro, & Liu, 2018; Z. Xie, 2003)]. Binding of cardiotonic steroids (specific inhibitors and ligands of Na/K-ATPase) such as ouabain

to the Na/K-ATPase α 1 subunit initiates different signaling pathways, including activation of c-Src kinase and Ras/Raf/ERKs cascade, and increases in ROS generation ((J. Liu et al., 2016; Sodhi et al., 2015; Sodhi et al., 2018; Sodhi et al., 2017; Z. Xie & Xie, 2005; Yan et al., 2013; Yan et al., 2016)). Recently, we further demonstrated that, while endogenous and/or exogenous factors stimulate ROS generation via Na/K-ATPase signaling, increases in ROS alone are also capable of stimulating the Na/K-ATPase signaling function (Yan et al., 2013). This forms a positive oxidant amplification loop in which the Na/K-ATPase signaling functions as an amplifier of oxidants through its downstream signaling cascade. Our lab has demonstrated that systemic administration of pNaKtide, a cell permeable peptide designed to inhibit Na/K-ATPase signaling, was able to reduce oxidative stress, obesity, nonalcoholic steatohepatitis (NASH), and atherosclerosis induced by a Western diet (Sodhi et al., 2015; Sodhi et al., 2017). Based on this, we were interested in examining the Na/K-ATPase signaling cascade in the specific cell involved in the pathogenesis of obesity: adipocytes.

Lentiviral vectors have evolved over the last decade as powerful, reliable, and safe tools for stable gene transfer in a wide variety of mammalian cells (Cao et al., 2012; Cao, Sodhi, Inoue, et al., 2011; Huentelman et al., 2005; Schambach & Baum, 2008). Lentivirus used for vector construction has been made replication deficient. Using lentivirus allows specific targeting of protein(s) to specific tissue depending on the specificity of the promoter; utilization of lentiviral vector is safe and highly effective, and has been demonstrated as an ideal tool for studies on obesity, hypertension, and related diseases (Cao et al., 2012).

Based on our previous observation that blockage of the Na/K-ATPase signaling cascade through systemic administration of pNaKtide decreased adipogenesis in 3T3-L1 cells by decreasing ROS (Sodhi et al., 2015), we aim to determine whether specific stimulation of the

Na/K-ATPase in adipocytes by administration of ouabain increases adipogenesis, and if this could be inhibited with pNaKtide administration. 3T3-L1, a murine preadipocyte cell line, has been widely used for studying mammalian adipogenesis (MacDougald & Lane, 1995; Rosen & MacDougald, 2006). These pre-adipocytes undergo a well-characterized process of differentiation upon induction with insulin, dexamethasone, and indomethacin, and differentiated 3T3-L1 cells exhibit many of the characteristics found in mature adipocytes from mammalian fat tissue (MacDougald & Lane, 1995). Further, we will explore whether lentiviral-mediated delivery of NaKtide (a non-cell permeable version of pNaKtide) will show a similar effect in adipogenesis as systemic administration of pNaKtide.

Results

Ouabain alone does not induce adipogenesis in 3T3-L1 cells but enhances the effects in adipogenic media

3T3-L1 cells were exposed to both maintenance media (MM) [Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic solution] and adipogenic media (AM) (MacDougald & Lane, 1995; Puri et al., 2012; Rosen, Walkey, Puigserver, & Spiegelman, 2000) and treated with increasing doses of ouabain. The adipogenic media consisted of DMEM-high glucose, 10% FBS, 10 $\mu\text{g}/\text{mL}$ insulin, 0.5 mM dexamethasone and 0.1 mM indomethacin. Our results showed that in MM there was no induction of adipogenesis at any concentration of ouabain, but when administered along with AM for 7 days ouabain enhance adipogenesis in a dose-dependent manner on top of AM (Figure 14 and 15). No cytotoxic effects of ouabain were noted at the lower doses used, but when dosing of ouabain reached 500 nM and above, there was a sharp increase in cytotoxicity; this was

anticipated as at high doses, ouabain can be toxic (Figure 16) (Aizman et al., 2001; Tian et al., 2006; Yan et al., 2012).

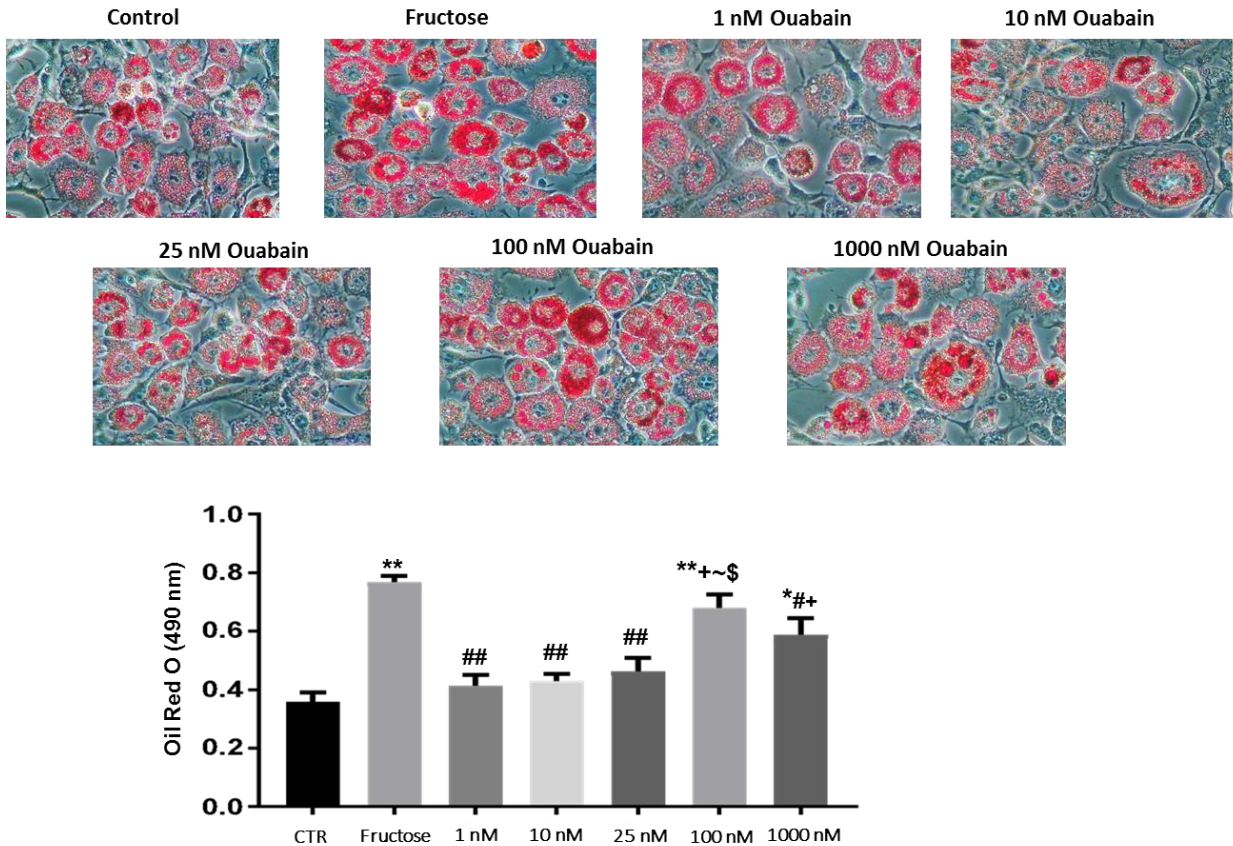


Figure 14. Ouabain increased adipogenesis in 3T3-L1 cells cultured in Adipogenic Media.

Oil Red O staining in 3T3 cells treated with varying concentrations of ouabain in adipogenic media. n=6 Per group. * p-value<0.05 vs CTR, ** p-value<0.001 vs CTR, # p-value <0.05 vs Fructose, ## p-value <0.001 vs Fructose, + p-value <0.05 vs 1 nM ouabain, ~ p-value <0.05 vs 10 nM ouabain, \$ p-value <0.05 vs 25 nM ouabain.

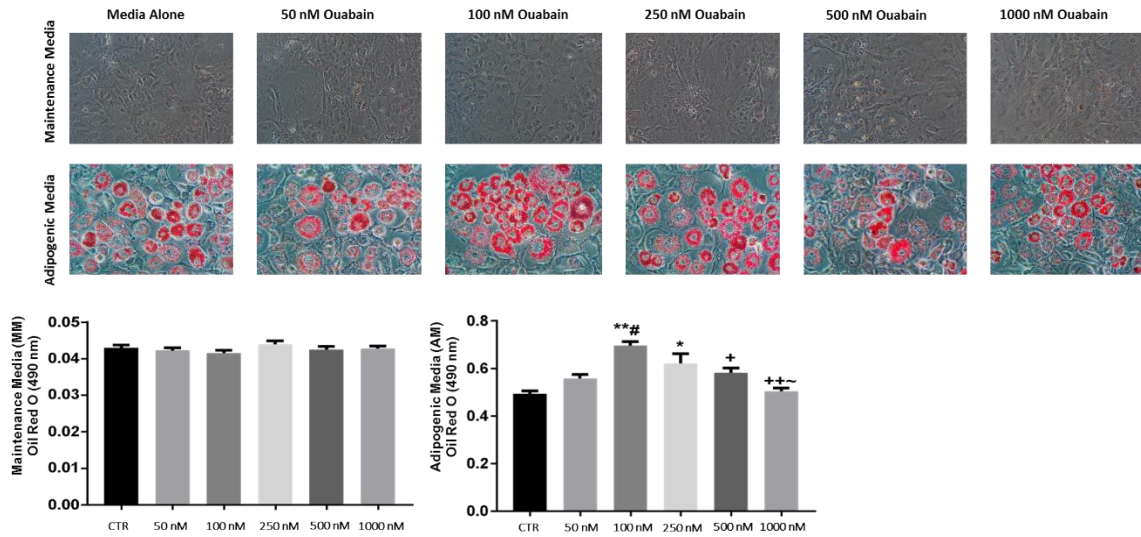
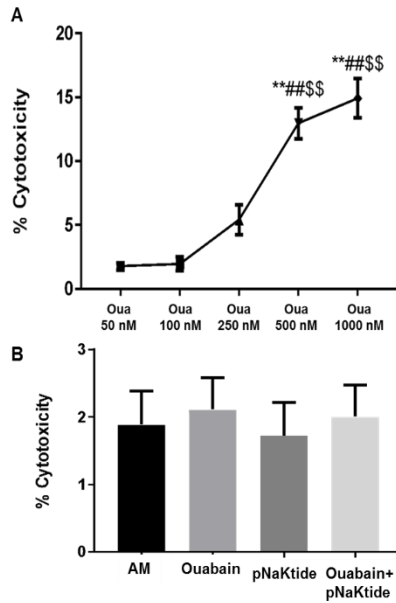


Figure 15. Ouabain alone did not induce adipogenesis but enhanced the effects in Adipogenic Media.

Oil Red O staining in 3T3-L1 cells treated with varying concentrations of ouabain in maintenance media and adipogenic media. n=6 Per group. * p-value <0.05 vs CTR, ** p-value<0.001 vs CTR, ## p-value <0.001 vs 50 nm ouabain ,++ p-value <0.001 vs ouabain 100 nM, ~ p-value <0.05 vs ouabain 250 nM



	LDH Activity	%Cytotoxicity
Spontaneous LDH Activity Control	0.0040	0.0
Maximum LDH Activity Control	0.1027	100.0
Oua 50	0.0057	1.8
Oua 100	0.0059	2.0
Oua 250	0.0093	5.4
Oua 500	0.0168	13.0
Oua 1000	0.0187	14.9

	LDH Activity	%Cytotoxicity
Spontaneous LDH Activity Control	0.0040	0.0
Maximum LDH Activity Control	0.1040	100.0
AM	0.0059	1.9
Ouabain 100 nM	0.0061	2.1
pNaKtide 700 nM	0.0057	1.7
Oua 100 nM + pNaKtide 700 nM	0.0060	2.0

Figure 16. Ouabain and pNaKtide on cytotoxicity in 3T3-L1 cells.

Measured LDH activity in (A) 3T3-L1 cells treated with varying concentrations of ouabain in AM and (B) 3T3-L1 cells treated with 100 nM ouabain, 700 nM pNaKtide, and both ouabain and pNaKtide in adipogenic media. n=6/group. ** p-value<0.001 vs ouabain 50 nM, ## p-value <0.001 vs ouabain 100 nM, \$\$p-value <0.001 vs ouabain 250 nM

pNaKtide decreases adipogenesis and expression of adipogenic and inflammatory markers in 3T3-L1 cells

3T3-L1 cells were exposed to AM, 100 nM of ouabain and 700 nM of pNaKtide for 7 days (Sodhi et al., 2015). As shown in Figure 16 there were no cytotoxic effects at these doses or with AM alone. Ouabain significantly increased adipogenesis as evidenced by Oil Red O staining, and pNaKtide decreased adipogenesis. Interestingly, even in the presence of ouabain, pNaKtide was able to decrease Oil Red O staining (Figure 17).

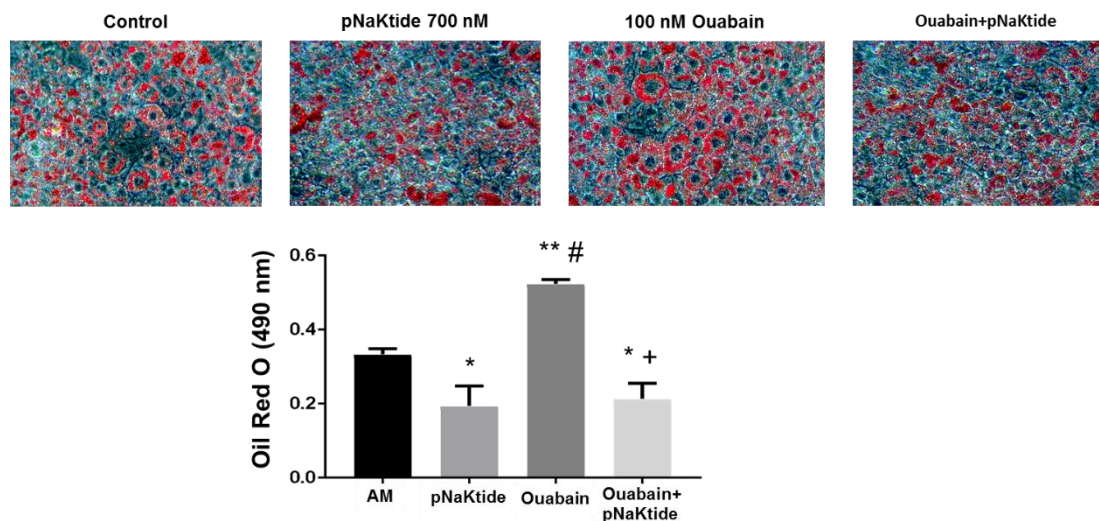


Figure 17. pNaKtide decreases adipogenesis.

Oil Red O staining of 3T3-L1 cells treated with 100 nM ouabain, 700 nM pNaKtide, and both ouabain and pNaKtide in adipogenic media. n=6/group. ** p-value<0.001 vs AM, # p-value <0.05 vs ouabain 100 nM, + p-value <0.05 vs pNaKtide

Administration of ouabain decreased adiponectin (a protein hormone involved in regulating glucose levels and fatty acid breakdown) levels, while significantly increasing the expression of adipogenic markers FAS, MEST, and PPAR γ and inflammatory markers TNF α , IL-6, and MCP-1 (Figure 18 and 19). Administration of pNaKtide, whether alone or in the presence of ouabain, increased adiponectin levels and reduced the expression of the adipogenic and inflammatory markers. This indicates that pNaKtide prevented or reversed adipocyte

dysfunction and inflammation associated with the dysfunction, even in the presence of specific stimulation of the Na/K-ATPase by ouabain.

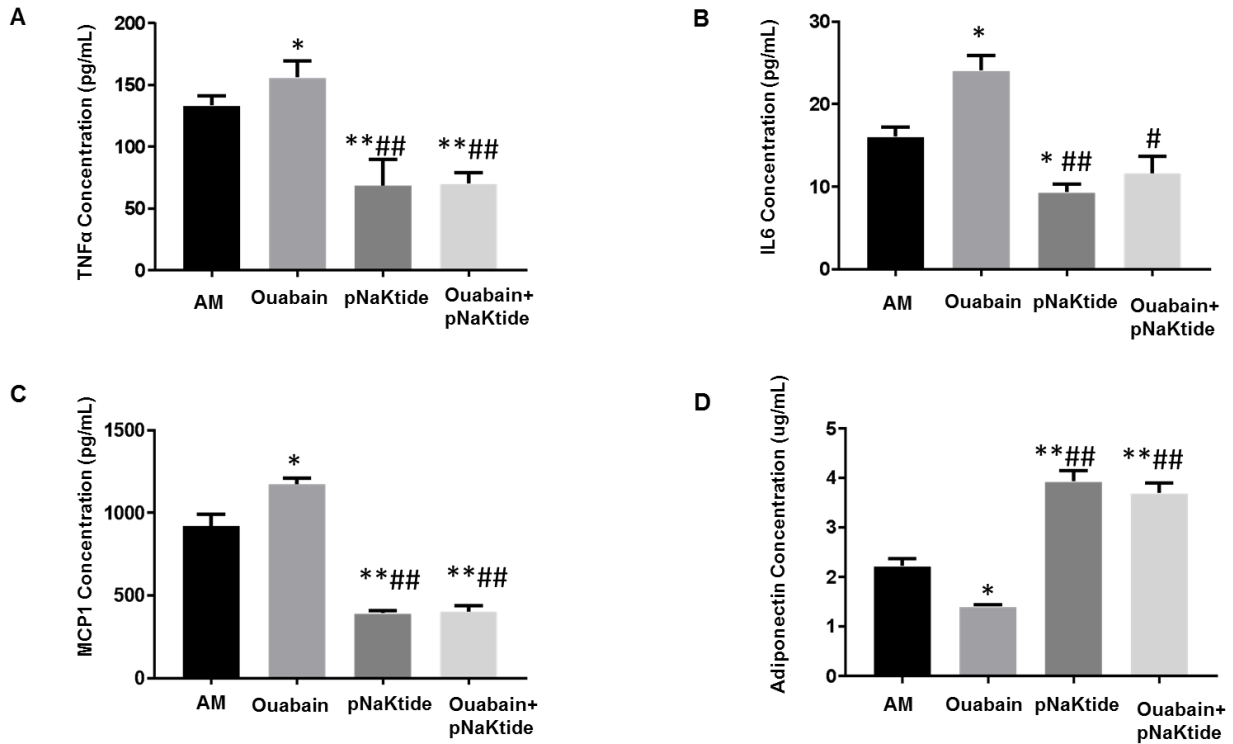


Figure 18. pNaKtide improves inflammatory profile of 3T3-L1 cells treated with AM and/or ouabain.

Measurement of (A) TNF α , (B) IL-6, (C) MCP-1 and (D) adiponectin in media of 3T3-L1 cells treated with 100 nM ouabain, 700 nM pNaKtide, and both ouabain and pNaKtide in adipogenic media. n=6/group. ** p-value<0.001 vs AM, ## p-value <0.001 vs ouabain

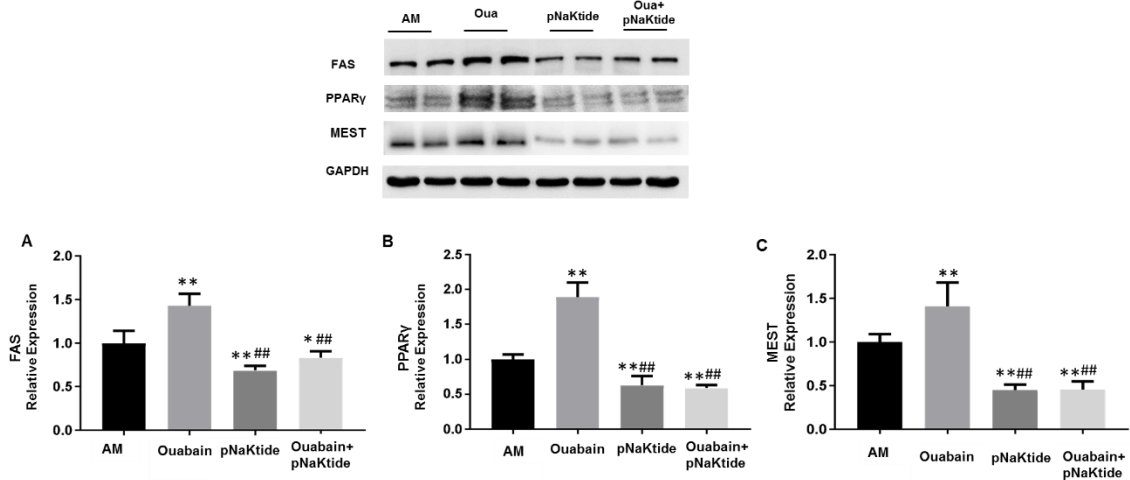


Figure 19. Ouabain increases expression of adipogenic markers in 3T3-L1 cells cultured in Adipogenic Media.

Western blot of (A) FAS, (B) PPAR γ , and (C) MEST in 3T3-L1 cells treated with 100 nM ouabain, 700 nM pNaKtide, and both ouabain and pNaKtide in adipogenic media. n=6/group. ** p-value<0.001 vs AM, ### p-value <0.001 vs ouabain

pNaKtide decreases c-Src phosphorylation and protein carbonylation in 3T3-L1 cells

When exposed to ouabain, c-Src phosphorylation significantly increased in 3T3-L1 cells (Figure 20). This was accompanied by an increase in protein carbonylation, which is a well-known marker of oxidative stress (Rebecca D. Pratt et al., 2019; P. T. Shah et al., 2016; Yan et al., 2013). Administration of pNaKtide reduced c-Src phosphorylation and decreased protein carbonylation; this suggests that pNaKtide has a positive role on cellular redox in the adipocyte, and can prevent adipogenesis-mediated oxidative stress through partially blocking the Na/K-ATPase signaling cascade.

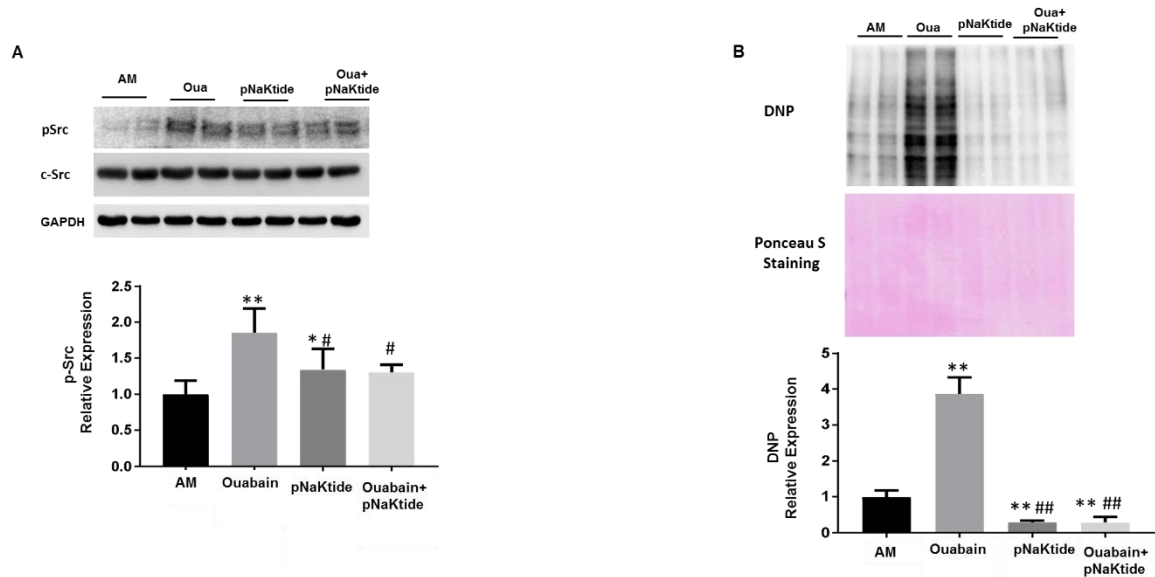


Figure 20. Ouabain stimulated c-Src phosphorylation and protein carbonylation in 3T3-L1 cells cultured in Adipogenic Media.

Western blot of (A) Src and (B) protein carbonylation in 3T3-L1 cells treated with 100 nM ouabain, 700 nM pNaKtide, and both ouabain and pNaKtide in adipogenic media. n=6/group. ** p-value<0.001 vs AM, ## p-value <0.001 vs ouabain.

pNaKtide increases expression of the $\alpha 1$ subunit of the Na/K-ATPase over the course of 7-day treatment window in 3T3-L1 cells

The $\alpha 1$ subunit of the Na/K-ATPase is regularly endocytosed, repaired, and recycled back to the plasma membrane to be re-integrated; it has been shown that increased levels of oxidative stress and ROS can impair this process and decrease the amount of endocytosed $\alpha 1$ subunit that is recycled (Cavalli et al., 2001; Chibalin et al., 1997; Comellas et al., 2006; Dada et al., 2003; J. Liu, 2005; J. Liu et al., 2004; J. Liu et al., 2005). To this end, we examined $\alpha 1$ expression at Days 1, 3, 5, and 7 of after treatment with MM and AM. In MM there is no change in $\alpha 1$ expression over the course of 7 days (Figure 21). Interestingly, both AM alone and AM with 100 nM of ouabain reduces $\alpha 1$ expression by approximately 50%, seen in Day 3 and by the end of treatment (Day7) (Figure 21 and 22). pNaKtide, prevented this decrease in $\alpha 1$ expression

(pNaKtide alone) or significantly slowed the process even in the presence of ouabain (ouabain+pNaKtide) (Figure 22).

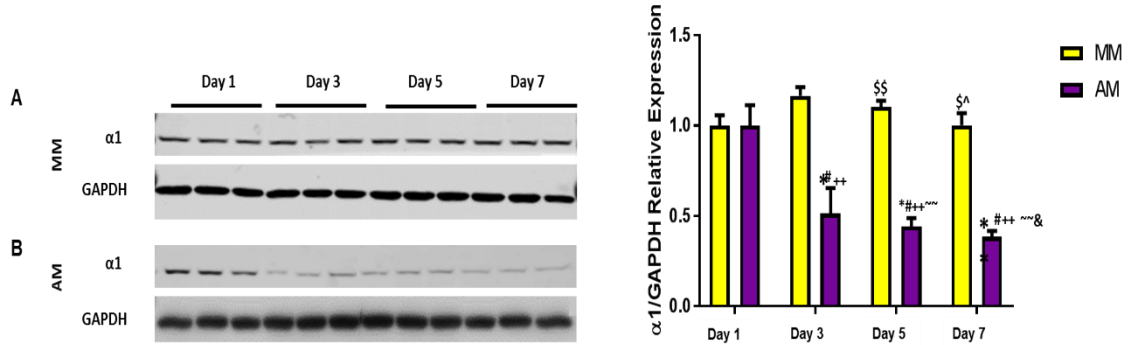


Figure 21. Adipogenic Media (AM) but not Maintenance Media (MM) decreased $\alpha 1$ expression during 7 day time course.

Western blot of $\alpha 1$ expression in 3T3-L1 cells in (A) maintenance media or (B) adipogenic media at time points of 1, 3, 5, or 7 days of exposure to media. n=6/group. *p-value<0.05 vs MM Day 1, **p-value<0.001 vs MM Day 1, #p-value<0.05 vs AM Day 1, ##p-value<0.001 vs AM Day 1, ++p-value<0.001 vs MM Day 3, \$p-value<0.05 vs AM Day 3, \$\$p-value<0.001 vs AM Day 3, ~p-value<0.001 vs MM Day 5, ^p-value<0.05 vs AM Day 5, &p-value <0.05 vs MM Day 7

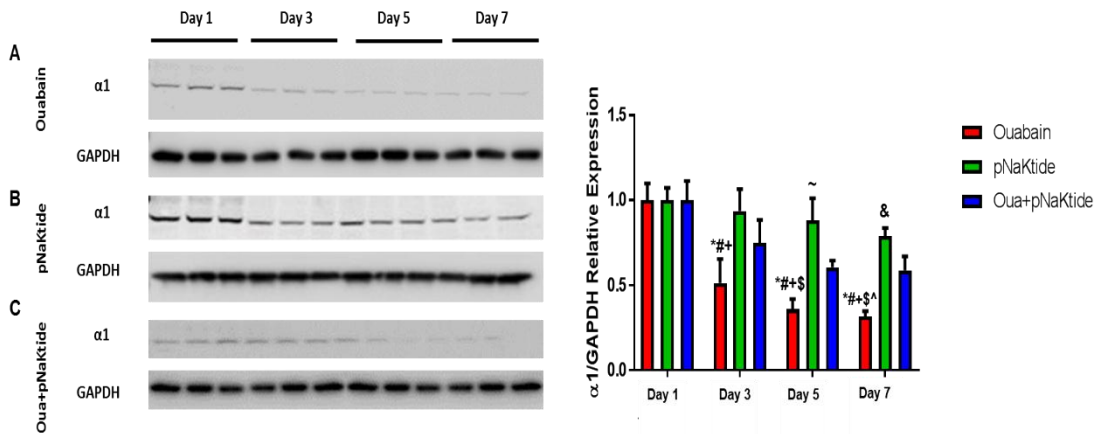


Figure 22. pNaKtide prevents decrease in $\alpha 1$ expression during 7 day time course.

Western blot of $\alpha 1$ expression in 3T3-L1 cells in (A) adipogenic media with 100 nM ouabain, (B) adipogenic media with 700 nM pNaKtide, or (C) adipogenic media with both ouabain and pNaKtide at timepoints of 1, 3, 5, or 7 days of exposure to media and treatment. n=6/group. * p-value<0.05 vs ouabain Day 1, # p-value<0.05 vs pNaKtide Day 1, +p-value<0.05 vs ouabain+pNaKtide Day 1, \$ p-value<0.05 vs pNaKtide Day 3, ~ p-value<0.05 vs ouabain Day 5, ^ p-value<0.05 vs pNaKtide Day 5, &p-value<0.05 vs ouabain Day 7

Transfection of NaKtide to 3T3-L1 cells effectively reduces AM-induced adipogenesis

Lentiviral constructs were used to deliver NaKtide specifically into adipocytes with an adiponectin promoter (Figure 23). Adiponectin mRNA transcripts are highly expressed in adipocytes as well as preadipocytes differentiating into adipocytes; therefore, adiponectin is the most suitable candidate for targeting adipocytes (Lara-Castro, Fu, Chung, & Garvey, 2007; Maeda et al., 1996; Matsuzawa, Funahashi, Kihara, & Shimomura, 2004).

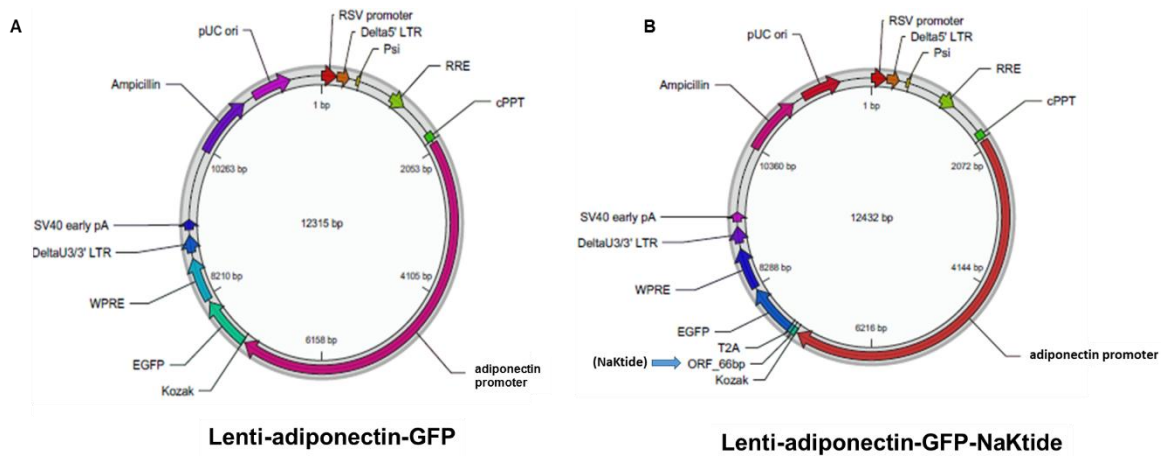


Figure 23. Lentiviral Constructs.

An adiponectin promoter drove expression of the NaKtide cassette linked by means of a 2A peptide to eGFP for bicistronic expression. (A) Lenti-adiponectin-GFP is the empty viral vector and (B) lenti-adiponectin-GFP-NaKtide contains the sequence for NaKtide.

After transfection with the lentivirus, NaKtide was shown to decrease adipogenesis in comparison to the AM alone and- expression of control vector (an empty lentiviral vector expressing GFP alone) (Figure 24). Transduction efficiency was calculated (see Materials and Methods) to determine the percentage of cells that were successfully infected with lentivirus; both the lenti-GFP and lenti-NaKtide group had transduction efficiencies of approximately 80% and there was no significant difference between the groups (Figure 24).

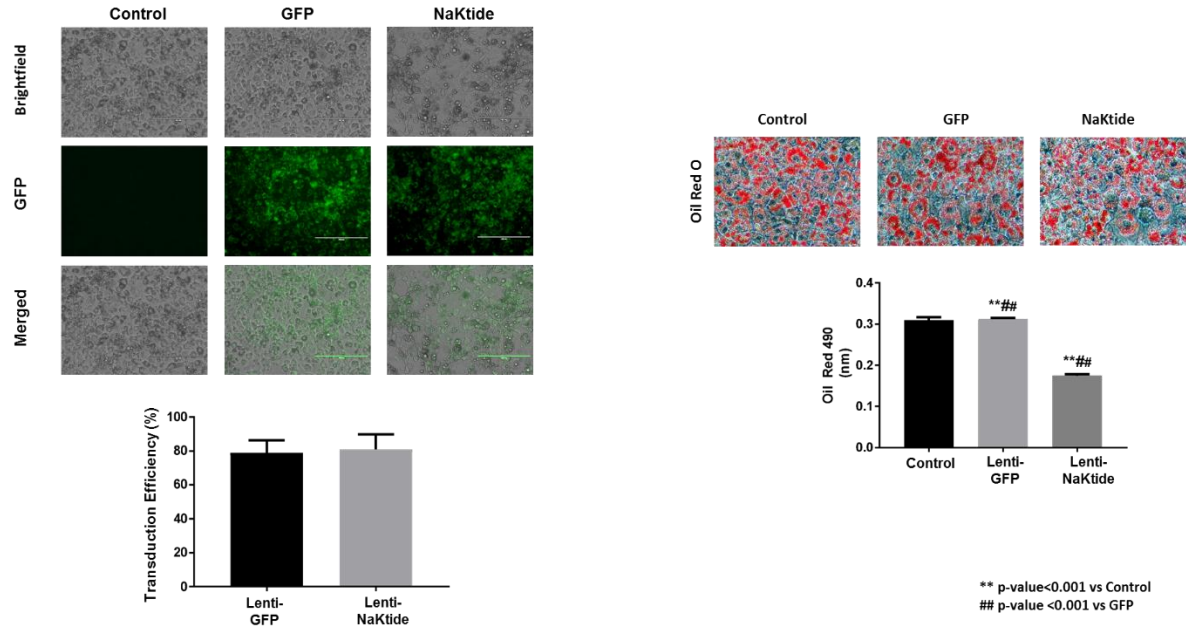


Figure 24. Transfection of NaKtide to 3T3-L1 cells effectively reduces adipogenesis. (A) Brightfield and GFP images taken of 3T3-L1 cells transfected with lenti-adiponectin-GFP and lenti-adiponectin-NaKtide, respectively. (B) Transduction efficiency of both lenti-adiponectin-GFP and lenti-adiponectin-NaKtide. (C) Oil Red O staining of cells transfected with lenti-adiponectin-GFP and lenti-adiponectin-NaKtide. n=8/group. ** p-value<0.001 vs Control, ## p-value <0.001 vs GFP

Discussion

This study highlights the therapeutic potential of targeting adipocytes in obesity. Oxidative stress has been implicated with the chronic inflammatory state that has been shown to be a crucial factor in obesity (Gutierrez et al., 2009; Trayhurn, 2007; Wellen & Hotamisligil, 2003). We have demonstrated in this report that pNaKtide effectively reduces adipogenesis *in vitro* by antagonizing Na/K-ATPase-mediated amplification of ROS signaling; our results with lenti-adiponectin-GFP-NaKtide show a similar trend as is shown with the reduction of adipogenesis.

We observed that in murine preadipocytes, exposure to pNaKtide attenuated oxidative stress, decreased lipid accumulation, and decreased presence of adipogenic regulators PPAR γ , CEBP α , and FAS. There was a significant upregulation in the production of adiponectin levels in the pNaKtide treated cells as well. The inflammatory profile in these cells (TNF α , MCP-1, and IL-6) showed significant reductions, indicating a decrease in oxidative stress. Interestingly, these effects were seen even in the presence of ouabain, a specific stimulator of the Na/K-ATPase. This implies that pNaKtide acts on the Na/K-ATPase oxidant amplification loop in the adipocytes; increased ROS and oxidative stress, increased Na/K-ATPase signaling, and increased adipogenesis seen with administration of ouabain in adipogenic conditions could contribute to a redox imbalance that is improved with treatment of pNaKtide.

It is important to note that the increase in adipogenesis and oxidative stress was only seen when combined with adipogenic media. 3T3-L1 cells in maintenance media did not see any adipogenesis, even in the presence of ouabain. This is because the growth arrest seen in *in vitro* adipogenesis occurs in tandem with the expression of transcription factors peroxisome proliferator activated receptor γ (PPAR γ) and CCAAT/enhancer binding protein α (CEBP α) and addition of a “pro-differentiative hormonal regimen”, in our case, adipogenic media (Rosen et al., 2000).

We also observed a marked decrease in $\alpha 1$ expression in 3T3-L1 cells treated with adipogenic media and ouabain, and this decrease was rescued upon addition of pNaKtide in the presence and absence of ouabain. Literature shows that there is an approximately 55% decrease in $\alpha 1$ expression upon differentiation in adipocytes; we saw this effect in adipogenic media and upon addition of ouabain. At higher doses of ouabain there was an increase in toxicity. The IC₅₀ of ouabain, or the half maximal inhibitory concentration (a measure of the potency of a substance

in inhibiting a specific biological or biochemical function) is 100 μM for $\alpha 1$ (Lelievre, Crambert, & Allen, 2001). However, the IC_{50} of ouabain for $\alpha 2$ is 1 μM ; in differentiated adipocytes the major isoform of the Na/K-ATPase present is $\alpha 2$. The increased concentration (and the isoform switch and pumping function from $\alpha 2$ seen in adipocytes) may explain the increased toxicity seen at higher doses. Another area of interest is where the majority of signaling of the Na/K-ATPase comes from; the endocytosed Na/K-ATPase versus the plasma membrane Na/K-ATPase. Increased oxidative stress causes an increase in endocytosis of the pump. The internalized Na/K-ATPase may still have some signaling function, but this has not yet been determined. This is an important future direction that could be crucial to further understanding Na/K-ATPase signaling in the adipocyte.

These observations are of budding interest for several reasons. First, we have confirmed the role of the Na/K-ATPase oxidant amplification loop in the process of adipogenesis. By utilizing ouabain, a known stimulator of Na/K-ATPase signaling, we have shown that in tandem with adipogenic conditions, increased Na/K-ATPase signaling increases adipogenesis and ROS; this process was not previously linked to cardiotonic steroids or the Na/K-ATPase signal cascade but our data shows a potentially larger role of the Na/K-ATPase oxidant amplification loop in adipocyte biology and obesity pathology.

Next, utilization of pNaKtide, even in the presence of specific stimulation of the Na/K-ATPase may present a therapeutic effect. pNaKtide alone decreased adipogenesis, ROS, and the inflammatory profile of the adipogenic cells, and the effect was able to surpass the effects of ouabain. This implies that not only does pNaKtide have an effect on adipogenesis *in vitro*, but it also suggests that the effect of pNaKtide is coming through direct action on the Na/K-ATPase oxidant amplification loop to decrease impaired redox biology of the adipocyte itself. This

further confirms the role of adipocytes in obesity and its comorbidities is much more important than previously determined.

Finally, we used a lentiviral vector to deliver NaKtide to the 3T3-L1 cells. NaKtide and pNaKtide are similar in structure, but NaKtide is non-cell permeable. pNaKtide contains a 13 amino acid TAT leader sequence that allows it to penetrate the cell membrane, and act inside the cell. NaKtide, until now, has been unable to permeate the cell membrane without some measure of toxicity (Z. Li et al., 2009). By utilizing a lentiviral vector, NaKtide is expressed inside the cells. Expression of lenti-adiponectin-GFP-NaKtide showed a promising decrease in adipogenesis, and will be used in a rodent model of obesity to see if it has similar effects to systemic administration of pNaKtide(Sodhi et al., 2015)

We have clearly demonstrated that the Na/K-ATPase amplification loop has an impact on adipogenesis. ROS amplification can occur through this pathway, and significantly increase adipogenesis and further increase Na/K-ATPase signaling. These results suggest that the Na/K-ATPase oxidant amplification loop may serve as a potential target in dysfunctional redox states and oxidative stress therapies associated with obesity.

Materials and Methods

Cell Culture Experiments

Frozen mouse preadipocytes (3T3-L1) were resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic solution. The cultures were maintained at 37°C in a 5% CO₂ incubator, and the medium was changed after 24 hours and every 3 to 4 days thereafter. When the 3T3-L1 cells were confluent, the cells were recovered by the addition of trypsin.

3T3-L1 cells (passages 2 to 3) were plated in 24-well plates at a density of 10,000 cells/cm² and cultured in DMEM until ~75% confluence was achieved. The medium was replaced with adipogenic medium (DMEM-high glucose, 10% FBS, 10 µg/mL insulin, 0.5 mM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), and 0.1 mM indomethacin (Sigma-Aldrich, St. Louis, MO, USA) and the cells were cultured for an additional 7 days. Cells were treated every day with either media alone, fructose, ouabain (concentrations 1 nM, 10 nM, 25 nM, 100 nM, 250 nM, 500 nM, or 1000 nM) or 700 nM pNaKtide (previously described by Sodhi et. al (Sodhi et al., 2015)). After 7 days, the cells were stained with Oil Red O solution to analyze adipogenesis as previously described by Sodhi *et al* (Sodhi et al., 2015; Sodhi et al., 2014). Media was collected for cytokine measurements, and cells were also collected from 75 cm² flasks for western blot

Lentivirus Construction

Lentiviral constructs with NaKtide driven by an adiponectin promoter were constructed by VectorBuilder Inc. and were used to achieve NaKtide expression. Lentiviral constructs featured the adiponectin promoter driving expression of the NaKtide cassette linked by means of a 2A peptide to eGFP for bicistronic expression.

The NaKtide sequence used for this vector was ATGAGCGCCACCTGGCTGGCCCTGAGCAGGATCGCCGGTCTTTGCAACAGGGCCGTGTTCCAG. Lentivirus with NaKtide and its counterpart Lenti-GFP (driven by adiponectin promoter) were mixed into DMEM (10 uL/mL) with polybrene (100 uL/mL) and added to approximately 60% confluent 3T3 L1 cells. After 24 hours, cells were placed in adipogenic media and left for 14 days. After 14 days, cells were stained with Oil Red O solution to analyze

adipogenesis. Media was collected for cytokine measurements, and cells were also collected from 75 cm² flasks for western blot analysis.

LDH Assay

Potential toxicity of ouabain was measured using a Pierce LDH Cytotoxicity Assay Kit according to manufacturer instructions (ThermoFisher Scientific, Waltham, MA). Briefly, at the end of treatment, 50 µl of medium solution was transferred to a 96-well plate and the LDH reaction was performed. To determine LDH activity, the absorbance at 680 nm (background signal) was subtracted from the absorbance at 490 nm. LDH activity was converted to percentages compared to the maximum LDH activity control and the spontaneous LDH activity control using the following formula:

$$\% \text{Cytotoxicity} = \frac{\text{Compound treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \times 100$$

Cytokine Measurements

TNF α , IL-6, and MCP-1 were measured in cell culture media using an ELISA assay kit according to manufacturer instructions (Abcam Cambridge, MA) as reported previously (Bartlett, Miller, Thiesfeldt, Lakhani, Khanal, et al., 2018; Sodhi et al., 2015).

Western Blot Analysis

Cell culture lysates were processed using TGH buffer. Homogenates were centrifuged, the supernatant was isolated, and immunoblotting performed. Lysates were used for

determination of $\alpha 1$ subunit of the Na/K-ATPase, FAS, PPAR γ , MEST as previously reported (Haller et al., 2012; Yan et al., 2013). Loading conditions were controlled for using GAPDH.

Measurement of c-Src phosphorylation

Cell culture lysates were processed using TGH buffer. Activation of c-Src was determined as described previously (Yan et al., 2013). Polyclonal anti-Src [pY418] phospho-specific antibody was from Invitrogen (Camarillo, CA). Monoclonal antibody against total c-Src was from Santa Cruz (Santa Cruz, CA). After immunoblotting for phospho-Src, the same membrane was stripped and blotted for total c-Src. Activation of c-Src was expressed as the ratio of phospho-Src/c-Src with measurements normalized to 1 for control samples.

Assessment of protein carbonylation

Cell culture lysates were processed using TGH buffer and western blotting for protein carbonylation assay was done as previously described (Yan et al., 2013). 2, 4-dinitrophenylhydrazine (DNPH) and antibody against 2, 4-dinitrophenyl (DNP) hydrazone derivatives were purchased from Sigma-Aldrich. The signal density values of control samples were normalized to 1 with Coomassie blue staining.

Transduction Efficiency

Transduction efficiency in lentivirus treated cells was performed using GFP images and brightfield images. 7 days after transfection cells were checked for GFP expression using a Nikon Eclipse 80i microscope equipped with a Nikon camera head DS-Fi1 (Nikon, Japan). Brightfield and GFP images were merged and counted using Image J. Images were changed to 8 bit, enhanced through Threshold, Dilate, and Watershed functions, converted to mask using the binary function, and enhanced. Black and white images were compared to the original image to

ensure accurate representation of cells. The total number of cells was calculated from the brightfield image, and the fluorescent cells were counted from the GFP image. This was consistent and used for all subsequent images. The ratio of cells was calculated (brightfield/GFP) and converted to a percentage.

Statistical analyses

Statistical significance between experimental groups was determined by the Tukey method of analysis of multiple comparisons ($P < 0.05$). For comparisons among treatment groups, the null hypothesis was tested by a one way analysis of variance (ANOVA). Data are presented as means \pm SE.

CHAPTER 5

THE ADIPOCYTE NA/K-ATPASE OXIDANT AMPLIFICATION LOOP IS THE CENTRAL REGULATOR OF WESTERN DIET-INDUCED OBESITY AND ASSOCIATED COMORBIDITIES

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Keywords: NaKtide, obesity, lentivirus, Na/K-ATPase signaling, ROS, adipocyte

Abstract

Obesity has become a worldwide epidemic. We have previously reported that systemic administration of pNaKtide which targets the Na/K-ATPase oxidant amplification loop (NKAL) was able to decrease systemic oxidative stress and adiposity in mice fed a high fat and fructose supplemented western diet (WD). As adipocytes are believed to play a central role in the development of obesity and its related comorbidities, we examined whether lentiviral-mediated adipocyte-specific expression of NaKtide, a peptide derived from the N domain of the $\alpha 1$ Na/K-ATPase subunit, could ameliorate the effects of the WD. C57BL6 mice were fed a WD, which activated Na/K-ATPase signaling in the adipocytes and induced an obese phenotype and caused an increase in plasma levels of leptin, IL-6 and TNF α . WD also decreased locomotor activity, expression of the D2 receptor and tyrosine hydroxylase in brain tissue, while markers of neurodegeneration and neuronal apoptosis were increased following the WD. Selective adipocyte expression of NaKtide in these mice fed a WD attenuated all of these changes including the brain biochemical alterations and behavioral adaptations. These data suggest that adipocyte derived cytokines play an essential role in the development of obesity induced by a WD and that targeting the adipocyte NKAL loop may serve as an effective therapeutic strategy.

Main Text

Obesity is a major international health challenge as it is often associated with other comorbidities including (but not limited to) metabolic syndrome, cardiovascular diseases, chronic kidney disease, non-alcoholic steatohepatitis (NASH), and neurodegenerative disorders (Pugazhenti, Qin, & Reddy, 2017; Sodhi et al., 2015; Sodhi et al., 2018; Sodhi et al., 2017). It is estimated that by the year 2030, 38% of the world's adult population will be overweight and 20% will be obese (Hruby & Hu, 2015). Although traditionally the adipocyte had been thought to play a passive role in the process of obesity, recent studies have established adipocyte malfunction as playing a causative role in the pathogenesis of obesity, associated systemic oxidant stress and the comorbidities of obesity including insulin resistance, accelerated cardiovascular disease and NASH [2-4]. Evidence suggests that deranged mitochondrial function and chronic inflammation in the adipocytes further contribute towards the pathogenesis of obesity (Cui, Kong, & Zhang, 2012; Oberg et al., 2004) .

Our group has demonstrated that the Na/K-ATPase functions as a scaffolding protein, affecting a signal cascade that also serves to amplify oxidants in a feed forward manner (Aperia, 2007; Bagrov & Shapiro, 2008; Bagrov et al., 2009; Z. Li & Xie, 2009; Liang, Cai, Tian, Qu, & Xie, 2006; J. Liu & Xie, 2010; Pierre & Xie, 2006; Tian et al., 2006; Z. Xie & Askari, 2002; Z. Xie & Cai, 2003; Z. Xie & Xie, 2005). Activated Na/K-ATPase signaling has been demonstrated in adipose, hepatic, renal, and cardiac tissues, thus implying a role for Na/K-ATPase signaling in various disease models (J. Liu et al., 2016; Sodhi et al., 2015; Sodhi et al., 2018; Sodhi et al., 2017). Specifically, we have demonstrated that reactive oxygen species (ROS) can both activate this Na/K-ATPase signal cascade as well as generate additional ROS through downstream consequences of this pathway (Y. Wang et al., 2014; Yan et al., 2013). NaKtide, a specific

peptide antagonist of Src kinase, was derived from the $\alpha 1$ subunit of the NaK-ATPase, consisting of Ser 415 to Gln 434. NaKtide or a cell permeant derivative, pNaKtide which was created by merging NaKtide with a 13 amino acid TAT leading sequence, prevents the activation of Src which is normally regulated by the $\alpha 1$ subunit of the Na/K-ATPase (Z. Li et al., 2009; J. Liu et al., 2016). Since we have demonstrated that systemic administration of pNaKtide was able to reduce oxidative stress, obesity and atherosclerosis induced by a western diet composed of high fat and supplemental fructose (J. Liu et al., 2016; Sodhi et al., 2015; Sodhi et al., 2017), we were interested in using this molecular strategy to examine the specific tissues involved in the pathogenesis of obesity.

Lentiviral vectors have evolved over the last decade as a promising approach to target a gene of interest using a promoter for a specific cell type (Cao, Sodhi, Inoue, et al., 2011). Utilization of the lentiviral vector has been safe and highly effective in experimental models (Ames & Lu, 2009; Bian et al., 2013; Cao, Sodhi, Inoue, et al., 2011; Eerola et al., 2013; W. Wu et al., 2018). We therefore chose this strategy to test the hypothesis that adipocyte oxidant stress caused by activation of adipocyte Na/K-ATPase signaling might play a central role in the development and maintenance of obesity and its associated comorbidities.

Results

Effect of lenti-adiponectin-GFP-NaKtide on adiposity, metabolic balance, and locomotion in C57BL6 mice fed a WD

The effectiveness and specificity of the NaKtide lentiviral-construct were evaluated by immunofluorescence studies in C57BL6 mice fed a WD. Since adiponectin is expressed specifically in adipocytes, the lentiviral construct with NaKtide driven by an adiponectin promoter was used to achieve NaKtide expression specifically in adipocytes (Figure 30). Results

showed that NaKtide expression was exclusively present in the adipose tissue and not visibly expressed in liver and brain tissues (Figure 31). Further, our results showed that mice fed a WD exhibited a significant increase in body weight over a period of 12 weeks compared to the mice on normal chow (Figure 25A). The increase in the body weight was significantly attenuated by the transduction of lenti-adiponectin-GFP-NaKtide (Figure 25A). Our results also demonstrated a significant increase in the visceral and subcutaneous fat, liver weight, and heart weight in the mice fed a WD, which was markedly reduced with lenti-adiponectin-GFP-NaKtide treatment (Table 1). Food intake and energy intake did not differ amongst the different experimental groups (Figure 25B and C). Energy expenditure was determined as heat production rate in units of kcal/kg/day (Sodhi et al., 2017). The results showed significant reduction in the energy expenditure of mice fed a WD compared to control group; this energy expenditure was significantly increased with lenti-adiponectin-GFP-NaKtide treatment (Figure 25D). Further, our results showed that oxygen consumption in mice fed a WD alone (2491 ± 149 mL/kg/hr) was significantly ($p < 0.01$) decreased as compared to control (3104 ± 88 mL/kg/hr). Treatment with lenti-adiponectin-GFP-NaKtide (3146 ± 232 mL/kg/hr) significantly ($p < 0.01$) improved the oxygen consumption as compared to WD alone. Since obesity has been associated with decreased locomotion (Massiera et al., 2001; Sodhi et al., 2017), we further looked at the effect of lenti-adiponectin-GFP-NaKtide on movement. Locomotor activity was measured as previously described (Sodhi et al., 2017). Mice fed a WD showed decreased locomotion, and this was normalized by treatment with the lenti-adiponectin-GFP-NaKtide (Figure 25E).

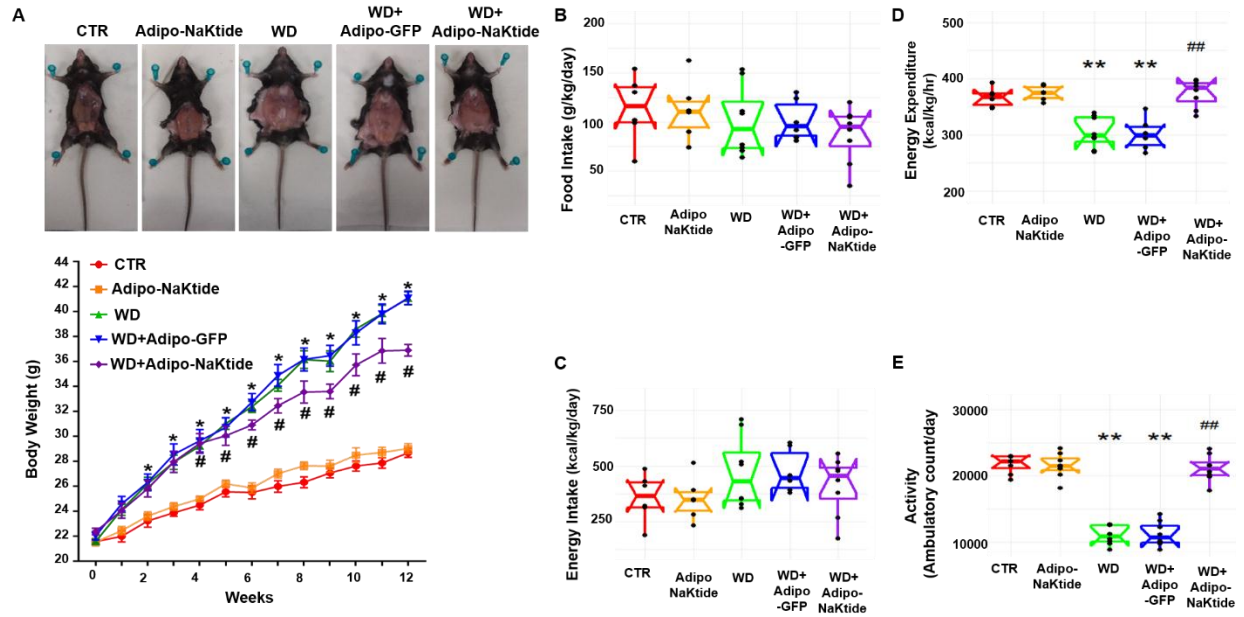


Figure 25. Effect of lenti-adiponectin-GFP-NaKtide on adiposity, metabolic balance, and locomotion in C57BL6 mice fed a WD.

Body weight (A). Food intake (B) Energy intake (C) Energy expenditure (D), and Locomotion (E) determined via CLAMS cages after 48 hours. Data are displayed as "scatter plots" showing data points and "box plots" showing the distribution of a continuous variable as described in the Methods section. N=12-14/group; * $p < 0.05$ vs. Control (CTR), ** $p < 0.01$ vs. CTR, # $p < 0.05$ vs. Western Diet (WD), ### $p < 0.01$ vs. WD.

	CTR (n=18)	Adipo- NaKtide (n=13)	WD (n=17)	WD+Adipo-GFP (n=14)	WD+Adipo-NaKtide (n=18)
Visceral Fat (g)	0.36±0.03	0.39±0.04	3.06±0.08**	3.10± 0.10**	1.65± 0.09***##
Subcutaneous Fat (g)	0.10±0.02	0.14±0.02	1.64±0.06**	1.61± 0.07**	0.52± 0.05***##
Liver (g)	1.57±0.04	1.34±0.05	2.90±0.15**	3.00± 0.16**	1.98± 0.08***##
Heart (g)	0.15±0.01	0.14±0.01	0.19±0.01*	0.17± 0.01*	0.15± 0.01#
RT-PCR Genes (mRNA Relative Expression in adipose tissue)					
MFN 1	1.02±0.07	1.27±0.16	0.27±0.11*	0.39±0.16	1.81±0.26***##
MFN 2	1.04±0.13	1.13±0.34	0.18±0.03*	0.28±0.07	1.87±0.25***##
TNF α	1.03±0.10	1.08±0.15	4.32±0.71**	4.76±0.46**	1.83±0.26#
Leptin	1.39±0.49	1.44±0.36	10.68±1.95*	10.78±2.05*	4.35±0.79#
Sirt3	1.03±0.12	1.20±0.14	0.35±0.06*	0.26±0.04*	1.49±0.21##

Table 1

Results are means \pm SEM, n = 13-18/group. *p < 0.05, **p < 0.01 vs. control; #<0.05, ## p < 0.01 vs WD

Effect of lenti-adiponectin-GFP-NaKtide on adipocyte phenotype and systemic inflammatory profile in C57BL6 mice fed a WD

The WD altered the morphological phenotype of visceral adipocytes with dramatic increases in the size and fat content of these cells. Mice treated with the lenti-adiponectin-GFP-NaKtide showed an improved adipocyte phenotype as evidenced by significantly increased numbers of adipocyte, but significant decreases in adipocyte cell area, when compared to mice fed a WD alone (Figure 26A, B & C). Mice injected with lenti-adiponectin-GFP-NaKtide and fed the normal chow diet showed no significant differences in any of the aforementioned measurements from the control mice. There was also no significant difference noted between mice injected with the empty vector control, the lenti-adiponectin-GFP lacking the NaKtide sequence that were fed a WD when compared to mice fed a WD alone. We next evaluated the markers directly tied to the altered adipocyte phenotype that plays a causal role in the

aggravation of systemic oxidative stress. Our Western blot analysis showed the upregulated expression of lipogenic marker, fatty acid synthase (FAS) and adipogenic markers, peroxisome proliferator-activated receptor gamma (PPAR γ) and mesoderm specific transcript gene (MEST) in WD fed mice. This upregulation was significantly attenuated in lenti-adiponectin-GFP-NaKtide transduced mice (Figure 26D-F). Since adipose mitochondria participate in energy expenditure, we measured genes of mitochondrial biogenesis in adipose tissue. Our results showed a significant down regulated expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), a major regulator of mitochondrial biogenesis, in mice fed a WD, as compared to control. This down regulation was prevented by lenti-adiponectin-GFP-NaKtide treatment (Figure 26G). Similarly, the expression of genes related to mitochondrial biogenesis and the “browning” phenomenon of adipose tissue, including expression of mitofusin (MFN) 1 and 2, and sirtuin 3 (Sirt3) were significantly down regulated in mice fed a WD. Importantly, the expression of the aforementioned genes were all significantly increased upon treatment with lenti-adiponectin-GFP-NaKtide (Table 1). Further, our results showed that the expression level of the inflammatory cytokine, tumor necrosis factor alpha (TNF α), was significantly attenuated by treatment with lenti-adiponectin-GFP-NaKtide (Table 1). Leptin, a hormone secreted by adipocytes, is a potent inducer of ROS generation by promoting inflammation and oxidative stress (Dubey & Hesong, 2006; Fernandez-Sanchez et al., 2011). Mice fed a WD exhibited upregulated levels of

leptin, as compared to control. This was significantly attenuated by lenti-adiponectin-GFP-NaKtide treatment (Table 1).

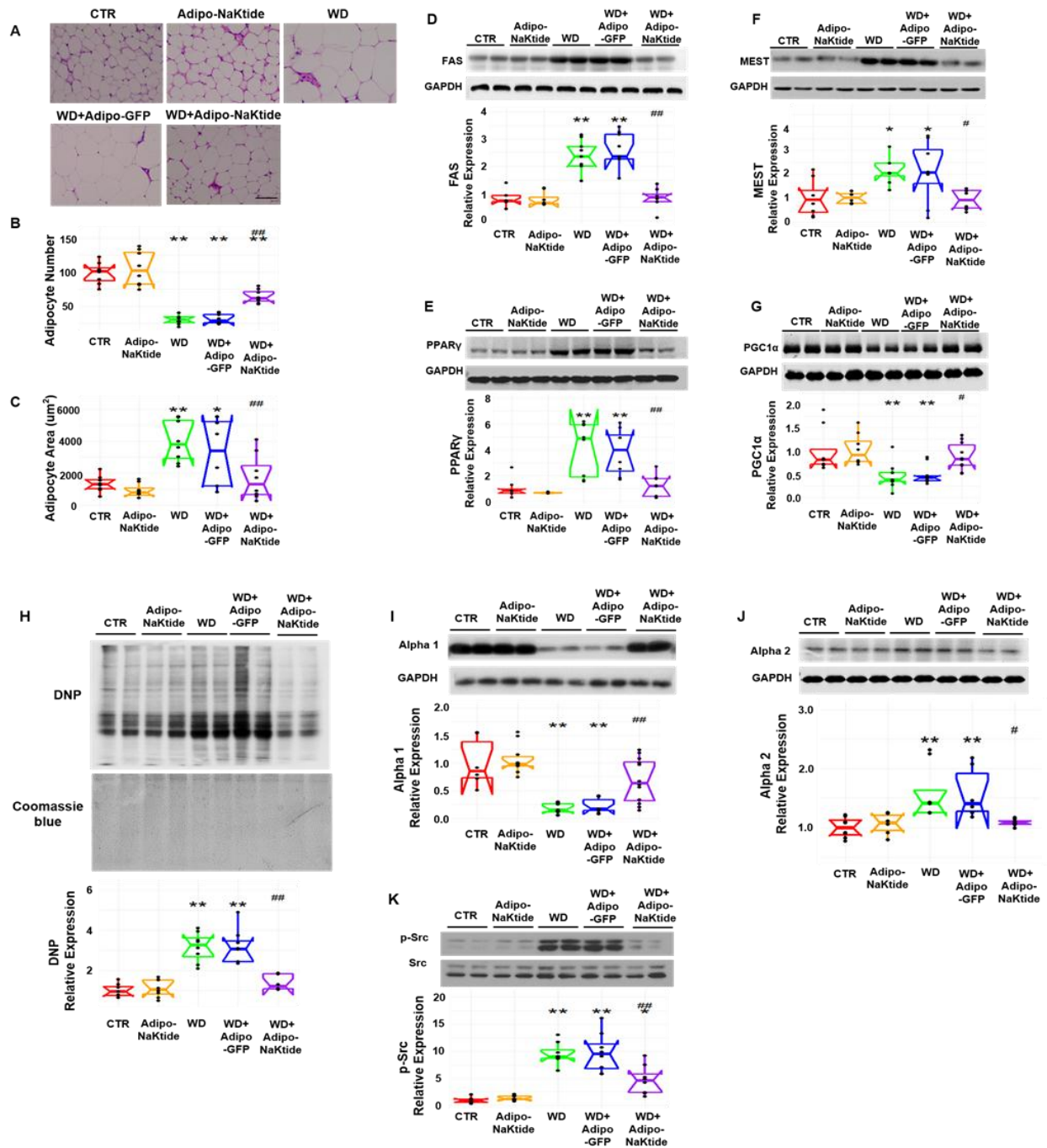


Figure 26. Effect of lenti-adiponectin-GFP-NaKtide on adipocyte phenotype, mitochondrial biogenesis, inflammatory markers, and Na/K-ATPase signaling cascade in C57BL6 mice fed a WD.

Representative H&E staining in visceral adipose tissue. Images taken with 20x objective lens; scale bar represents 100 μ m (A). Quantitative analysis of the adipocyte number (B) and adipocyte area (C) in visceral adipose tissue. Western blot analysis of visceral adipose tissue homogenates, with data shown as mean band density normalized to GAPDH, for fatty acid synthase (FAS) (D), peroxisome proliferator-activated receptor gamma (PPAR γ) (E), mesoderm specific transcript (MEST) (F) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) (G). Quantitative analysis of protein carbonylation levels (shown as 2, 4-dinitrophenylhydrazine (DNP) expression) with Coomassie staining as a loading control (H). Immunoblot analysis of α -1 subunit (I) and α -2 subunit (J) with data shown as mean band density normalized to GAPDH. pSrc immunoblot analysis with data shown as mean band density normalized to total Src (K). All gels have been cropped above and below the band, and the full blots have been included in Supplementary Figure S5 D-K. N=12-14/group; *p<0.05 vs. CTR, **p<0.01 vs. CTR, #p<0.05 vs. WD, ###p<0.01 vs. WD.

The insulin resistance in mice fed a WD was reversed by treatment with lenti-adiponectin-GFP-NaKtide (Figure 27A). Inflammatory cytokines are both indicative of high levels of oxidative stress as well as contribute to the production of oxidants (Sodhi et al., 2015). Mice fed a WD showed significantly upregulated plasma levels of the inflammatory cytokines TNF α , monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6) (Figure 27B-D). Treatment with the lenti-adiponectin-GFP-NaKtide ameliorated the increases in the plasma concentrations of these inflammatory cytokines. Our results showed increases in plasma leptin concentrations in mice fed a WD. This was attenuated by treatment with lenti-adiponectin-GFP-NaKtide (Figure 27E).

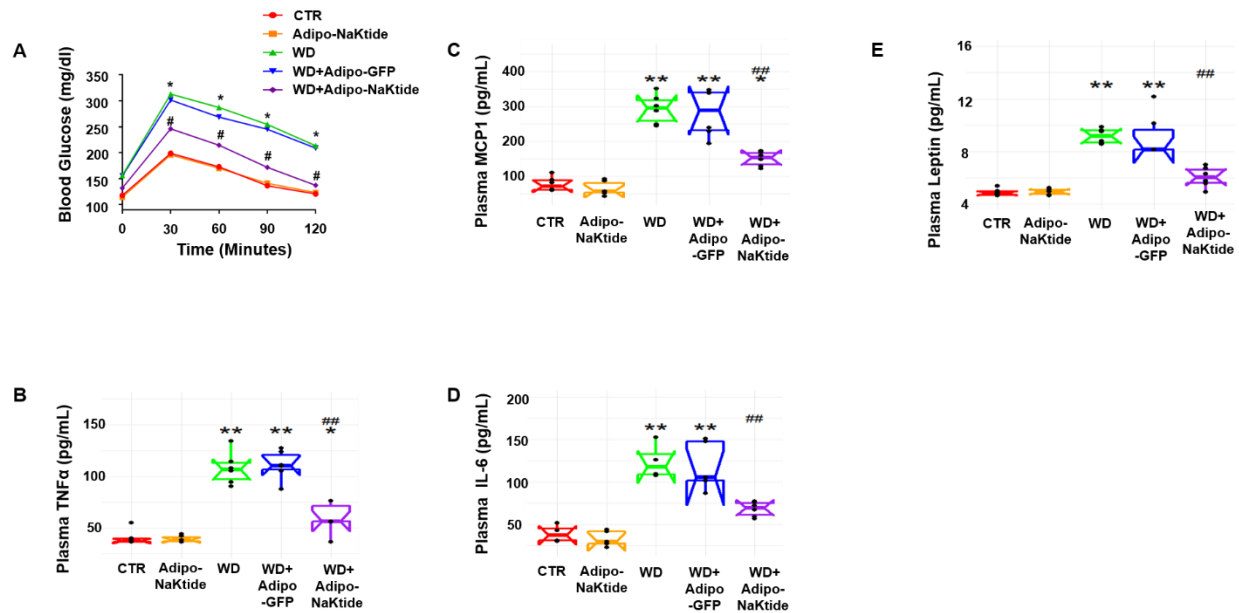


Figure 27. Effect of lenti-adiponectin-GFP-NaKtide on systemic inflammatory cytokines, and metabolic profile in C57BL6 mice fed a WD. Glucose tolerance test (A), Plasma levels of TNF α (B), monocyte chemoattractant protein-1 (MCP-1) (C) interleukin-6 (IL-6) (D) and leptin (E) assessed by ELISA assay. N=12-14/group; *p<0.05 vs. CTR, **p<0.01 vs. CTR, # p<0.05 vs. WD, ## p<0.01 vs. WD.

Effect of lenti-adiponectin-GFP-NaKtide on Na/K-ATPase/Src signaling cascade in C57BL6 mice fed a WD

Protein carbonylation is an established method for assessing oxidative stress (Bartlett, Miller, Thiesfeldt, Lakhani, Shapiro, et al., 2018; Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003). Our results showed a significant increase in protein carbonylation, measured by 2, 4-dinitrophenylhydrazine (DNP), in mice fed a WD which was attenuated by treatment with the lenti-adiponectin-GFP-NaKtide (Figure 26H). Expression of the α 1 subunit of the Na/K-ATPase was significantly down regulated in visceral fat of mice fed a WD. This was prevented by lenti-adiponectin-GFP-NaKtide treatment (Figure 26I). Conversely, the up-regulated expression of the α 2 subunit in mice fed a WD was significantly attenuated by lenti-adiponectin-

GFP-NaKtide (Figure 26J). Treatment with lenti-adiponectin-GFP-NaKtide also prevented Src activation in mice fed a WD (Figure 26K).

Effect of lenti-adiponectin-GFP-NaKtide on neurodegeneration in C57BL6 mice fed a WD

Obesity and systemic oxidative stress have been implicated with neurodegenerative disorders, so we next examined the effect of lenti-adiponectin-GFP-NaKtide on these parameters. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the production of dopamine; the expression of both TH and the type 2 dopamine receptor (D2) correlate with locomotor activity (Mundorf, Joseph, Austin, Caron, & Wightman, 2001). TH staining showed decreased expression of TH in the prefrontal cortex of the brain in mice fed a WD; western blot analysis of TH showed significant decrease in brains of mice fed WD (Figure 28A and B). Treatment with lenti-adiponectin-GFP-NaKtide negated these changes. Furthermore, expression of D2 receptor in the brain was significantly down regulated in mice fed a WD. This was also improved by the treatment with the lenti-adiponectin-GFP-NaKtide (Figure 28B). Further, our results demonstrated an increase in protein carbonylation in the brain tissues of mice fed a WD; this was attenuated by the lenti-adiponectin-GFP-NaKtide (Figure 28C). Our results also showed decreased expression of postsynaptic density protein 95 (PSD95), a marker of synaptic plasticity (Kothari et al., 2017) in the brain tissues of WD mice. Treatment with lenti-adiponectin-GFP-NaKtide improved this PSD95 expression (Figure 28D). Tau, a marker of tangled neuron tracks and a hallmark of neurodegenerative disease (Kothari et al., 2017), was up regulated in the brains of mice fed WD and markedly reduced by the lenti-adiponectin-GFP-NaKtide (Figure 28E). The TUNEL assay demonstrated that apoptosis was increased in the brain tissue of mice fed WD. Apoptosis was also attenuated by the lenti-adiponectin-GFP-NaKtide treatment (28F-G).

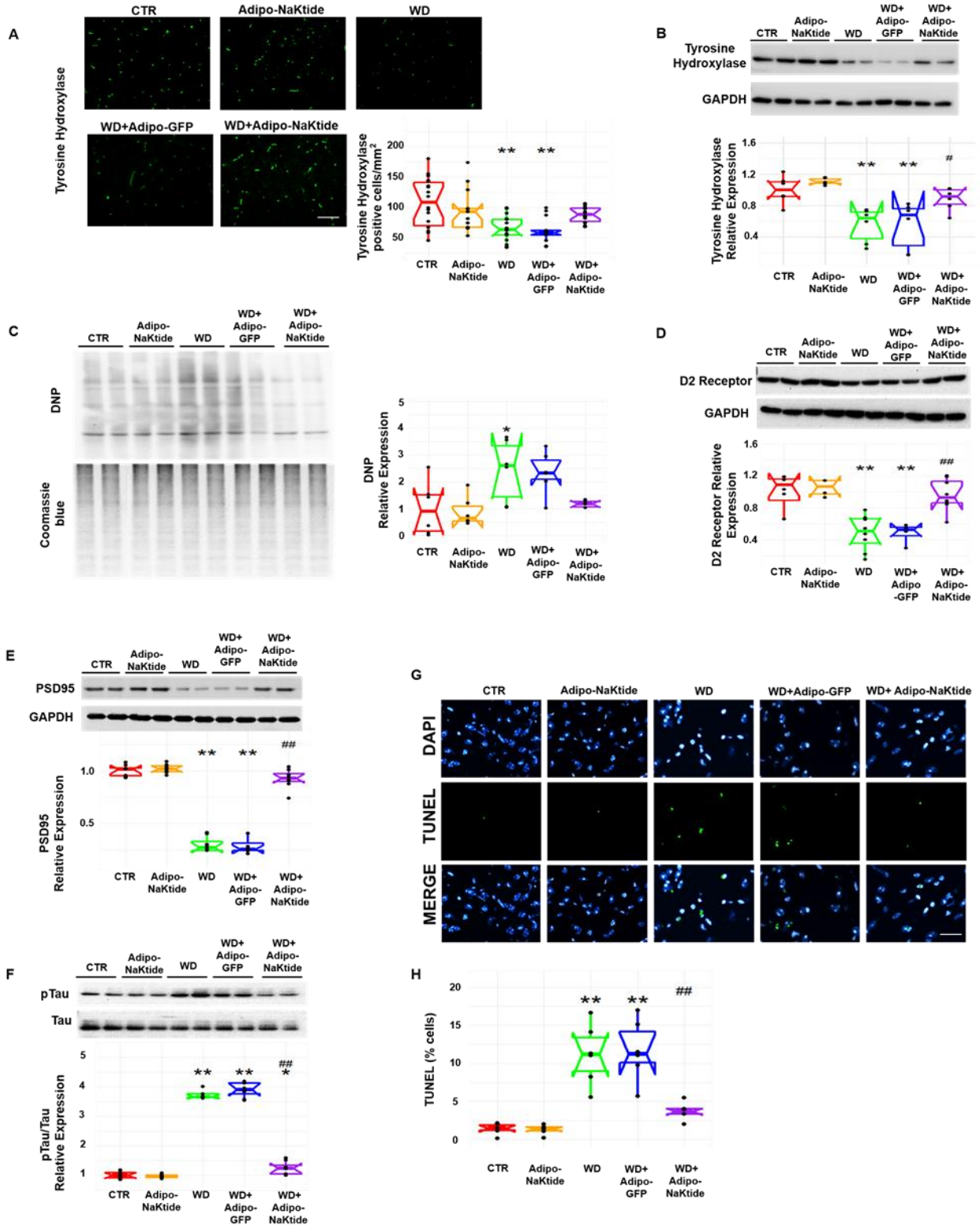


Figure 28. Effect of lenti-adiponectin-GFP-NaKtide on neurodegeneration in brain tissue in C57BL6 mice fed a WD.

Representative and quantitative analysis based on tyrosine hydroxylase staining (A), images taken with 20X objective lens; scale represents 100 μ m. Immunoblot analysis for tyrosine hydroxylase (TH) (B). Protein carbonylation levels with Coomassie staining as a loading control (C). Immunoblot analysis of D2 receptor with data shown as mean band density normalized to GAPDH (D). Immunoblot analysis for marker of synaptic plasticity, PSD95 (E), and marker of tangles in neuron tracks, Tau (F). Representative images and quantification of the TUNEL assay in brain tissue (G-H). All gels have been cropped above and below the band, and the full blots have been included in Supplementary Figure S6 B-F. N=12-14/group; * $p < 0.05$ vs. CTR, ** $p < 0.01$ vs. CTR, # $p < 0.05$ vs. WD, ## $p < 0.01$ vs. WD.

Effect of lenti-adiponectin-GFP-NaKtide on hepatic histology, inflammation, and fibrosis in C57BL6 mice fed a WD

Next, we aimed to determine whether NaKtide targeted specifically to adipocytes was able to attenuate the development of nonalcoholic steatohepatitis (NASH). H&E staining of liver sections from C57BL6 mice fed a WD showed inflammation and increased lipid accumulation in the liver as compared to the control group (Figure 32A). Treatment with lenti-adiponectin-GFP-NaKtide exhibited decreased lipid and inflammatory cell infiltration. We note that the viral transduction did not lead to demonstrable hepatic NaKtide expression (Figure 31) implying an indirect effect. Mice fed a WD had significantly increased lipid accumulation in the liver compared to mice fed a normal chow diet as demonstrated by Oil Red O staining. Administration of lenti-adiponectin-GFP-NaKtide decreased lipid accumulation in mice fed a WD (Figure 32B). CD36, a fatty acid transport protein, contributes to the progression of NASH (Sodhi et al., 2017). Our results showed that CD36 mRNA expression was decreased by lenti-adiponectin-GFP-NaKtide treatment as compared to the mice fed a WD (Figure 32C). Similarly, TNF α and F4/80 mRNA expression, markers of inflammation and macrophage/kupffer cells infiltration, were also increased in mice fed a WD as compared to control mice (Figure 32D-E). These changes were

attenuated by the lenti-adiponectin-GFP-NaKtide treatment. Furthermore, mRNA expression of hepatic matrix metalloproteinases (MMP) 2 and 9, genes related to fibrogenesis, were also elevated in mice fed a WD, and these increases were also attenuated with lenti-adiponectin-GFP-NaKtide treatment (Figure 32F-G). These findings indicate that the lenti-adiponectin-GFP-NaKtide not only improves the metabolic profile in adipocytes, but also has profound effects on the liver through indirect mechanisms.

Correlational analysis of markers associated with reprogramming of adipocyte phenotype, NASH and neurodegeneration in C57BL6 mice fed a WD

First, we examined the degree of correlation between the various measurements performed in our study. These data are summarized in Figure 29A. Our results showed a number of strong correlations. In particular, it was clear that the plasma levels of inflammatory cytokines correlated with locomotor activity (Figure 29A). When we performed multiple linear regression analysis, it was clear that a model consisting of plasma TNF α and IL-6 predicted the locomotor activity with excellent accuracy ($R^2=0.84$) (Figure 29B). We have also included a webpage illustrating heat map and corresponding box plates as Figure 33.

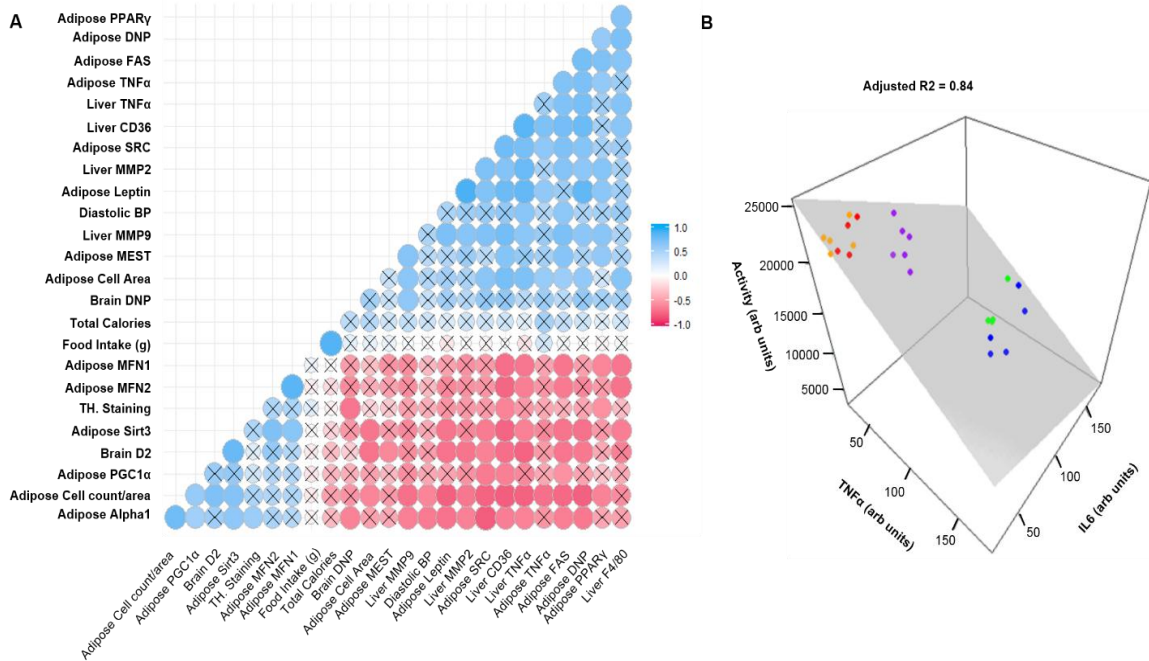


Figure 29. Correlational analysis of the markers associated with reprogramming of adipocyte phenotype in C57BL6 mice fed a WD.

Correlational analysis of the markers associated with reprogramming of adipocyte phenotype in C57BL6 mice fed a WD using the R package Psycho software (A). Multiple linear regression analysis between the plasma inflammatory cytokines, IL-6 and TNF- α , predicting the locomotor activity (B).

Discussion

In this study, we targeted the Na/K-ATPase signaling antagonist NaKtide specifically to adipocytes in mice fed a WD using a lentivirus employing adiponectin to drive its expression, thus expressing the NaKtide only in adipocytes. In our previous studies, systemic pNaKtide treatment attenuated obesity, as well as aging, NASH, and atherosclerosis (Sodhi et al., 2015; Sodhi et al., 2018; Sodhi et al., 2017). In this study, our results showed that targeting of NaKtide specifically to adipocytes was still able to reverse WD-induced changes in metabolic profile, oxidative stress, and inflammation. This highlights the importance of the Na/K-ATPase oxidant amplification loop within the adipocyte as well as the importance of adipocyte biology itself. There is accumulating evidence suggesting that adipocyte mitochondria might play an important

role in the development of dysfunctional adipocytes (Boudina & Graham, 2014). Our results showed that PGC1 α levels, the master regulator of mitochondrial biogenesis and adaptive thermogenesis (Villena, 2015), increased upon adipocyte-targeted NaKtide expression. Furthermore, the expression of genes associated with mitochondrial biogenesis and “browning” of fat, including MFN1, MFN2, and SIRT1, were increased by NaKtide treatment. Adipocytes also showed increased protein carbonylation in mice fed a WD. This was decreased by NaKtide treatment, indicating a reduction in oxidative stress within the adipose tissue.

Our results also showed that expression of the α 1 subunit of the Na/K-ATPase was significantly downregulated in visceral fat by the WD. We have shown previously that oxidative stress can induce endocytosis of α 1; we therefore propose that WD increases oxidative stress, increasing α 1 endocytosis (H. Cai et al., 2008; J. Liu et al., 2004; Yan et al., 2016). Some of the endocytosed α 1 is recycled to the surface, while some is destroyed through endosomal and proteolytic pathways. Previous evidence has shown that ROS can accelerate the degradation of the Na/K-ATPase (W. H. Huang et al., 1992; W. H. Huang, Y. Wang, A. Askari, N. Zolotarjova, & M. Ganjeizadeh, 1994; Kim & Akera, 1987; J. Liu et al., 2012; Thevenod & Friedmann, 1999). Hence, we propose that increased oxidative stress induced by WD leads to a decrease in total adipocyte α 1 expression in mice fed a WD. Interestingly, expression of α 2 subunit of Na/K-ATPase was noted to move in the opposite direction as α 1; this may be related to a compensatory mechanism that has yet to be elucidated. Treatment with pNaKtide also blocked Na/K-ATPase-regulated Src activation in mice fed a WD.

Studies in mouse models of obesity have shown that increased systemic oxidative stress is due to an altered adipocyte phenotype (Furukawa et al., 2004; Le Lay et al., 2014). Comorbidities such as insulin resistance and dyslipidemia also contribute to systemic oxidative

stress (Vincent & Taylor, 2006). Attenuation of the NKAL in the adipocytes during exposure to the WD ameliorated oxidant stress within these cells as well as the WD-induced hypertension, dyslipidemia, and insulin resistance. In particular, the increases in plasma levels of leptin and inflammatory cytokines (i.e., IL-6, TNF α , and MCP-1) in mice fed a WD were dramatically attenuated by NaKtide expression in adipocytes. This suggests a central role of the Na/K-ATPase signaling loop within adipocytes in the development of obesity and systemic oxidative stress in response to a WD.

Perhaps of greater interest, we observed profound changes in the locomotor activity of animals with the switch to a WD, which were dramatically attenuated by adipocyte NaKtide treatment. Specifically, animals given the WD demonstrated marked reductions in activity level and calculated energy expenditure. Such changes were also observed in previous reports, even in a mouse strain which did not gain excessive amounts of weight (Sodhi et al., 2017). Previously, we found that systemic pNaKtide prevented these decreases in activity, and we found that NaKtide expression limited to the adipocytes accomplished the same effect. We found that these behavioral changes were strongly associated with changes in brain D2 receptor expression and tyrosine hydroxylase expression, confirmed through both immunofluorescence and western blot. All of these changes appeared to be linked to adipocyte cytokine production. In fact, mouse activity could be predicted with a linear combination of plasma TNF α and IL-6 levels with an R² of 84%. In addition to these functional effects, marked changes in brain biochemistry and morphology were also noted with the western diet, and again, adipocyte NaKtide treatment ameliorated these, which included protein carbonylation, expression of PSD95 and pTau as well as neuronal apoptosis. The implications of these observations to cognitive and emotional disturbances associated with obesity and other neurodegenerative disorders including aging

related cognitive impairment have yet to be explored. We also note that virtually all consequences of obesity such as dyslipidemia, insulin resistance and NASH were also attenuated by adipocyte NaKtide expression.

Together, our results suggest a central role for the Na/K-ATPase oxidant amplification loop within adipocytes in the pathogenesis of obesity induced by a WD as well as the commonly associated comorbidities. Somewhat surprisingly, behavioral changes could be attributed to this deranged adipocyte metabolism which, in turn, were very strongly associated with the levels of cytokines known to be produced by adipocytes. As we review these data, it appears not only that targeting the Na/K-ATPase oxidant amplification loop may be promising in terms of efficacy as well as limiting off-target effects. If these findings can be confirmed in humans, a number of therapeutic strategies might be envisioned ranging from surgical control of fat mass, molecular biological strategies described in this report to simple dietary manipulations.

Materials and Methods

Experimental Design

All animal studies were approved by the Marshall University Animal Care and Use Committee in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. C57BL6 mice (Male, 8 weeks) were purchased from Hilltop Lab Animals. After arriving to the Robert C. Byrd Biotechnology Science Center Animal Resource Facility (ARF) mice were placed in cages, and fed normal chow diet with *ad libitum* access to water. Animals were randomly divided into five groups (12-18/group) as follows: 1. Control (normal chow) 2. NaKtide (normal chow+ lenti-adipo-GFP-NaKtide) 3. Western Diet (WD) 4. WD+GFP (WD+lenti-adiponectin-GFP) 5. WD+NaKtide (WD + lenti-adiponectin-GFP-NaKtide). Western diet (WD) containing fructose is a well-known inducer of diet-induced obesity (Andre et al.,

2014; Cao et al., 2012; Sodhi et al., 2015). WD was purchased commercially from Envigo (Indianapolis, IN), and contained 42% fat, 42.7% carbohydrate, and 15.2% protein, yielding 4.5 KJ/g specific energy. Fructose was purchased commercially from Alfa Aesar (Ward Hill, MA). Fructose water was made at a concentration of 42g/L, yielding 0.168 KJ/mL of specific energy. Mice in the WD group were given WD and *ad libitum* access to fructose water.

Lentiviral construct with NaKtide driven by an adiponectin promoter were constructed by VectorBuilder Inc. and were used in mice to achieve NaKtide expression specifically in adipose tissue (Figure S2). Lentiviral construct featured the adiponectin promoter driving expression of the NaKtide cassette linked by means of a 2A peptide to eGFP for bicistronic expression. The NaKtide sequence used for this vector

was ATGAGCGCCACCTGGCTGGCCCTGAGCAGGATCGCCGGTCTTTGCAACAGGGCC GTGTTCCAG. Lentivirus (100 uL, 2×10^9 TU/mL in saline) with NaKtide and its counterpart Lenti-GFP (driven by adiponectin promoter) were injected into mice intraperitoneally (IP) at week 0. Groups 2 and 5 received an injection of lenti-adiponectin-GFP-NaKtide, and group 4 was given an injection of lenti-adiponectin-GFP. Body weight was measured weekly for 12 weeks, as well as food and water intake. At the time of sacrifice, the body weight, visceral and subcutaneous fat weight, liver weight, heart weight, kidney weights, and brain weights were measured. Blood samples were collected for determination of leptin and inflammatory cytokine levels. Tissues were flash frozen in liquid nitrogen and maintained at -80°C , preserved in OCT for sectioning, or placed in paraformaldehyde for paraffin embedding.

Indirect calorimetry and locomotor activity

At the end of the 12-week period, energy expenditure, oxygen consumption, and locomotor activity were measured as described previously (Sodhi et al., 2017). All mice were

acclimatized to the cages for 24 hours prior to an additional 48 hours of recordings under the regular 12- hour light/dark cycle.

Glucose Tolerance Test

At the end of the 12-week period, mice were fasted for 8 hours. After the fasting period, a 10% glucose solution (2g/kg body weight) was injected intraperitoneally. Samples were taken from the tail vein at 0, 30, 60, 90, and 120 minutes after glucose injection. Blood glucose was measured using the Accutrend Sensor glucometer.

Cytokine and Leptin Measurements

Plasma TNF α , IL-6, MCP-1, leptin were measured using an ELISA assay kit according to manufacturer instructions (Abcam Cambridge, MA) as reported previously (Sodhi et al., 2017).

RNA extraction and real time PCR

Total RNA was extracted from adipose and hepatic tissue using RNeasy Protect Mini Kit (QIAGEN, Maryland) as described previously (Sodhi et al., 2018). Total RNA was analyzed by a quantitative real time polymerase chain reaction. Real time PCR was done using SYBR Green PCR Master Mix on a 7500 HT Fast Real-Time PCR System (Applied Biosystems, US). Each reaction was done in triplicate. All experimental samples were normalized using GAPDH. Specific primers were used for MFN 1 and 2, Sirt3, TNF α , Leptin, CD36, MMP2 and 9 and F4/80.

Western Blot Analysis

Visceral adipose tissue and brain tissue were pulverized with liquid nitrogen and placed in RIPA homogenization buffer. Homogenates were centrifuged, the supernatant was isolated, and immunoblotting performed. Adipose tissue was used for determination of α 1/2 subunits of the Na/K-ATPase, FAS, PPAR γ , MEST, and PGC1 α as previously reported (Haller et al., 2012;

Yan et al., 2013). Brain tissue was used for expression of tyrosine hydroxylase (TH), dopamine 2 (D2) receptors, PSD95, p-Tau, and Tau expression. D2 receptor antibody and tyrosine hydroxylase was from Abcam (Cambridge, MA) and PSD95, p-Tau, and Tau from Cell Signaling (Danvers, MA).

Measurement of c-Src phosphorylation

Whole cell lysates from visceral adipose tissue were prepared with RIPA buffer. Activation of c-Src was determined as described previously (Yan et al., 2013). Polyclonal anti-Src [pY418] phospho-specific antibody was from Invitrogen (Camarillo, CA). Monoclonal antibody against total c-Src was from Santa Cruz (Santa Cruz, CA). After immunoblotting for phospho-Src, the same membrane was stripped and blotted for total c-Src. Activation of c-Src was expressed as the ratio of phospho-Src/c-Src with measurements normalized to 1 for control samples.

Assessment of protein carbonylation

Whole cell lysates from visceral adipose tissue and brain tissue were prepared with RIPA buffer and western blotting for protein carbonylation assay was done as previously described (Yan et al., 2013). 2, 4-dinitrophenylhydrazine (DNPH) and antibody against 2, 4-dinitrophenyl (DNP) hydrazone derivatives were purchased from Sigma-Aldrich. The signal density values of control samples were normalized to 1 with Coomassie blue staining.

Immunofluorescence studies in adipose, hepatic, and brain tissues

Adipose tissue, hepatic tissue, and brain tissue were frozen in OCT compound, cut into 6 μ m sections and mounted on slides. The sections were fixed with 4% PFA for 15 minutes, washed once with PBS, probed with 1:100 primary antibody dilution and 1:1000 secondary antibody (Alexa 455 Red), and then mounted with DAPI solution and coverslips. Expression of

lenti-adiponectin-GFP was determined using a Nikon Eclipse 80i microscope equipped with a Nikon camera head DS-Fi1 (Nikon, Japan). Expression of lenti-adiponectin-GFP-NaKtide was determined using an RFP filter on Nikon Eclipse 80i microscope equipped with a Nikon camera head DS-Fi1 (Nikon, Japan). Protocol was adapted from Alexa-fluor-555 Cell Analysis from Thermo Fisher, and IHC protocols (IHC WORLD Life Science Products).

Hematoxylin and Eosin Staining

Visceral adipose tissue and hepatic tissue was cut into 6 μm sections and stained with hematoxylin and eosin for histological analysis as previously described (Sodhi et al., 2018; Sodhi et al., 2017). Adipocyte number and area was determined using ImageJ software (NIH) (Parlee, Lentz, Mori, & MacDougald, 2014). In brief, H&E stained adipose tissue images (20X magnification) were opened in ImageJ. Images were changed to 8 bit, enhanced through Threshold, Dilate, and Watershed functions, converted to mask using the binary function, and enhanced. Black and white images were compared to the original image to ensure accurate representation of adipocytes. The total number and size of adipocytes was calculated using the “analyze particles” command. To calculate the number of adipocytes, the size of the sample was normalized based on the number of pixels present in the image and the magnification. This was consistent and used for all subsequent images. The number of total adipocytes in the distribution is calculated from this for scale.

Oil Red O Staining

Liver tissues, frozen in OCT compounds, were cut into 6 μm sections and Oil Red O staining was done as described previously (Sodhi et al., 2017). Images were taken on a Nikon Eclipse 80i microscope equipped with a Nikon camera head DS-Fi1 (Nikon, Japan). For quantitative analysis, total area of the red pixels on stained tissue was measured by Image J

software (NIH). The data was expressed as percentage of the Oil Red O stained area with respect to total area.

Tyrosine Hydroxylase Staining

Brain tissue preserved in paraffin was cut into 6 μ m sections and mounted on slides. Deparaffinization, rehydration, and antigen retrieval were performed on the slides. After drying, slides were stained with anti-Tyrosine Hydroxylase (Abcam, US) overnight, following modified manufacturer protocol (Becker et al., 2018). Imaging was done with a Nikon camera head DS-Fi1 (Nikon, Japan). For quantitative analysis, total area of the green pixels on stained tissue was measured by Image J software (NIH). The data was expressed as percentage of the green stained area with respect to total area.

TUNEL assay for DNA damage detection

DNA double-strand breaks were detected in frozen mouse brain tissue using the Click-iT® Plus TUNEL Assay (Thermo Fisher Scientific Inc., US) according to the manufacturer's protocol. Specimens were mounted using VECTASHIELD® mounting medium and counterstained with 50 mg/ml DAPI. Images were collected using a digital inverted microscope that spanned the entire area of the tissues on the cover slip. The percentage of TUNEL-positive cells was calculated based on the number of positively stained cells divided by the total number of cells multiplied by 100 as reported previously (Sodhi et al., 2018).

Statistical analyses

The plots used were combination of scatter and boxplots. The boxplot displays the distribution of the data. It visualizes five summary statistics (the median, two hinges and two whiskers), and all "outlying" points individually (van Raalte & Egorov, 2015). The boxplot was defined by:

Notch lower = median - 1.58 * interquartile range (IQR) / sqrt (n) (A. Stolarczyk, Horvath, Szczechura, Kaminska, & Dziechciarz)

Middle = median

Notch upper = median + 1.58 * IQR / sqrt (n);

Lower hinge is the 25th percentile

Upper hinge is the 75th percentile

Data were analyzed and presented with the statistical program R using the packages ggplot2 and doBy. Multiple groups of parametric data (passed Shapiro-Wilk normality test) were compared with one way analysis of variance (ANOVA) and individual group means compared with an unpaired t-test employing the Holm correction for multiple comparisons. Nonparametric data were analyzed with the Wilcoxon rank sum test, also employing the Holm correction for multiple comparisons. All data comparisons are presented at the NS, p<0.05 and p<0.01 levels. Correlation analysis was performed using the R package Psycho. The fit of a plane based on TNF α and IL-6 to locomotion was performed using linear regression and displayed with the R package plot3D.

Acknowledgements

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Authors Contributions

Rebecca D. Pratt: Performed the experiments and participated in the writing of the manuscript

Cameron Brickman: Performed the experiments

Athar Nawab: Performed the experiments

Cameron Cottrill: Performed the experiments

Brian Snoad: Performed the experiments

Hari Vishal Lakhani: Performed the experiments

Austin Jelcick: Constructed lenti-adiponectin-GFP-NaKtide promoter

Brandon Henderson: Edited the manuscript

Niharika N. Bhardwaj: Analyzed data

Juan R. Sanabria: Edited the manuscript

Jiang Liu: Edited the manuscript

Zijian Xie: Edited the manuscript

Nader G. Abraham: Edited the manuscript

Joseph I. Shapiro: Designed the experiments, analyzed data, and wrote the manuscript

Komal Sodhi: Designed the overall project as well as individual experiments, analyzed data and served as senior author in the writing of the manuscript

Competing Interests All the authors declare that there is no competing interests.

Supplementary Information

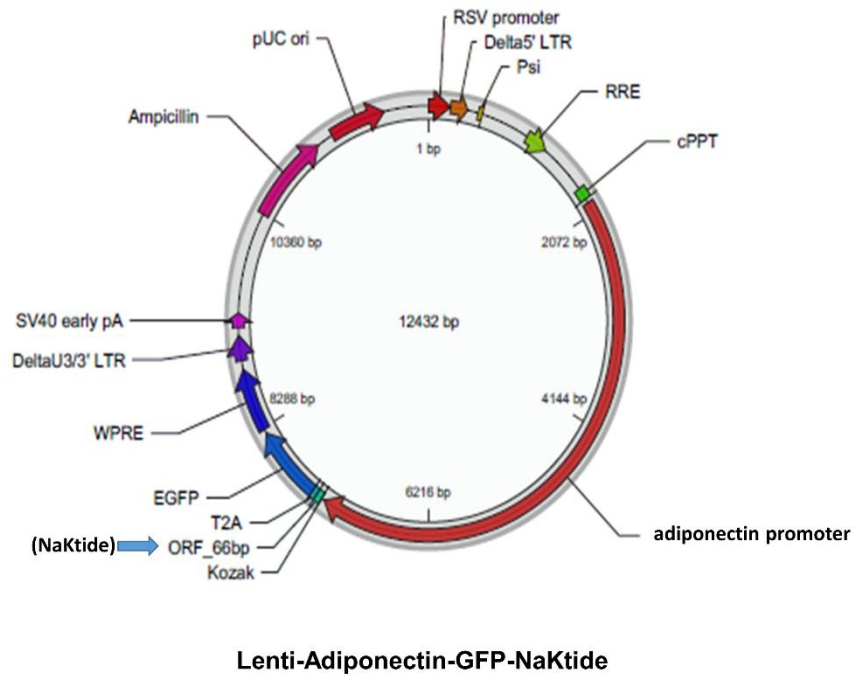


Figure 30. Lentiviral construct of lenti-adiponectin-GFP-NaKtide.
An adiponectin promoter drove expression of the NaKtide cassette linked by means of a 2A peptide to eGFP for bicistronic expression.

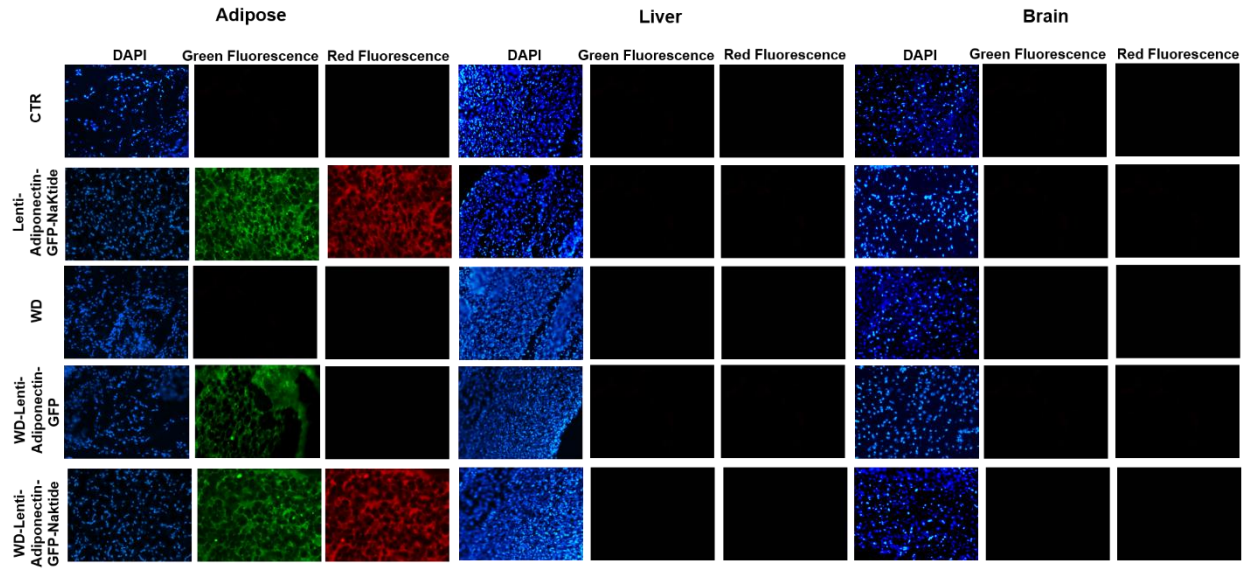


Figure 31. Immunofluorescence analysis of GFP and NaKtide in adipose, hepatic, and brain sections from mice transduced with lenti-adiponectin-GFP and lenti-adiponectin-GFP-NaKtide.

Representative images taken with 20X objective lens; scale represents 100 μ m.
n=6/group.

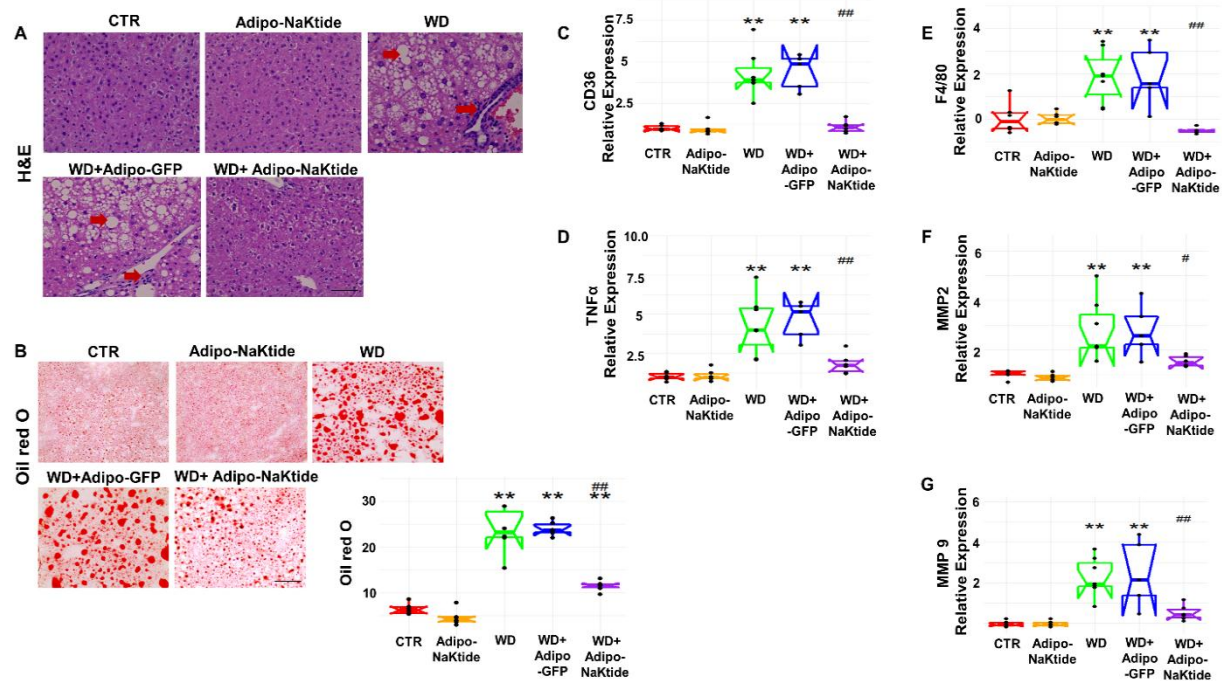


Figure 32. Effect of lenti-adiponectin-GFP-NaKtide on hepatic tissue in C57BL6 mice fed a WD.

Representative H&E staining of the liver tissue. Arrows mark indicative of increased lipid accumulation and inflammation. (A) Lipid accumulation measured as the relative absorbance of Oil Red O staining (B). Images taken with 40X objective lens, scale bar represents 100 μ m. qRT-PCR analysis of CD36 (C), TNF α (D), F4/80 (E), matrix metalloproteinases (MMP) 2 (F), and MMP9 (G). N=12-14/group; *p<0.05 vs. CTR, **p<0.01 vs. CTR, #p<0.05 vs. WD, ###p<0.01 vs. WD.

Figure S4: Webpage illustrating heat map and corresponding box plots.

https://public.tableau.com/profile/joseph.shapiro#!/vizhome/Sodhi_Rebecca_v2/Dashboard1?publish=yes

Note: Data shown as heat map with data shown in scientific notation as well as boxplot showing actual data points. Heat map has color-coding showing marked decreases or increases relative to first column. This can be changed using dropdown filters in webpage.

Figure 33. Webpage illustrating heat map and corresponding box plots.

Data shown as heat map with data shown in scientific notation as well as boxplot showing actual data points. Heat map has color-coding showing marked decreases or increases relative to first column. This can be changed using dropdown filters in webpage.

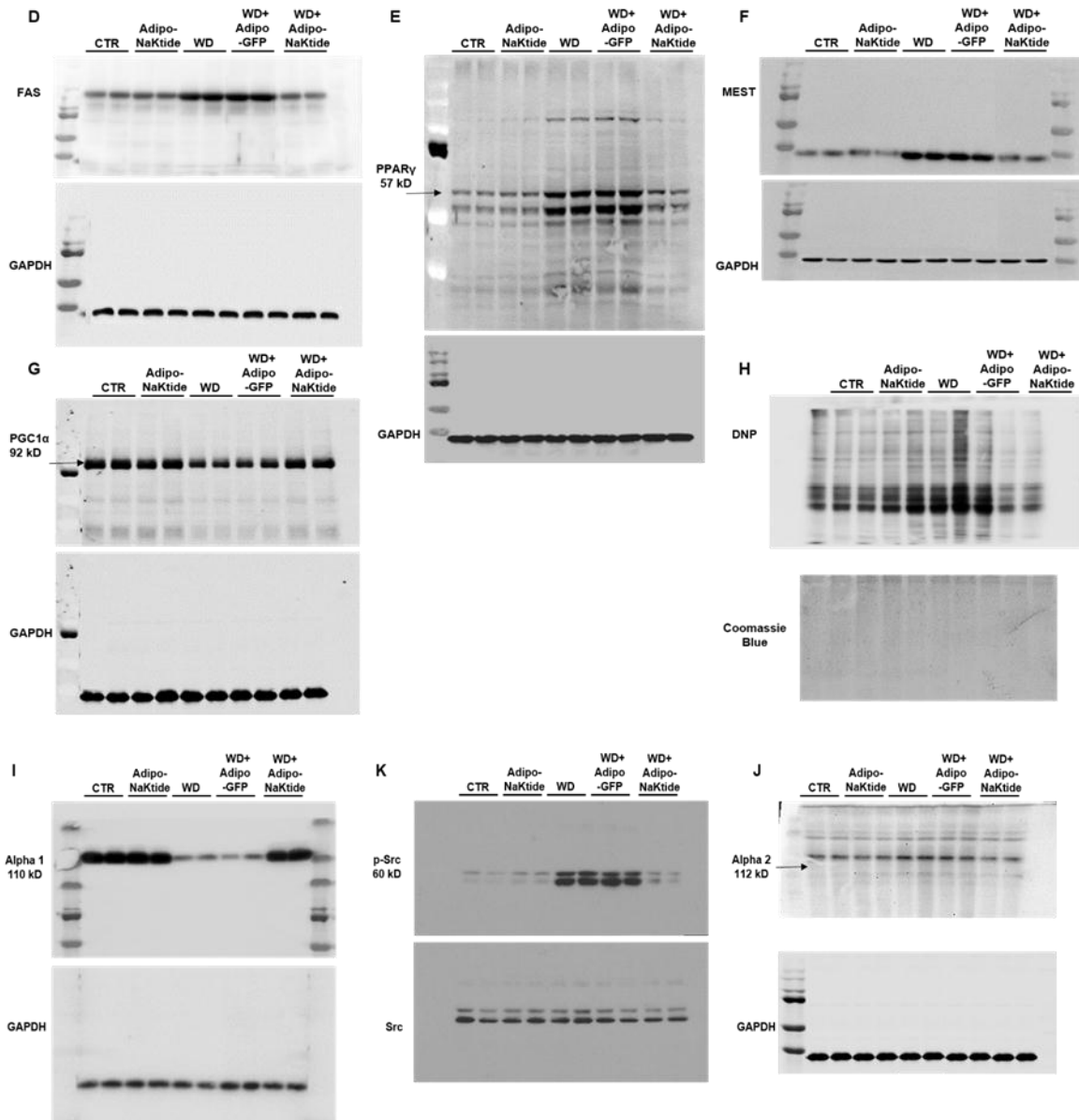


Figure 34. Uncropped Western Blots for Figure 26.
 Uncropped western blots for membranes shown in Figure 26

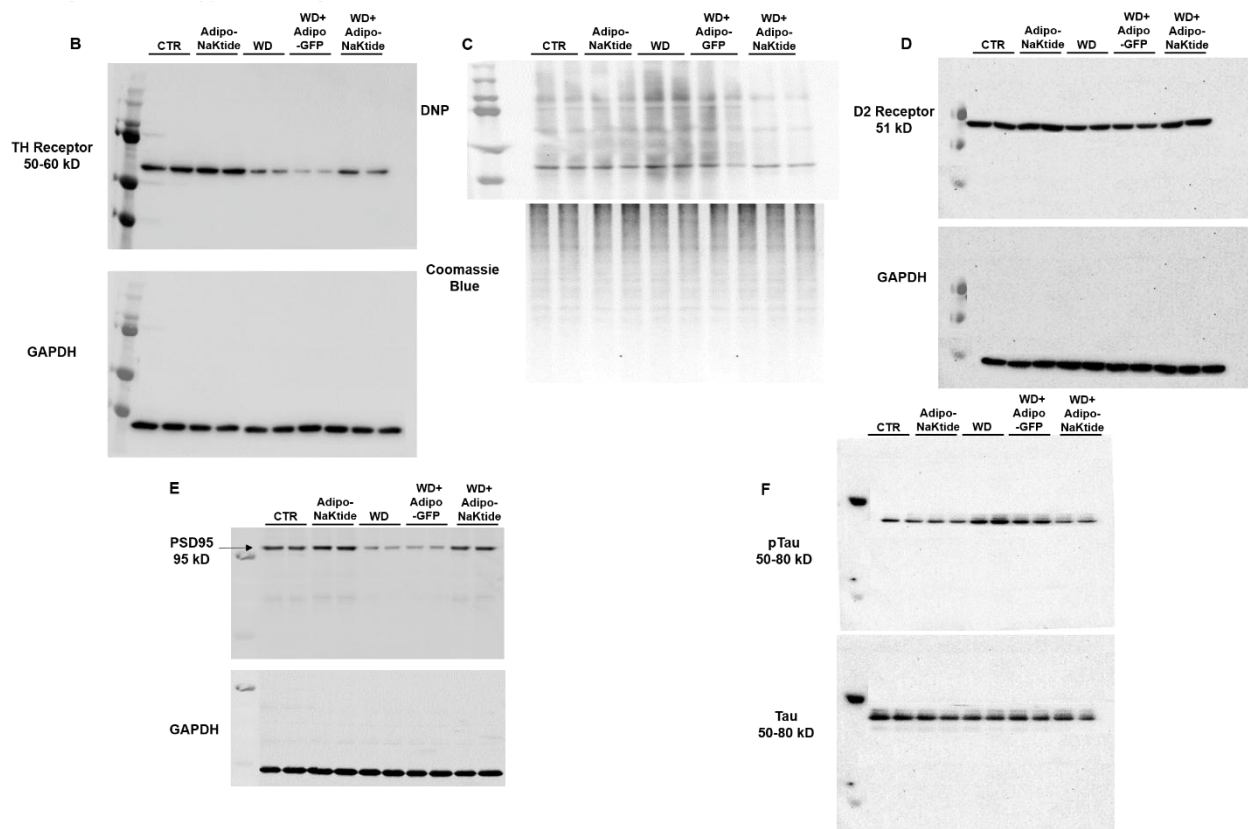


Figure 35. Uncropped Western Blots for Figure 28.
 Uncropped western blots for membranes shown in Figure 4.

CHAPTER 6

DISCUSSION

Collectively, this work explores the hypothesis that specific NaKtide expression in adipocytes modulates the adipocyte redox state which accomplishes two specific ends: amelioration of both local oxidative stress (inside the adipose tissue itself) and systemic inflammation and prevention of the dysfunctional adipocyte phenotype in both *in vitro* and *in vivo* models of obesity.

As has been mentioned, obesity is a disease characterized by chronic inflammation. Obesity not only increases oxidative stress, but it simultaneously decreases expression and activity of key cytoprotective systems while increasing the inflammatory cytokines released by adipocytes; obviously, obesity pushes the adipocyte towards a severely pro-inflammatory state and causes the adipocyte to secrete proinflammatory adipokines and cytokines that are detrimental on peripheral organs and tissues and can severely impact their function(Cao et al., 2012). WD and high fat diet (HFD) specifically can induce metabolic inflammation in both central and peripheral organs by this mechanism, which further contributes to the pathogenesis of obesity(Gutierrez-Tenorio et al., 2017; Kothari et al., 2017). Consumption of these diets induces immune cell responses and tissue inflammation in adipose tissue, which can also change the secretory profile of the adipocytes (Naznin et al., 2018).

The data presented here supports the hypothesis that expression of NaKtide in adipocytes would reduce adipogenesis and obesity by inhibiting the Na/K-ATPase-mediated oxidant amplification loop. Previous work on the subject has extensively shown that the Na/K-ATPase signaling is a regulator of oxidant amplification, and when the Na/K-ATPase is oxidized it forms

an amplification loop that causes a sharp rise in the presence of ROS and consequently oxidative stress (J. Liu et al., 2016; Rebecca D. Pratt et al., 2019; Sodhi et al., 2015; Sodhi et al., 2018; Sodhi et al., 2017). The previously done work has not taken into account the specific power held by adipocytes. For decades adipocyte biology has been a growing field, and it is believed that the adipocyte plays a much larger role in metabolism and health than just being an energy reservoir that are dysfunctional in obesity. It has been reported that adipose tissue is responsible for secreting hormones, cytokines, and adipokines that can potentially negatively impact the redox state of not only the adipocytes but other tissues as well (Lafontan, 2014; Okuno et al., 2018; Victorio & Davel, 2019). Part of our hypothesis was that by reducing oxidative stress with NaKtide treatment in the adipocytes would be able to reduce systemic oxidative stress; an apparent reduction in oxidative stress has been shown, and it is believed that this change in redox biology in the adipocytes is responsible for the amelioration of the obese phenotype and the reduction in systemic oxidative stress.

Dysfunctional adipocytes are characterized by inflammation, altered adipokine secretion and increased pro-inflammatory cytokine production (Goossens & Blaak, 2015); all these factors were observed in our models and all of these factors were attenuated with either pNaKtide or lenti-adiponectin-GFP-NaKtide treatment. This suggests that the Na/K-ATPase oxidant amplification loop can indeed exacerbate the obese phenotype and further lead to adipocyte dysfunction (Figures 14, 18, 19, and 20) as we saw this increase in dysfunction in our cells treated with ouabain). By hindering this loop, we are able to reduce inflammation and adipocyte dysfunction in our models (Figures 14, 18, 19, and 20).

A lentiviral vector delivered NaKtide in both our *in vitro* and *in vivo* models. By utilizing a lentiviral vector, NaKtide was able to enter the cell. Our hypothesis involves specific targeting

to adipocytes, so the use of the lentiviral vector was to ensure we had expression of NaKtide in the adipocytes only, and the risk of exocytosis of NaKtide to neighboring tissues would be prevented since NaKtide is cell membrane impermeable. This is confirmed in Figure 31; immunofluorescence analysis for GFP and NaKtide in adipose tissue, liver tissue, and brain tissue showed there was no expression of NaKtide outside the adipose tissue. Expression of lenti-adiponectin-GFP-NaKtide showed a promising decrease in adipogenesis, and was used in a rodent model of obesity to see if it has similar effects to systemic administration of pNaKtide(Sodhi et al., 2015).

There is a strong link between obesity and metabolic imbalance, and oxidative stress has been implicated with the chronic inflammatory state seen in obesity (Gutierrez et al., 2009; Trayhurn, 2007; Wellen & Hotamisligil, 2003). Here, it has been demonstrated that administration of pNaKtide effectively reduces adipogenesis *in vitro* by antagonizing specific Na/K-ATPase-mediated amplification of ROS signaling (i.e. treatment with ouabain) (Figure 20). Our results with lenti-adiponectin-GFP-NaKtide, specifically targeted to the adipocytes themselves, show a similar effect *in vivo* (Figure 26).

In 3T3-L1 cells, exposure to pNaKtide and transfection with lenti-adiponectin-GFP-NaKtide decreased adipogenesis. pNaKtide treatment attenuated oxidative stress, decreased lipid accumulation, and decreased expression of major adipogenic markers such as PPAR γ , MEST, and FAS. There was a significant upregulation of adiponectin level in the pNaKtide treated cells as well. In cells treated with pNaKtide, inflammation was reduced and protein carbonylation was decreased, indicating a decrease in oxidative stress and inflammation. These effects were seen even when signaling was activated by ouabain.

These observations are promising and offer novel information for Na/K-ATPase research in adipocytes. By utilizing ouabain we have confirmed the role of the Na/K-ATPase oxidant amplification loop in the process of adipogenesis. Combined with the activation of transcriptional regulators of adipogenesis, activation of Na/K-ATPase signaling enhanced adipogenesis and increased ROS generation. Our data shows a potentially larger role of the Na/K-ATPase oxidant amplification loop in adipocyte biology and obesity pathology which we further explored *in vivo*.

Based on our *in vitro* data, we applied lenti-adiponectin-GFP-NaKtide to a rodent model of obesity. In this study, our results showed that targeting of NaKtide specifically to adipocytes was able to prevent WD-induced changes related to metabolic profile, oxidative stress, and inflammation, highlighting the importance of the Na/K-ATPase oxidant amplification loop within the adipocyte. We report that the obesity phenotype, evidenced by significant weight gain, increased fat deposits, and altered metabolic profile, was markedly improved with lenti-adiponectin-GFP-NaKtide treatment, which we believe attenuated Na/K-ATPase oxidant amplification in the adipocyte. Even though lenti-adiponectin-GFP-NaKtide was targeted specifically to the adipocytes, we present several peripheral effects of our treatment (i.e. decreases in NASH and neurodegeneration discussed in Chapter 5), which suggests adipocyte phenotype has a larger role in obesity than previously recognized.

Treatment with pNaKtide in our *in vitro* model showed a hopeful therapeutic effect. pNaKtide alone decreased adipogenesis, ROS, and the inflammatory profile of the adipogenic cells, and was able to supersede the increases in inflammation and adipogenesis caused by ouabain. This suggests that the effect of pNaKtide on adipogenesis *in vitro* is through direct action on the Na/K-ATPase oxidant amplification loop, improving the redox biology of the

adipocyte. This subsequently changes adipokine and cytokine secretion from the adipocyte, which may have ramifications on systemic inflammation in our *in vivo* model. This further confirms the role of adipocytes in obesity and its comorbidities is much more important than previously determined.

Obesity is characterized as a disease of inflammation and is often characterized with high levels of oxidative stress and inflammation. Plasma levels of inflammatory markers TNF α , IL-6, and MCP-1 (along with leptin) were significantly reduced in lenti-adiponectin-GFP-NaKtide treated animals (Figure 27), indicative of a peripheral effect from treating adipocytes specifically. Treatment with lenti-adiponectin-GFP-NaKtide resulted in significant phenotypic changes of adipocytes in visceral fat. First, there was a significant increase in cell number and decrease in cell size in animals treated with lenti-adiponectin-GFP-NaKtide. These morphological changes point to healthier adipocytes. Western blot analysis of visceral fat treated with lenti-adiponectin-GFP-NaKtide showed decreases in FAS, PPAR γ , and MEST, indicating a decrease in adipogenesis. mRNA levels of leptin and TNF α were also improved in the adipose tissue itself, further indicating a more metabolically healthy phenotype.

While it has been shown before that Na/K-ATPase signaling has an impact in obesity and adipogenesis (Bartlett, Miller, Thiesfeldt, Lakhani, Shapiro, et al., 2018; Sodhi et al., 2015; Sodhi et al., 2018; Sodhi et al., 2017), this work is novel in the fact that it investigates specifically Na/K-ATPase signaling in the adipocyte itself by use of the lentiviral vector and ouabain. Specific stimulation of the pump had not yet been investigated, and this information lends to the hypothesis that the adipocyte Na/K-ATPase plays a larger role in the prognosis of obesity.

In our *in vivo* model, genes and proteins related to mitochondrial biogenesis and “browning” of adipose tissue were significantly downregulated in mice fed a WD, but upon treatment with lenti-adiponectin-GFP-NaKtide peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1 α), mitofusion 1 and 2 (MFN1 and 2), and NAD-dependent deacetylase sirtuin-3 (Sirt3) were all significantly increased. There is accumulating evidence suggesting that adipocyte mitochondria might play an important role in the development of dysfunctional adipocytes and our results suggest this is at play, as well as increased mitochondrial biogenesis (Boudina & Graham, 2014). Third, energy expenditure and oxygen consumption were significantly improved in the lenti-adiponectin-GFP-NaKtide treated group.

Obesity is a known inducer of oxidative stress, and increases in ROS are known to increase Na/K-ATPase signaling and thus the Na/K-ATPase oxidant amplification loop (Bartlett, Miller, Thiesfeldt, Lakhani, Khanal, et al., 2018; P. T. Shah et al., 2016; Yan et al., 2013). Protein carbonylation is a marker of ROS, and in WD-fed mice there are significant increases of ROS (Figure 26). In the lenti-adiponectin-GFP-NaKtide treated mice there is a reduction in ROS and activation of c-Src. Phosphorylated Src is a marker of Na/K-ATPase signaling and is responsible for downstream signaling pathways and activation of other signaling cascades such as Raf/Ras, NOX, and increased ROS as well (Z. Xie, 2003; Yan et al., 2016).

Our results showed that expression of the $\alpha 1$ subunit of the Na/K-ATPase was significantly downregulated in visceral fat. It has been shown previously that oxidative stress can induce endocytosis of $\alpha 1$ and increases in ROS oxidize the $\alpha 1$ subunit which also promotes degradation of the Na/K-ATPase (H. Cai et al., 2008; J. Liu et al., 2004; Yan et al., 2016). Some of the endocytosed $\alpha 1$ is recycled to the surface, while some is destroyed through endosomal, proteolytic, and proteosomal pathways; previous evidence has shown that oxidative damage

increases the degradation of the Na/K-ATPase in these pathways and impairs the cells ability to recycle $\alpha 1$, and ROS can accelerate degradation of the Na/K-ATPase (W. H. Huang et al., 1992; W. H. Huang et al., 1994; Kim & Akera, 1987; J. Liu et al., 2012; Thevenod & Friedmann, 1999). Hence, we propose that increased oxidative stress induced by WD leads to impaired ability of the adipocyte to successfully regenerate or recycle previously endocytosed $\alpha 1$ and in turn increases the amount of oxidized $\alpha 1$ further promoting endocytosis and degradation, leading to a decrease in total $\alpha 1$ expression in mice fed a WD. As NaKtide expression attenuated the decrease in $\alpha 1$ expression as well as the oxidant stress associated with WD, it is suggested that the aforementioned oxidant stress plays a role in the observed decrease in $\alpha 1$ expression seen with WD (Figure 26). This role, as stated before, is to temper the oxidant stress of the adipocyte and turn the adipocyte towards a more balanced pro-and anti-inflammatory state. Treatment with lenti-adiponectin-GFP-NaKtide also blocked Na/K-ATPase-regulated Src activation in mice fed a WD (Figure 26). Further, as discussed previously, there may be a phenotypic switch from $\alpha 1$ to $\alpha 2$ in the adipocytes to compensate for impaired pumping function; this switch is supported by increased $\alpha 2$ in our *in vivo* study (Figure 26). Our findings suggest a crucial role of the Na/K-ATPase oxidant amplification loop in adipocytes that can be implicated in obesity.

Studies in mouse models of obesity have shown that increased oxidative stress in plasma is due to increased ROS and cytokine production from dysfunctional adipocytes (Furukawa et al., 2004; Le Lay et al., 2014). Furthermore, several metabolic parameters that are dysregulated in obesity, including hyperglycemia, insulin resistance, and hyperlipidemia, also contribute to and amplify systemic oxidative stress (Vincent & Taylor, 2006). Blocking Na/K-ATPase signaling in the adipocytes ameliorated WD-induced dyslipidemia and insulin resistance (Gutierrez et al., 2009; Sodhi et al., 2017). Moreover, the increase in plasma levels of leptin and

inflammatory cytokines (i.e., IL-6, TNF α , and MCP-1) in mice fed a WD was attenuated by NaKtide expression in adipocytes (Figure 27). This suggests a central role of the Na/K-ATPase signaling loop within adipocytes in the development of obesity and systemic oxidative stress, and the potential to improve obesity-related conditions in other organs by blocking this signaling.

Our results suggest a central role for the Na/K-ATPase oxidant amplification loop within adipocytes in the pathogenesis of obesity induced by a WD. Moreover, expression of NaKtide prevented WD-induced neurodegenerative effects (Figure 28); this is the first study implicating the role of obesity and impaired Na/K-ATPase signaling in neurodegenerative disorders and may shed light on the complex interplay between obesity and disorders such as Alzheimer's disease. It appears that targeting the Na/K-ATPase oxidant amplification loop may be promising in terms of efficacy for treatment of adiposity and many related diseases while limiting off-target effects. We also note that other consequences of obesity such as insulin resistance were attenuated by adipocyte NaKtide expression. If these findings can be confirmed in humans, a number of therapeutic strategies might be envisioned ranging from surgical control of fat mass, molecular biological strategies described in this report to simple dietary manipulations. These data suggest that adipocyte derived cytokines play an essential role in the development of obesity induced by a WD and that targeting adipocyte Na/K-ATPase signaling may serve as an effective therapeutic strategy.

Conclusions and Future Directions

Together, our results suggest a central role for the Na/K-ATPase oxidant amplification loop within adipocytes in the pathogenesis of diet induced obesity. It appears not only that targeting the Na/K-ATPase oxidant amplification loop may be promising but restricting interventions to the adipocyte may have important clinical implications. If these findings can be

confirmed in humans, several therapeutic strategies might be envisioned ranging from surgical control of fat mass, molecular biological strategies described in this report to simple dietary manipulations. This area of research is crucial given the prevalence of obesity currently seen not only in the United States, but worldwide; as the prevalence of obesity increases, it is paramount to note that the incidence of comorbidities such as insulin resistance, metabolic syndrome, NASH, etc., are going to increase as well. By finding a suitable therapeutic target that can limit the off-hand effects seen with some current obesity treatments, we will not only be able to limit negative side effects, but also limit the incidence of comorbidities as well.

Future directions will explore the implications of manipulating adipocyte redox biology in other disease states that have been explored in our lab, such as uremic cardiomyopathy and atherosclerosis. Further, more specific attention will be given to investigate other changes (i.e. NASH and neurodegeneration) noticed in the *in vivo* models with treatment of lenti-adiponectin-GFP-NaKtide to add more to the story of the adipocyte's role on whole body homeostasis and redox signaling.

We have clearly demonstrated that the Na/K-ATPase amplification loop has an impact on adipogenesis. ROS amplification can occur through this pathway, and significantly increase adipogenesis and further increase Na/K-ATPase signaling. These phenomena suggest that the Na/K-ATPase oxidant amplification loop may serve as a potential target in dysfunctional redox states and oxidative stress therapies associated with obesity.

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APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER



Office of Research Integrity

June 12, 2019

Rebecca Pratt
6249 Beech Dr.
Huntington, WV 25705

Dear Ms. Pratt:

This letter is in response to the submitted dissertation abstract entitled "*NaKtide Targeted to Adipocytes Ameliorates Western Diet Induced Obesity.*" After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #648. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director
Office of Research Integrity

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APPENDIX B: LIST OF ABBREVIATIONS

- $[Ca^{2+}]_i$: Intracellular calcium concentration
- $[K^+]_i$: Intracellular potassium concentration
- $[Na^+]_i$: Intracellular sodium concentration
- ACE: angiotensin converting enzyme
- ADP: adenosine diphosphate
- AM: Adipogenic Media
- Ang II: angiotensin II
- ANOVA: Analysis of variance
- ApoE: Apolipoprotein E
- ARF: Animal Resource Center
- AT1: Angiotensin Type 1
- AT2: Angiotensin Type 2
- ATMs: Adipose tissue macrophages
- ATP: Adenosine triphosphate
- BAPTA-AM: 1,2-Bis(2-aminophenoxy)ethane
- BAT: brown adipose tissue/brown adipocytes
- Ca^{2+} : calcium
- Cav-1: caveolin-1
- CC BYNC 4.0: Creative Commons Attribution Non Commercial License
- CEBP α : CCAAT Enhancer Binding Protein Alpha
- CD₂: $\alpha 1$ CD₂ segment
- CD36: cluster of differentiation 36

- CDC: Center for Disease Control
- CTS: cardiotonic steroids
- Cys-46: cystein residue 46
- D2: dopamine receptor 2
- DMEM: Dulbecco's Modified Eagle Medium
- DNA: deoxyribonucleic acid
- DNP: 2,4, dinitrophenyl
- DNPH: 2,4, dinitrophenylhydrazine
- EBF2: Early B Cell Factor 2
- EGTA: ethylene glycol-bis(β -aminoethyl ether)
- ELISA: enzyme linked immunosorbent assay
- ER: Endoplasmic reticulum
- F4/80: EGF-like module-containing mucin-like hormone receptor-like 1
- FAS: fatty acid synthase
- FBS: fetal bovine serum
- FFA: free fatty acids
- FGF21: Fibroblast growth factor 21
- GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
- GFP: green fluorescent protein
- Gln 434: glutamine residue 434
- GLUT4: glucose transporter type 4
- H^+ : Hydrogen ions
- H&E: hematoxylin and eosin staining

- H₂O₂: hydrogen peroxide
- HFD: high fat diet
- HO-1: heme oxygenase 1
- IHC: immunohistochemistry
- IL-1 β : interleukin 1 beta
- IL-4: interleukin-4
- IL-6: interleukin-6
- IL-10: interleukin 10
- IL-13: interleukin-13
- IL-1R: interleukin 1 receptor
- iNOS: Nitric oxide synthase family
- IP: intraperitoneally
- IP₃R: inositol 1,4,5-trisphosphate receptors
- IRS1: insulin receptor substrate 1
- K⁺ : potassium
- KD: cSrc Kinase Domain
- LDH: lactate dehydrogenase
- LKK: Binding site for α 1 and IP₃R NH₂ terminuses
- LPL: lipoprotein lipase
- MBG: marinobufagenin
- MCP-1: monocyte chemoattractant protein-1
- MEK: methyl ethyl ketone
- MEST: mesoderm specific transcript gene

- MFN1: Mitofusin 1
- MFN2: Mitofusin 2
- Mg^{+} : magnesium
- $mitoK_{ATP}$: mitochondrial ATP-sensitive K^{+} channels
- MM: Maintenance Media
- MMP2: matrix metalloproteinase 2
- MMP9: matrix metalloproteinase 9
- mRNA: Messenger RNA
- Myf5+: myogenic factor 5
- Na^{+} : sodium
- NAC: *N*-acetylcysteine
- NaCl: Sodium Chloride
- NADPH: nicotinamide adenine dinucleotide phosphate
- NASH: nonalcoholic steatohepatitis
- NCX: Na^{+}/Ca^{2+}
- ND₁: $\alpha 1$ ND₁ segment
- NF κ B: nuclear factor kappa light chain enhancer of activated B cells
- NHE₃: Sodium–hydrogen antiporter 3
- NIH: National Institutes of Health
- NKAL: Na/K-ATPase oxidant amplification loop
- NO: Nitric oxide
- NOS: Nitric oxide synthase family
- Nos2: Nitric oxide synthase family

- OCT: optimal cutting temperature compound
- OSA: Obstructive Sleep Apnea
- Pax 3: Paired box protein 3
- Pax7+: paired box protein
- PBS: phosphate buffered saline
- PDGFR α : platelet derived growth factor receptor alpha polypeptide
- PFA: paraformaldehyde
- PGC1 α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
- PLC- γ : phospholipase C- γ
- PNx: partial nephrectomy
- PPAR- γ : Peroxisome proliferator-activated receptor gamma
- PRDM16: PR-domain contain 16 protein
- Pro222: Proline 222
- Pro224: Proline 224
- PSD95: postsynaptic density protein 95
- R²: R-squared
- RAAS: Renin-angiotensin aldosterone system
- RBCs: Red blood cells
- RIPA: Radioimmunoprecipitation assay buffer
- RNA: ribonucleic acid
- RNS: reactive nitrogen species
- ROS: reactive oxygen species
- RT-PCR: Reverse transcription polymerase chain reaction

- SE/SEM: Standard Error/Standard Error of the Mean
- Ser 415: serine residue 415
- SERCA: sarco-endoplasmic reticulum Ca^{2+}
- SH₂: cSrc SH₂
- Sirt1: Sirtuin 1
- Sirt 3:NAD-dependent deacetylase sirtuin-3
- SNS: Sympathetic Nervous System
- TAT: twin-arginine translocation
- TH: Tyrosine hydroxylase
- Thr224: Threonine 224
- TLR4: Toll like receptor 4
- TNF α : tumor necrosis factor α
- TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling
- Tyr 418: Tyrosine residue 418
- UCP1: uncoupling protein 1
- UUU: unilateral ureteral obstruction
- WAT: White adipose tissue/white adipocytes
- WD: western diet
- WHO: World Health Organization

APPENDIX C: CURRICULUM VITAE

Rebecca Pratt (Martin prior to March 2018)

6249 Beech Dr
Huntington, WV 25705
martin570@live.marshall.edu

Education

Marshall University: August 2014-July 2019

Major: Biomedical Research PhD

Lab: Dr. Jiang Liu/Dr. Shapiro

Department: Pharmacology, Physiology, Toxicology

Research Cluster: Cardiovascular Disease, Obesity, and Diabetes (CODRC)

Davis and Elkins College: August 2010-May 2014

Major: Biology | Minor: Psychology

Cumulative GPA: 3.7 [Cum Laude]

Degree Date: May 17, 2014

Laboratory Skills

- Western Blotting
- Cell Culture Techniques
- Lysate Preparation
- Animal Handling/Care
- Histology

Professional Experience

Publications

- Martin, Rebecca, and Joseph I. Shapiro. "Role of adipocytes in hypertension." *World J Hypertens* 6.2 (2016): 66-75.
- Liu, Jiang, et al. "Attenuation of Na/K-ATPase mediated oxidant amplification with pNaktide ameliorates experimental uremic cardiomyopathy." *Scientific reports* 6 (2016).
- Shah, Preeya T., et al. "Carbonylation Modification Regulates Na/K-ATPase Signaling and Salt Sensitivity: A Review and a Hypothesis." *Frontiers in physiology* 7 (2016).
- Shah PT, Martin R, Sanabria J, Khitan Z, Santhanam P, et al. (2016) Adiposity Predicts Pulse Pressure in Subjects with Chronic Kidney Disease: Data from the Modification of Diet in Renal Disease. *J Cardiol Curr Res* 7(2): 00240. doi: 10.15406/jccr.2016.07.00240

- Sodhi, Komal, et al. "pNaKtide Attenuates steatohepatitis and atherosclerosis by blocking Na/K-ATPase/ROS amplification in C57Bl6 and ApoE knockout mice fed a western diet." *Scientific Reports* 7 (2017).
- Sodhi, K., et al. (2018). "The Na/K-ATPase Oxidant Amplification Loop Regulates Aging." *Scientific Reports* 8(1): 9721.
- Pratt RD, Brickman CR, Cottrill CL, Shapiro JI, Liu J. The Na/K-ATPase Signaling: From Specific Ligands to General Reactive Oxygen Species. *Int J Mol Sci.* 2018 Sep 1;19(9). pii: E2600. doi: 10.3390/ijms19092600. Review. PubMed PMID: 30200500; PubMed Central PMCID: PMC6163532.
- Bartlett DE, Miller RB, Thiesfeldt S., et. al. "Uremic Toxins Activates Na/K-ATPase Oxidant Amplification Loop Causing Phenotypic Changes in Adipocytes in In Vitro Models". *Int J Mol Sci.* 2018 Sep 10;19(9). pii: E2685. doi: 10.3390/ijms19092685. PubMed PMID: 30201874; PubMed Central PMCID: PMC6164729.
- Pratt, Rebecca, et al. "The Adipocyte Na/K-ATPase Oxidant Amplification Loop is the Central Regulator of Western Diet-Induced Obesity and Associated Comorbidities." *Scientific Reports* 9, Article number: 7927 (2019)

Abstracts

- Successful abstract submissions to Research Day 2016-2018
- Submission of abstract to Humanities Research Day 2017
- Successful abstract submissions to ARCC, AHA and ASN 2017

Presentations

- July 2013 HSTA presentation of INBRE research, Marshall University
 - Presentation to high school students interested in STEM field
- October 2013, Presentation of INBRE Research at Chi Beta Phi National Conference
 - Research findings from summer 2013 (Effect of Capsaicin on Human Small Cell Lung Cancer Cells) presented at National conference, awarded third place of 14.
- January 2014, Participation in Undergraduate Research Day at the Capitol
 - Research findings presented in Charleston, WV
- February 2014, Senior Seminar Presentation, Davis and Elkins College
 - Effect of Capsaicin on Human Small Cell Lung Cancer Cells
- March 2017, Research Day, Marshall University
 - The Na/K-ATPase signaling in obesity development in lentivirally transfected pNaKtide in C57BL6 mice
- April 2017, Forum Presentation, Davis and Elkins College
 - Invited by Shawn Stover to be part of the Biology and Environmental Science Forum
- June 2017, MU State of the University, Washington, DC

- pNaKtide targeted to adipocytes inhibits Na/K-ATPase reactive oxygen species, systemic inflammation, and obesity development in mice fed a western diet
- August 2017, Marshall University Research Retreat, Huntington, WV
 - NaKtide targeted to adipocytes inhibits Na/K-ATPase reactive oxygen species, systemic inflammation, and obesity development in mice fed a western diet
- September 2017, AHA Joint Scientific Sessions 2017, San Francisco, CA
 - NaKtide targeted to adipocytes inhibits Na/K-ATPase reactive oxygen species, systemic inflammation, and cardiac hypertrophy in mice fed a western diet
- November 2017, ASN Kidney Week, New Orleans, LA
 - NaKtide targeted to adipocytes inhibits Na/K-ATPase reactive oxygen species, systemic inflammation, and obesity development in mice fed a western diet with no renal toxicity
- November 2017, Appalachian Regional Cell Conference, Athens, OH
 - NaKtide targeted to adipocytes inhibits Na/K-ATPase reactive oxygen species, systemic inflammation, and obesity development *in vivo*
- March 2018, Marshall University Research Day, Huntington, WV
 - Na/K-ATPase signaling in and secreted factors of adipocytes mediate obesity development and may contribute to comorbid conditions
- March 2019, Marshall University Research Day, Huntington, WV
 - The Adipocyte Na/K-ATPase Oxidant Amplification Loop is the Central Regulator of Western Diet-Induced Obesity and Associated Neurodegeneration
- April 2019, Forum Presentation, Davis and Elkins College
 - Invited by Shawn Stover to be part of the Biology and Environmental Science Forum