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# The Role of NADPH oxidase 2 (NOX2) in High-Fat Diet-Induced Adiposopathy and Brain Dysfunction in a Mouse Model

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# THE ROLE OF NADPH OXIDASE 2 (NOX2) IN HIGH-FAT DIET-INDUCED ADIPOSOPATHY AND BRAIN DYSFUNCTION IN A MOUSE MODEL

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## Abstract

Obesity can have numerous detrimental consequences, namely metabolic syndrome, type 2 diabetes, cardiovascular disease, cancer, and Alzheimer's disease. The pathogenesis and physiologic consequences of obesity are unknown, but they are often associated with increased inflammation and oxidative stress in both the body and in the brain. One factor that has been implicated in causing inflammation associated with a high fat diet is the enzyme NADPH oxidase, or NOX, specifically the subunit NOX2. Two studies were performed in order to assess the effects of a high fat diet in combination with a universal NOX2 deficiency and a NOX2 deficiency targeted to macrophages. The results of the first study indicate that the NOX2 knockout (NOX2KO) mice on a high fat diet do not experience all the deleterious metabolic and inflammatory effects in the body and brain to the same degree as wild-type mice. This suggests that a deficiency in NOX2 does offer protection from some of the deleterious effects of a high fat diet. It was also determined from the first study that NOX2 expression is localized to macrophages in the visceral adipose tissue. In order to target these macrophages, a second study was conducted. For this second study, a mouse model was genetically engineered with the intent of inhibiting NOX2 solely within macrophages. Similar to the first study, these macrophage-deficient NOX2 knockout (macNOX2KO) mice were placed on a high fat along with the NOX2-flox wild-type (WT-FL) mice. The results suggest that the macrophage-deficient NOX2 knockout mice were protected from the deleterious effects of a high fat diet.

In summary, the deletion of NOX2 appears to offer a protective benefit against the deleterious effects of obesity in the context of a high fat diet. Specifically, the deletion of NOX2 in macrophages also offers this protection with the added benefit of targeting the deletion so as to not affect NOX2 functioning in other cells in the body.

# Chapter 1. Introduction and Literature Review

## 1.1 Obesity and Its Consequences

Over the past 20 years, there has been a dramatic rise in the incidence of obesity in the United States. According to the CDC, in 2010, every state had a prevalence of obesity of at least 20%, and 36 states had a prevalence of 25% or more. In comparison, in 2000, the obesity prevalence range was 10-24%. More than one-third of U.S. adults are obese, and about 17% of children and adolescents are obese, indicating that obesity is a condition that affects all ages of people (Ogden, 2014). One of the problematic consequences of obesity is that it is costly. In 2008 the estimated annual medical cost of obesity was $147 billion, and these medical costs were $1,429 higher for obese people than for those of normal weight (Finkelstein, 2009). In addition to the United States, obesity has become a prominent issue in other parts of the world as evidenced by at least 300 million adults being obese on a global scale (World Health Organization, 2000).

It is difficult to pinpoint exactly what causes obesity, since there are a multitude of factors that contribute to its incidence, including genetic predisposition, diet, and reduced exercise. Determining the various causes of obesity is important, since it has been shown to be a risk factor for a multitude of diseases including heart disease, stroke, dementia, and cancer, and it is the most important risk factor for the development of type 2 diabetes (Chan, 1994). In recent years, obesity has been suggested as being linked to cognitive dysfunction and diseases such as Alzheimer's disease (Olufadi, 2008). For example, studies have reported that increased adiposity or obesity is associated with decreased cognitive performance (Smith, 2011), and

regression studies have shown that higher body weight is associated with smaller brain volume (Ward, 2005). Clinical obesity is associated with reductions in focal gray matter volume and enlarged white matter, particularly in the frontal lobe (Pannacciulli, 2006). There is also evidence that specifically indicates that there is an association between obesity and type II diabetes and Alzheimer's disease, and that individuals who are obese are at greater risk for Alzheimer's disease (Luchsinger, 2009).

Alzheimer's disease (AD) is the most common form of dementia among the elderly, and the CDC estimates that as many as 5 million Americans have the disease. Alzheimer's disease usually begins at age 60, and the risk increases with age. About 50 percent of those 85 years and older may have the disease. Although uncommon, younger people can get the disease as well. It has been determined that the progression of cognitive decline is associated with neuropathological hallmarks that include senile plaques that consist of extracellular amyloid beta (Abeta) and intracellular neurofibrillary tangles that are comprised of hyperphosphorylated tau protein (Wilkinson, 2006). It has also been suggested that four-hydroxynonenal (HNE), an aldehyde product of lipid peroxidation, plays a role in the pathogenesis of neuron degeneration in AD, thus indicating that increased oxidative stress is involved in the pathogenesis of AD (Markesbery, 1998). Additional research is needed to fully elucidate the causes of cognitive decline in AD.

**1.2 Obesity Versus Adiposopathy**

Obesity in and of itself is not necessarily deleterious or always implicated in contributing to diseases. Obesity can be thought of as having an abnormal amount of body fat, which may or may not bring about deleterious health consequences. In fact,

some obese people are metabolically healthy and do not develop any of the various diseases that can result from obesity (Karelis, 2004). Adiposopathy or "sick fat" specifically addresses the characteristics of the adipocytes, indicating pathological, dysfunctional changes (Bays, 2005). Despite gaining weight or even fat, some individuals do not exhibit the negative metabolic effects which impact health and are indicative of adiposopathy. This is what distinguishes adiposopathy from obesity, in that obesity is simply having an extreme amount of body fat, whereas adiposopathy pertains to pathological changes in the fat that have an adverse impact on health. It is important to differentiate the two, because adiposopathy is ultimately what can lead to diseases such as diabetes and Alzheimer's disease (Bays, 2005). Obesity, a sedentary lifestyle, and a genetic predisposition are the factors which will often lead to development of adiposopathy (Bays, 2005).

An individual can be obese and not have adiposopathy and vice versa. Individuals can be obese and not suffer from type 2 diabetes mellitus, hypertension or dyslipidemia. These individuals appear to be protected from the adverse metabolic consequences typically associated with obesity (Bays, 2005). Lipodystrophy is a situation in which adiposopathy can occur without obesity. In lipodystrophy, there is a deficiency of adipose tissue, which is also associated with insulin resistance (Ganda, 2000). Both of these examples indicate that the amount of adipose tissue is not the driving factor in causing detrimental health consequences. In cases of metabolic derangement, that is linked with adipose tissue (obesity and too much adipose tissue or lipodystrophy and too little or no adipose tissue), there is an impaired capacity to store fat in adipose tissue, and this is what can lead to ectopic fat storage and adiposopathy

(Goossens, 2008). This is truly an endocrine disease in that adipose tissue as an organ can lead to hormonal disturbances when it is diseased. Previously, adipose tissue was simply viewed as a fat depot and was not considered to play a crucial role in facilitating disease conditions. Or it was thought that adipose tissue played a minor or secondary role in the development of disease, while other organs such as the heart, liver, pancreas, etc. were thought of as the key contributors. Now, adipose tissue is regarded as a complex endocrine organ that secretes and responds to hormones and plays a complex role in many metabolic pathways (Wells, 2012).

### 1.3 Pathological Changes in Adipose Tissue

In the context of a high fat diet and obesity, normally functioning adipose tissue is triggered to undergo adipogenesis, which is the production of additional adipocytes. If adipogenesis is impaired, then already existing adipocytes will undergo further adipocyte hypertrophy which may lead to metabolic disease (Bays, 2008). If hypertrophied adipocytes outgrow their blood supply they can become dysfunctional or die. This leads to an increase in adipose tissue hypoxia, production of chemotactic factors, and increased free fatty acid (FFA) fluxes (Sun, 2011). This results in an increase in recruitment of macrophages to the adipose tissue, and the macrophages form crown-like structures (CLSs) around the dead adipocytes, which are then phagocytosed. The macrophages also produce pro-inflammatory cytokines, which lead to the development of insulin resistance (Cinti, 2012). Insulin resistance along with the other conditions of metabolic syndrome, such as visceral adiposity, hypertension,

hyperglycemia, and hypertriglyceridemia, are what can lead to diseases such as diabetes, cardiovascular disease, and neurodegenerative diseases such as Alzheimer's.

When the adipose tissue becomes overloaded, the excess fat can be stored as triacylglycerols in non-adipose tissues such as skeletal muscle, liver, and pancreas (Goossens, 2008). This accumulation of triacylglycerols may lead to insulin resistance. This ectopic fat deposition is called lipotoxicity and can also occur in individuals with lipodystrophy who have a lack of adipose tissue (Bays, 2008). In the liver, this accumulation of triacylglycerols and fatty acids can lead to higher glucose production, elevated very low-density lipoprotein-triacylglycerol output, and lower insulin clearance. Decreased glucose uptake and glucose oxidation can occur in the skeletal muscle and hyperinsulinemia can ensue in the pancreas (Goossens, 2008).

In addition to the deleterious metabolic effects and subsequent diseases of expanding, dysfunctional adipose tissue, there can also be detrimental effects on the brain. Diet-induced obesity has the potential to induce higher levels of reactive oxygen species in the brain (Freeman, 2013). This oxidative stress can then lead to cognitive impairment. The major inflammatory cells of the CNS are microglia, and these have been shown to play a role in the development of diseases such as Alzheimer's Disease (Griffin, 1989). Microglia serve a critical phagocytic function in the brain, similar to peripheral tissue macrophages (Ransohoff, 2009). Macrophages in the adipose tissue and microglia in the brain are upregulated during obesity. Additionally, it has been shown that high-fat diet induced obesity can result in the recruitment of peripheral immune cells to the CNS.

These cells display characteristics of microglia/macrophages, indicating a possible relationship between adipose tissue and brain inflammation (Buckman, 2014).

### 1.4 Inflammation, Oxidative Stress, and NADPH oxidase in the Body and Brain

While the molecular link(s) between excess adiposity and inflammation have not yet been identified, activation of the proinflammatory enzyme NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase or NOX) has been implicated in the detrimental effects of diet-induced obesity. NADPH oxidase is a membrane bound enzyme complex that consists of membrane components (gp91phox and p22phox) and cytosolic components (p47phox, p67phox, and p40phox) that assemble at the plasma membrane to form the active oxidase (Babior, 1991 and DeLeo, 1996). The catalytic subunit of NOX is gp91phox (also known as NOX2). This enzyme complex has the function of generating superoxide and other reactive oxygen species by transferring electrons across cell membranes in a process referred to as the 'respiratory burst'. Usually the electron acceptor is oxygen and the product of the electron transfer is superoxide (Bedard, 2007). Reactive oxygen species (ROS) are involved in the killing of bacteria, fungi, and other pathogens, making ROS an important component in host defense. But despite this beneficial contribution to host defense, ROS are also involved in contributing to host damage through interaction with proteins, lipids, carbohydrates, and nucleic acids. In 1956, the role of ROS in the aging process was discovered (Harman, 1956). Since then, the role of ROS as contributors to cellular damage in the process of aging has been accepted (Beckman, 1998).

Under normal circumstances, NOX generation of ROS in the adipose tissue occurs in response to insulin (Krieger-Brauer and Kather, 1992; Krieger-Brauer and Kather, 1997; Mahadev et al., 2001; Mahadev et al., 2004). This is a physiologically important response, because ROS enhance adipocyte differentiation thus allowing for glucose uptake (Krieger-Brauer and Kather, 1995). But in obese animals, in addition to ROS production occurring in response to insulin, it can also occur spontaneously. NOX2, specifically, appears to be upregulated in obese rats (Furukawa et al., 2004). A study using obese rats found that generation of ROS promoted generation of factors involved in metabolic syndrome including plasminogen activator inhibitor 1 (PAI-1) and TNF-alpha (Bedard, 2007). ROS were also found to decrease generation of adiponectin, which is a protein involved in glucose regulation and insulin sensitization (Bedard, 2007; Furukawa et al., 2004). Additionally, if there is prolonged exposure to ROS, glucose transporter expression may be decreased and glucose uptake may be compromised (Rudich et al., 1998). A study using rats showed that ROS generated from NOX2 contributed to the deterioration of beta-cell function. This study also showed that suppression of NOX2 reverses the glucose-induced dysfunction of pancreatic NIT-1 cells (Yuan et al., 2010).

In addition to adipocytes, NOX generated ROS have also been recognized in microglia, and most recently in astrocytes and neurons (Noh, 2000). The nervous system produces large quantities of ROS due to the fact that the nervous system accounts for over 20% of the oxygen consumed by the body. The nervous system is also very sensitive to oxidative stress because of polyunsaturated fatty acid enrichment in the membranes (Bedard, 2007). Studies have suggested that ROS generated by

NOX can contribute to a variety of diseases of the CNS. Specifically, numerous studies have indicated that microglia respond to neuropathology due to Alzheimer's disease by secreting molecules which activate NOX, thus leading to the production of ROS (Wilkinson, 2006).

Not only has it been clearly shown that NOX is involved in the pathogenesis of AD, studies have also shown increased NOX activation in AD brains through the demonstration of translocation of the cytosolic factors p47-*phox* and p67-*phox* to the plasma membrane (Shimohama, 2000). In addition to AD brains, it has also been shown that there is higher NOX activity in mild cognitive impairment (MCI) brains, indicating that NOX may contribute to the early pathogenesis of AD (Bruce-Keller, 2010). One study used transgenic mice overexpressing the Swedish mutation of the human amyloid precursor protein as a model for AD that also had a NOX subunit deleted, and the results showed that the mice did not develop oxidative stress or cognitive deficits (Park, 2008). In a study using APP x PS1 knockin mice, which are a model for A-beta pathogenesis, it was found that NOX activity is significantly higher in aged APP x PS1 mice and shares a linear relationship with cognitive impairment (Bruce-Keller, 2011). In addition to Alzheimer's Disease, the effects of NOX have also been implicated in Parkinson's disease, atherosclerosis, diabetic nephropathy, cancer, and others (Lambeth, 2007).

Despite there being a multitude of studies and data that indicate the contributions of NOX2 in the pathogenesis of inflammation and subsequent development of adiposopathy, there has not been a study conducted that specifically investigates the effects of NOX2 deletion. Since it has been clearly shown that NOX2 plays a role in

these deleterious processes, it is implied that complete removal or inhibition of NOX2 would have a protective effect. Because this has not been tested directly, we set out to determine if in fact complete deletion of NOX2 in the context of high-fat diet induced obesity would result in attenuation of adiposopathy and brain injury. To accomplish this goal, we utilized a mouse model that has a whole-body deletion of NOX2 (NOX2KO mice).

**1.5 Mouse Model of Chronic Granulomatous Disease**

Chronic granulomatous disease or CGD is a group of recessive hereditary diseases in which there is chronic inflammation resulting in the formation of granulomas (Curnutte, 1993). These granulomas are often sterile and can involve skin, lung, liver, lymph nodes, spleen, and the lining of the gastrointestinal and genitourinary tract (Curnutte, 1993; Gallin, 1993). Individuals with this disease also have increased susceptibility to microbial infections due to a mutation in any of the four subunits of NOX, which results in absence of respiratory burst activity. The microbes that are particularly problematic are *Staphylococcus aureus*, Gram-negative enteric bacilli, *Aspergillus fumigatus*, and other fungi (Curnutte, 1993; Gallin, 1983). The majority of these individuals are males hemizygous for mutations in the X-linked gene for gp91phox (Royer-Pokora, 1986).

There is a mouse model of chronic granulomatous disease that has a non-functional allele for the gp91phox, or NOX2, subunit. This knockout mouse was created using targeted homologous recombination in murine embryonic stem cells (Pollock, 1995). These mice are the first animal model for this disease, and the model closely

resembles patients with CGD since they have absence of respiratory burst activity and have increased susceptibility to infection with *Aspergillus fumigatus* and *Staphylococcus aureus* (Pollock, 1995).

**1.6 The Beneficial, Physiological Role of NOX**

Although NOX has been shown to be involved in the pathogenesis of cognitive decline and other diseases, it should also be noted that NOX plays a role in carrying out normal, physiological functions, which include innate immunity, signal transduction, and biochemical reactions (Lambeth, 2007). NOX has also been shown to be a key regulator of ROS generation in synaptic plasticity and memory formation (Kishida, 2007). Although it may seem counterintuitive that NOX could contribute to both necessary physiological functions and harmful pathological processes, it can be explained in terms of antagonistic pleiotropy, in which the production of ROS by NOX early in life is beneficial but becomes harmful due to overexpression later in life (Lambeth, 2007). This is further exemplified by the fact that the majority of diseases that result from increased ROS generation due to NOX are chronic in nature and occur later in life (Lambeth, 2007). The use of drugs that target or inhibit specific NOX enzymes could potentially be useful in stopping the progression of these diseases that occur later in life due to an overabundance of NOX expression (Wilkinson, 2006 Lambeth, 2007).

Because of the beneficial, physiologic roles of NOX, it is necessary to limit the NOX2 deletion to those cells in which NOX2 appears to exhibit the greatest contribution to adiposopathy.

But first it must be determined that NOX2 deletion does indeed result in a protective effect. Once that is determined, then the mechanism by which to target the necessary cells will be utilized.

### 1.7 Cre-Lox NOX2 Knockout Mouse Model

As alluded to previously, knockout mouse models are produced through homologous recombination. This process involves altering a DNA sequence and then inserting it into embryonic stem cells to make the gene non-functional. These embryonic stem cells are then injected into host blastocysts eventually resulting in targeted mutant mice. The knockout model is advantageous, because the deletion is targeted, not random, and the impact on other genes would likely be minimal. The problem is that, while this whole-body deletion targets NOX2, the deletion occurs systemically, thus affecting cells in which NOX2 plays an important, beneficial role. It will be helpful to limit the deletion of NOX2 to a specific cell in order to better elucidate its role in high-fat diet-induced adiposopathy. The Cre-lox system is advantageous in that respect, because cell-specific knockouts can be created. This is done by placing loxP sites around the *cybb* gene and then using a Cre recombinase under the control of a LysM promoter, which will result in excision of the *cybb* gene only in LysM positive cells, which are those of the monocytic origin.

### 1.8 The Role of NOX2 in Macrophages

Resident macrophages are necessary in order to maintain adipose tissue homeostasis, but in the scenario of obese adipose tissue, the macrophages can take on a pathologic inflammatory nature, which can subsequently contribute to pathologies such as insulin resistance. These different populations of adipose tissue macrophages

have been referred to as M1 and M2, with M1 referring to the inflammatory macrophages and M2 referring to the resident macrophages (Hill et al., 2014). In addition to these different populations, it has been shown that total macrophage number increases in the context of obesity. Macrophages may initiate inflammatory pathways in the adipose tissues (Weisberg, 2003) and can disrupt function by inducing insulin resistance, inducing adipocyte chemokine and cytokine production, or by impacting adipose tissue expansion capacity during obesity (Hill et al., 2014).

Microglia share many characteristics with peripheral tissue macrophages (Ransohoff and Perry, 2009). Microglia in the central nervous system play a critical role in the pathology of conditions such as Alzheimer's Disease (Griffin et al., 1989). In the context of high-fat diet-induced obesity, there is an increased activation of microglia (Thaler et al., 2012; Yi et al., 2012). There is also an increased recruitment of bone marrow-derived monocytes into the central nervous system (Buckman, 2013), thus indicating that infiltration of macrophages into the visceral adipose tissue may induce systemic effects leading to increased macrophages in the brain.

NOX2 can be found in a wide assortment of cells, but the majority of NOX2 expression occurs in phagocytes, namely macrophages (Bedard, 2007). In reference to NOX2's expression in adipose tissue specifically, it has been shown that expression occurs in both rodents and humans (Sakurai et al., 2009; Catalan et al., 2011). Unlike NOX4, NOX2 expression in adipocytes is quite low (Han et al., 2012), suggesting that NOX2 expression may occur in the macrophage cells in adipose tissue. In light of this evidence, it will be advantageous to target myeloid lineage cells, which includes monocytes, macrophages, and microglia, when designing a cre-lox mouse model.

## 1.9 Objectives

The first objective of this project was designed to elucidate the effects of NOX2 deletion on both the body and the brain in the context of a high-fat diet. This was done in order to examine if deletion of NOX2 would provide protection against metabolic and brain impairments. The second objective was to determine where the majority of NOX2 expression occurs in the visceral adipose tissue. The third objective was to target NOX2 deletion to macrophages. By doing this, the physiological functioning of NOX2 in other cells can be preserved. This will help pave the way for investigating potential ways of blocking NOX2 in macrophages, which will aid in the prevention of the development of adiposopathy.

Specific Aims

1. Utilized a mouse model with a global knockout of NOX2 and evaluated these mice on a high fat diet along with the wild-type counterparts. Various parameters were measured in order to assess the degree of inflammation in the adipose tissue and the brain. This is necessary in order to validate that NOX2 deletion does indeed offer protection in the face of high-fat diet induced obesity. Our hypothesis is that NOX2 knockout (NOX2KO) mice will be protected from the high-fat diet as compared to wild-type mice.
2. Determined through immunohistochemistry, where the majority of NOX2 expression occurs in the visceral adipose tissue.
3. Designed a cre-lox NOX2 knockout mouse model. This is necessary in order to target the NOX2 deletion to macrophages specifically.

4. Placed these genetically engineered macrophage-deficient NOX2 knockout (macNOX2KO) mice on a high fat diet along with their NOX2-flox wild-type (WT-FL) counterparts. Various parameters were measured in order to assess the degree of inflammation in the adipose tissue and the brain. This is necessary in order to elucidate the protective effects that occur when NOX2 deletion is targeted to macrophages. Our hypothesis is that macrophage-specific NOX2 deletion plays a key role against protection of adiposopathy and cognitive impairment while preserving the beneficial roles of NOX.

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# Chapter 2. NOX2 Deficiency Attenuates Markers of Adiposopathy and Brain Injury Induced by High-Fat Diet

"This chapter, NOX2 Deficiency Attenuates Markers of Adiposopathy and Brain Injury Induced by High-Fat Diet, previously appeared as Jennifer K. Pepping, Linnea R. Freeman, Sunita Gupta, Jeffrey N. Keller and Annadora J. Bruce-Keller, NOX2 deficiency attenuates markers of adiposopathy and brain injury induced by high-fat diet, American Journal of Physiology Endocrinology and Metabolism, 304: E392-E404, 2013. It is reprinted by permission of the American Physiological Society."

## 2.1 Introduction

Diet-induced obesity may be the primary cause of metabolic syndrome, which is associated with dramatically enhanced risk for many diseases, including type 2 diabetes, cardiovascular disease, stroke, and cancer (Haslam and James, 2005). In recent years, diet-induced obesity has also been linked to brain pathology, cognitive dysfunction, and Alzheimer's disease (Olufadi and Byrne, 2008). For example, studies have reported deficits in learning, memory, and cognitive processing in obese compared with nonobese patients (Elias et al., 2003; Elias et al., 2005; Waldstein and Katzel, 2006), and regression studies have demonstrated that higher body weight is associated with smaller brain volume (Ward et al., 2005). Additionally, clinical obesity is associated with reductions in focal gray matter volume and enlarged white matter, particularly in the frontal lobe (Pannacciulli et al., 2006). While it is not fully understood how obesity destabilizes health, obesity is closely associated with a pattern of chronic inflammation thought to originate in adipose tissue (Shoelson et al., 2007), resulting in enhanced cytokine production, increased acute-phase reactants, and other inflammatory mediators (Berg and Sherer, 2005; Chandalia and Abate, 2007; Hotamisligil 2006).

Activation of these inflammatory markers correlates tightly with insulin resistance (Pickup and Crook, 1998), cardiovascular disease (Rader, 2000), and cognitive impairment (Dziedzic, 2006; Trollor et al., 2012).

While the molecular link(s) between excess adiposity and inflammation has not yet been identified, recent reports have implicated activation of the proinflammatory enzyme NADPH oxidase in the detrimental effects of diet-induced obesity. NADPH oxidase is a superoxide-producing complex consisting of membrane (gp91phox and p22phox) and cytosolic (p47phox, p67phox, and p40phox) components which assemble at the plasma membrane to form the active oxidase (Babior, 1991; DeLeo and Quinn, 1996). NADPH oxidase is expressed in many types of immune cells including macrophages, dendritic cells, and neutrophils, as well as in adipocytes, endothelial cells, and neurons. With regard to the participation of NADPH oxidase in obesity, studies from our laboratory show that NADPH oxidase activity/expression is increased in the brains of mice given a high-fat diet (Bruce-Keller et al., 2010), a scenario also observed in rats (Zhang et al., 2005). Obesity-associated alterations in both endothelial cells (Chinen et al., 2007; Silver et al., 2007) and leukocytes (Patel et al., 2007) have been linked to NADPH oxidase. Once activated in the brain, NADPH oxidase is thought to trigger inflammatory processes that can contribute to CNS disease (Block, 2008; Lambeth, 2007; Shimohama, 2000). For example, data from our laboratory and others' show the critical role that NADPH oxidase plays in directing brain inflammation, particularly in the release of proinflammatory cytokines, including TNFα, IL-6, and IL-1β (Clark and Valente, 2004; Robertson et al., 1990; Turchan-Cholewo et al., 2009).

Release of all of these cytokines has the potential to underlie neurological impairment (Akiyama et al., 2000; Arvin et al., 1996; Lane et al., 1996; Parnet et al., 2002; Tyor et al., 1992).

Despite the evidence of a role for NADPH oxidase in the adverse effects of obesity, the effects of NADPH oxidase subunit deletion have not yet been tested in models of diet-induced obesity. To address this issue, this study employed a mouse model of chronic granulomatous disease (CGD), an inherited deficiency in NADPH oxidase based on deletion of the catalytic gp91phox subunit (also known as NOX2). The effects of high-fat diet were compared in wild-type C57Bl/6 (WT) mice and NOX2-deficient (NOX2KO) mice. Our hypothesis is deletion of NOX2 will offer significant protection from the detrimental neurological and/or physiological consequences of diet-induced obesity.

**2.2 Materials and Methods**

Animal treatments

The Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center approved all experimental protocols, which were compliant with NIH guidelines on the use of experimental animals. Four-month-old male C57Bl/6 (WT) and B6.129S-*Cybbtm1Din*/J (NOX2KO) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed in standard caging with a 12:12-h light-dark cycle and *ad libitum* access to food and water. C57Bl/6 mice were housed in standard conventional rooms, whereas NOX2KO mice were housed under sterile conditions to accommodate the CGD phenotype, which is characterized by recurrent bacterial and fungal infections leading to excessive inflammation (Morgenstern, 1997). Both WT and

NOX2KO mice were separated into groups given either a high-fat diet (HFD) or a low-fat control diet (CD) for 14 weeks. The HFD was composed of 60% fat (pork lard), and the control diet was composed of 10% fat. Both diets were open source, were purchased from Research Diets (New Brunswick, NJ; HFD: D12492; CD: D12450B), and were provided in pelleted form. Data were compiled from two separate experiments, each composed of both WT and NOX2KO mice given CD or HFD, with 9–10 total animals in each group.

Body weight, food intake, and body composition [measured using a Bruker minispec LF90 time domain Nuclear Magnetic Resonance (NMR) analyzer; Bruker Optics, Billerica MA] were measured twice per month. Fasting blood glucose was measured in tail blood using a glucometer (Ascensia Elite, Bayer, Mishawaka, IN), and glucose tolerance was measured using a modified oral glucose tolerance test (OGTT). Briefly, mice were fasted for 4 h, baseline glucose was measured, and then mice were immediately administered glucose (2 g/kg) via oral gavage. Blood glucose was measured at 15, 30, 60, and 120 min, and area under the curve (AUC) was recorded as an index of glucose disposal. To measure circulating nonesterified fatty acids (NEFA) in the context of hyperglycemia, additional blood samples (~50 µl) were collected at 0 and 60 min by lancing the submandibular vein (Fernandez et al., 2010), and NEFA were analyzed as described below. All mice were humanely euthanatized via isoflurane inhalation and cardiac exsanguination after a brief (6 h) fast, and blood, brain (anterior 1/3 of cerebral cortex), and visceral (epididymal) and subcutaneous (inguinal) adipose tissue depots were collected.

Clinical chemistry

Whole blood was collected by cardiac exsanguination of anesthetized mice and was allowed to clot at 4°C overnight and then centrifuged at 3,000 x *g* for 30 min. Serum was collected and either analyzed immediately or aliquoted and stored at −80°C. Levels of total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, and NEFA in sera were measured colorimetrically using commercially available kits (Wako Chemicals, Richmond, VA). Insulin levels were evaluated by ELISA in accordance with the manufacturer's assay protocol (Crystal Chem, Downers Grove IL).

Histological and biochemical analyses of adipose tissue

The inguinal and epididymal adipose depots were collected for histological and biochemical analyses. For histology, the tissues were immersion-fixed in 10% neutral buffered formalin for 2–3 days, after which they were processed for paraffin embedding. Sections (5 μm) were then cut, stained with hematoxylin and eosin, and digitized. Adipocyte size was measured by an investigator blinded to the experimental grouping in ×40 microscope fields by counting the total number of adipocytes within predefined area grids and then dividing the area by the total number of adipocytes within the grids to calculate average adipocyte size. For each sample, three tissue sections were analyzed, with three fields counted in each section, for an average of nine fields per sample.

For Western blot, adipose tissue samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer (G biosciences, St. Louis, MO) and then cleared by centrifugation at 5,000 x *g* for 10 min at 4°C. Samples were denatured in SDS, and equivalent amounts of protein were electrophoretically separated in

polyacrylamide gels and blotted onto nitrocellulose. Blots prepared from adipose tissue were processed using anti-Iba-1 (1:500, Wako Chemicals, Richmond, VA), anti-PPARγ1/2 (1:1,000, Abcam, Cambridge, MA), anti-adiponectin (1:1,000, Abcam), anti-GADD153/CHOP (1:5,000, Abcam), anti-GRP78 (1:500, Novus Biologicals), and anti-tubulin (1:1,000, Wako Chemicals). After incubation with primary antibodies, blots were washed and exposed to horseradish peroxidase-conjugated secondary antibodies and visualized using a chemiluminescence system (Amersham Biosciences, Pittsburgh, PA). Blot images were scanned and densitometrically analyzed for quantification. To ensure accurate quantification across multiple blots, samples from all groups (HFD and CD in both WT and NOX2KO) were included in each individual blot. Data were calculated as a ratio of expression over tubulin expression, which was included as an internal loading control. Protein expression in HFD mice was then calculated and presented as percent expression relative to CD mice of the same genotype.

For immunohistochemical analyses of adipose depots, tissue sections were processed using anti-Iba-1 (1:100, Wako Chemicals) and anti-NOX2 (1:100, Santa Cruz Biotechnology). Sections were incubated with biotinylated or peroxidase-linked secondary antibodies and then visualized using diaminobenzidine (for Iba1) or NOVAred (for NOX2) as chromagens following the manufacturer's instructions (Vector Laboratories, Burlingame, CA). To document nonspecific staining, the primary antibodies were omitted from the staining protocol. To visualize the cellular distribution of NADPH oxidase subunit expression, sections were first labeled for NOX2 and then double-labeled using Iba-1 as a macrophage cell marker.

Measures of brain injury by western blot

Tissue samples generated from frontal cortices were homogenized and processed for Western blot with chemiluminescence, as described in previous reports (Bruce-Keller et al., 2011; Pistell et al., 2010). Blots were processed using the following primary antisera: anti-claudin-5 (1:400, Abcam), anti-ZO-1 (1:100, Abcam), anti-occludin (1:8,000, Abcam), anti-matrix metalloproteinase-2 (MMP2; 1:1,000, Abcam), anti-MMP9 (1:1,000, Abcam), anti-synapsin 1 (1:10,000, Thermo Fisher Scientific, Pittsburg, PA), anti-phospho(Ser$^{553}$)-synapsin 1 (1:10,000, Abcam), anti-synapse-associated protein-97 (SAP97; 1:2,500, Abcam), anti-glial fibrillary acidic protein (GFAP; 1:5,000, Abcam); anti-Iba-1 (1:500, Wako Chemicals), and anti-tubulin (1:1,000, Wako Chemicals). To ensure accurate quantification across multiple blots, samples from all groups were included in each individual blot. Data were first calculated as a ratio of expression over tubulin expression, which was included as an internal loading control, and then protein expression in HFD mice was calculated and presented as percent expression relative to CD mice of the same genotype.

Statistical analyses

All data are shown as means ± SE. Body weight and composition data, adipocyte size, and all metabolic data were all analyzed with two-way analyses of variance (ANOVA) followed by planned Bonferroni posttests to determine the effects of HFD in both WT and NOX2KO mice. Additional planned comparisons of WT and NOX2KO mice under both CD and HFD conditions were carried out to determine the effects of genotype under both diet conditions. Significant effects of diet are indicated with "*" (*$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001), while significant effects of genotype are indicated

with "#" (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Protein expression values generated by Western blot (ratios of expression over tubulin) were normalized to percent CD for each genotype to reconcile data from multiple blots and were analyzed by two-tailed, unpaired *t*-tests to determine if statistically significant differences existed between HFD and CD groups within each genotype.

**2.3 Results**

Effects of HFD on body weight and composition in WT and NOX2KO mice

Four-month-old male WT and NOX2KO mice were fed either HFD or CD for 14 wk as described in Materials and Methods. During that time, body weights progressively diverged such that, by the end of the diet exposure period, the weights of the HFD-fed mice were significantly higher than CD mice, although all mice gained weight (Fig 2.1-A). Statistically, ANOVA for genotype X diet revealed a significant main effect of diet on body weight ($F_{(1,35)} = 82.82$, $p < 0.0001$). The effect of genotype on body weight was also significant ($F_{(1,35)} = 2.27$, $p = 0.030$), but the interaction was not. Planned comparisons of NOX2KO and WT mice revealed that, although there were no significant differences between body weights in mice given CD, HFD-fed NOX2KO mice had significantly higher weights than the HFD-fed WT mice after 4 and 6 wk of diet consumption (Fig 2.1-A). Analysis of food intake during this period did not reveal any differences between WT and NOX2KO mice with regard to diet (CD or HFD) ingestion (data not shown), indicating that NOX2 is not involved in feeding behavior and that the increased body weight in NOX2KO mice was not caused by increased food intake. In addition to body weight, total body fat was measured using NMR as described in Materials and Methods. Similar to what was observed for body weight, the amount of

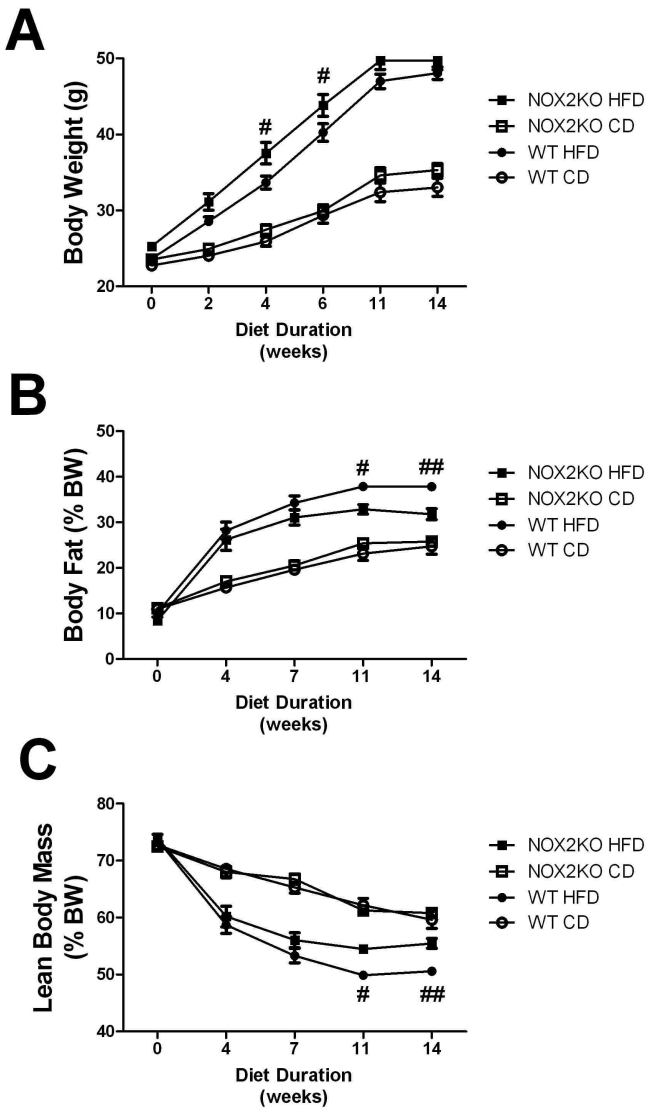


Figure 2.1: Effects of high-fat diet (HFD) on body weight and composition in WT and NADPH oxidase subunit 2 knockout (NOX2KO) mice. Four-month old male C57Bl/6 (WT) mice and B6.129S-*Cybb*$^{tm1Din}$/J (NOX2KO) mice were placed for 14 weeks on HFD or the nutritionally matched low-fat control diet (CD) with 10 mice in each group. Significant effects of genotype are indicated with "#" (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Figure 2.1-A: Body weight in WT and NOX2KO mice over time following administration of CD or HFD. Significantly (#$p < 0.05$) higher body weight noted in NOX2KO vs. WT mice after 4 and 6 weeks of diet. Figure 2.1-B: Body fat as %total body weight in WT and NOX2KO mice over time following administration of CD or HFD. Significantly (#$p < 0.05$, ##$p < 0.01$, respectively) lower body fat percentage in NOX2KO vs. WT mice after 11 and 14 wk of HFD. Figure 2.1-C: Lean body mass as %total body weight in WT and NOX2KO mice over time following administration of CD or HFD. Significantly (#$p < 0.05$, ##$p < 0.01$, respectively) higher lean body percentage in NOX2KO vs. WT mice after 11 and 14 weeks of HFD.

body fat (expressed as %total body weight) was higher in all mice over the 14-wk feeding trial, but mice given HFD had the greatest amounts of body fat (Fig 2.1-B). Statistically, ANOVA to measure the effects of genotype and diet on percent body fat revealed a significant main effect of diet ($F_{(1,33)} = 53.68$, $p < 0.0001$). The effect of genotype on body fat was also significant ($F_{(1,33)} = 4.41$, $p = 0.033$), with a significant interaction between diet and genotype ($F_{(1,33)} = 8.63$, $p = 0.0038$). Planned comparisons of NOX2KO and WT mice revealed no genotype-based differences in percent body fat in mice given CD but did reveal higher body fat in WT compared with NOX2KO mice after 11 and 14 wk of HFD (Fig 2.1-B). NMR-based measures of lean body mass (expressed as %total body weight) revealed a lower lean body mass percentage in all mice over the 14-wk feeding trial, with mice given HFD having the least amount of lean body mass by the end of the feeding trial (Fig 2.1-C). Statistically, two-way ANOVA revealed a significant main effect of diet on lean body mass ($F_{(1,35)} = 52.10$, $p < 0.0001$). The effect of genotype on body weight was also significant ($F_{(1,35)} = 9.27$, $p = 0.0038$), but the interaction was not. Planned comparisons of NOX2KO and WT mice revealed no differences in percent lean mass in mice given CD but did reveal more lean mass in NOX2KO mice compared with WT mice after 11 and 14 wk of HFD (Fig 2.1-C).

After the mice were euthanized, the subcutaneous inguinal and visceral epididymal adipose depots were collected for analysis. Comparison of the wet weights of the individual fat pads revealed that mice on the HFD had a significantly heavier subcutaneous inguinal fat pad. Specifically, ANOVA for the effects of genotype X diet on the weight of the inguinal fat pad revealed a significant main effect of diet ($F_{(1,35)} = 78.19$, $p < 0.0001$), but no effect of genotype and no interaction (Fig 2.2-A), whereas

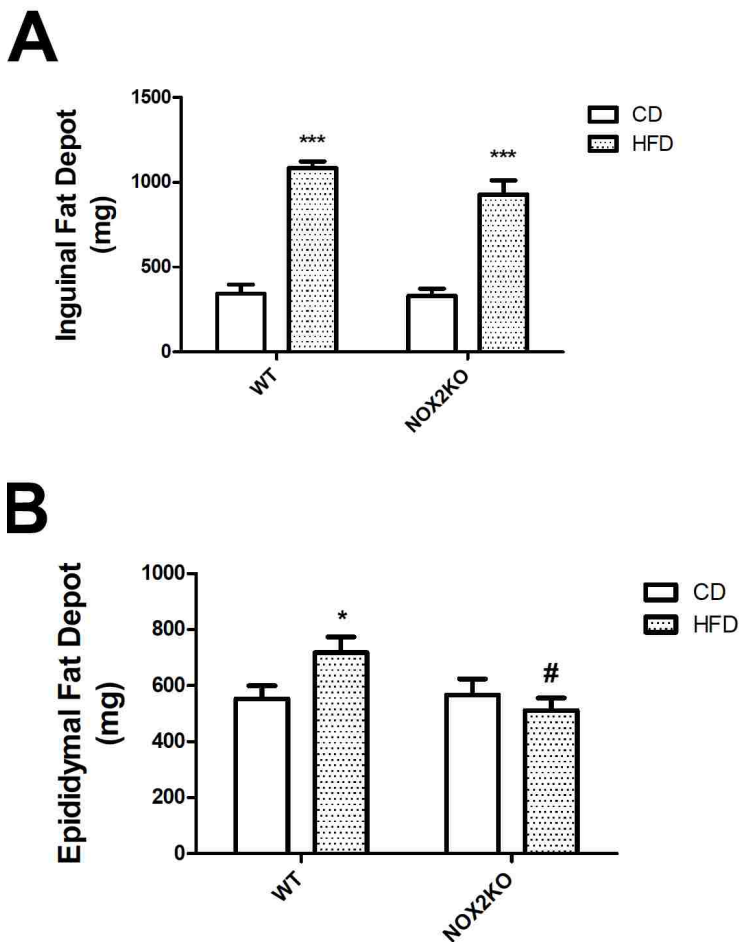


Figure 2.2: Effects of HFD on adipose depot weights in WT and NOX2KO mice. Subcutaneous inguinal and visceral epididymal fat pads were collected from WT and NOX2KO mice at the end of the 14-week feeding trial and weighed. Data were collected from 10 mice in each group except for the NOX2KO/HFD group, which had 9 samples. Significant effects of diet are indicated with "*" (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$), while significant effects of genotype are indicated with "#" (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Figure 2.2-A: Inguinal fat depot weight in WT and NOX2KO mice following administration of CD or HFD. Significantly (***$p < 0.001$) higher weight of inguinal fat depot in both WT and NOX2KO mice on HFD vs CD. Figure 2.2-B: Epididymal fat depots in WT and NOX2KO mice following administration of CD or HFD. Significantly (*$p < 0.05$) higher weight of epididymal fat depot in WT mice given HFD vs. WT mice on CD. Significantly (#$p < 0.05$) lower weight of epididymal fat depot in NOX2KO mice vs. WT mice on HFD.

post hoc tests confirmed that HFD significantly increased the weight of the inguinal fat pad in both WT and NOX2KO mice. Planned comparisons of NOX2KO and WT mice revealed no differences in the weight of the inguinal adipose depot in mice given CD;

likewise, the inguinal adipose depot in HFD-fed WT mice was not statistically different in size from the inguinal depot isolated from HFD-fed NOX2KO mice (Fig 2.2-A). Visceral adipose was likewise analyzed, and ANOVA for the effects of genotype X diet on the weight of the epididymal fat pad showed no main effect of either diet or genotype but did reveal a significant interaction ($F(1,35) = 10.51$, $p = 0.0401$; (Fig 2.2-B). Post hoc tests showed that HFD significantly increased epididymal adipose depot weight in WT mice but not in NOX2KO mice, whereas planned comparisons of NOX2KO and WT mice revealed that the epididymal adipose depot in HFD-fed NOX2KO mice was significantly lower than that isolated from HFD-fed WT mice (Fig 2.2-B).

Diet-induced adipocyte hypertrophy and inflammation in WT and NOX2KO mice

As obesity and metabolic dysfunction are frequently associated with adipocyte enlargement or hypertrophy (Bays et al., 2008; Heilbronn et al., 2004), the size of individual adipocytes within subcutaneous inguinal and visceral epididymal adipose depots was evaluated in tissue sections as described in Materials and Methods. Such measurements revealed that HFD-fed mice had significantly larger subcutaneous inguinal adipocytes (Fig 2.3-A). Specifically, ANOVA for the effects of genotype and diet on inguinal adipocyte size revealed a significant main effect of diet ($F_{(1,33)} = 47.79$, $p < 0.0001$) but no effect of genotype and no interaction. Planned comparisons revealed no differences in the size of inguinal adipocytes in CD-fed NOX2KO and CD-fed WT mice and likewise showed that both WT and NOX2KO HFD-fed mice had larger subcutaneous adipocytes (Fig 2.3-A). With respect to visceral adipocytes, two-way ANOVA on the effects of genotype and diet on epididymal adipocyte size revealed a significant main effect of diet ($F_{(1,34)} = 38.40$, $p < 0.0001$). Although there was no

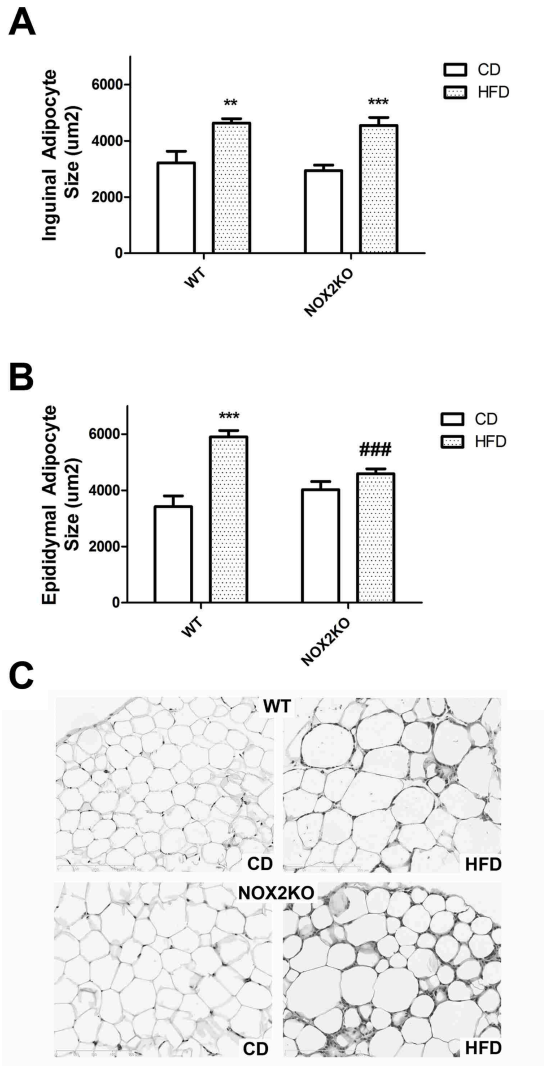


Figure 2.3: Effects of HFD on adipocyte hypertrophy in WT and NOX2KO mice. Subcutaneous inguinal and visceral epididymal fat pads were collected from WT and NOX2KO mice at the end of the 14-week feeding trial and processed for histological analyses of adipocyte size as described in Materials and Methods. Data were collected from 10 mice in each group except for the NOX2KO/HFD group, which had 9 samples. Significant effects of diet are indicated with "*" (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$), while significant effects of genotype are indicated with "#" (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Figure 2.3-A: Size of inguinal adipocytes in WT and NOX2KO mice after administration of CD or HFD. Significantly (**$p < 0.01$) larger adipocytes in WT mice on HFD vs. WT mice on CD; Significantly (***$p < 0.001$) larger adipocytes in NOX2KO mice on HFD vs. NOX2KO mice on CD. Figure 2.3-B: Size of epididymal adipocytes in WT and NOX2KO mice after administration of CD or HFD. Significantly (***$p < 0.001$) larger adipocytes in WT mice on HFD vs. WT mice on CD; Significantly (###p < 0.05) smaller adipocytes in HFD-fed NOX2KO mice vs. HFD-fed WT mice. Figure 2.3-C: Representative images of H&E stained adipocytes from which adipocyte size measures were based likewise revealed smaller adipocytes in HFD-fed NOX2KO mice compared with HFD-fed WT mice.

significant effect of genotype on visceral adipocyte size, there was a significant interaction of diet and genotype ($F_{(1,34)}$ = 15.18, $p$ < = 0.0015), and post hoc tests showed that HFD-fed WT mice had larger epididymal adipocytes (Fig 2.3-B). Planned comparisons revealed that HFD-fed NOX2KO mice had significantly smaller epididymal adipocytes than HFD-fed WT mice (Fig 2.3-B), with no differences in visceral adipocyte size between CD-fed mice. Representative images of hematoxylin and eosin-stained tissue sections prepared from visceral epididymal adipose depots (Fig 2.3-C).

Obesity is frequently accompanied by chronic low-grade inflammation (Berg and Scherer, 2005; Hotamisligil, 2006), and evidence suggests that macrophages may mediate inflammatory pathways initiated within adipose tissues (Weisberg et al, 2003). Thus, macrophage infiltration into inguinal and epididymal adipose depots was evaluated by measuring expression of Iba-1, a calcium-binding protein specifically expressed in macrophages that is upregulated with activation (Hilton et al, 2008; Lee et al, 2008; Zecca et al, 2008) and can be used in Western blot and immunohistological analyses in paraffin-embedded tissues (Ahmed et al, 2007; Vega-Avelaira et al, 2007). Evaluation of Western blots indicated lower Iba-1 expression in NOX2KO mice, but not WT mice, adipose following HFD (Fig 2.4-B). Quantification and two-tailed, unpaired *t*-test analyses of Iba-1 expression over multiple blots likewise revealed that HFD-fed mice had significantly higher Iba-1 expression in subcutaneous inguinal fat depots collected from both WT ($t_{(18)}$ = 4.43, $p$ = 0.0003) and NOX2KO mice ($t_{(18)}$ = 3.17, $p$ = 0.0053; Fig 2.4-A)). Conversely, HFD-fed mice had higher visceral epididymal Iba-1 expression only in WT ($t_{(18)}$ = 2.75, $p$ = 0.0132) but not NOX2KO mice (Fig 2.4-A). Representative images of hematoxylin and eosin-stained tissue sections prepared from

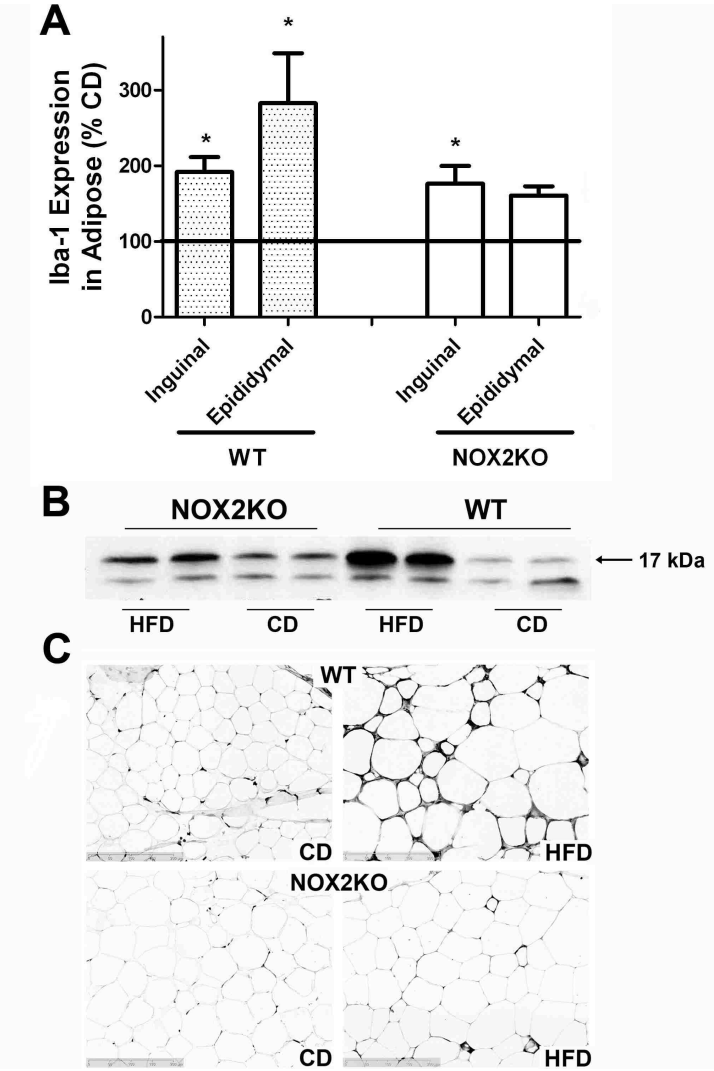


Figure 2.4: Effects of HFD on macrophage infiltration into adipose depots in WT and NOX2KO mice. Iba-1 expression was used to measure macrophage infiltration/activation in inguinal and epididymal fat. Data were collected from 10 mice in each group except for the NOX2KO/HFD group, which had 9 samples. Significant effects of diet are indicated with "*" (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Figure 2.4-A: HFD-induced Iba-1 expression in inguinal and epididymal adipose tissue in WT and NOX2KO mice as percentage of expression in CD mice. Significant (*$p < 0.05$) HFD-induced higher Iba-1 expression in inguinal and epididymal fat in WT mice and inguinal fat in NOX2KO mice. Figure 2.4-B: Representative Western blot images showing Iba-1 (17 kDa) expression in epididymal fat taken from WT and NOX2KO mice given CD or HFD. Figure 2.4-C: Representative images of Iba-1 immunostaining showing macrophage activation and infiltration into epididymal fat taken from WT and NOX2KO mice given CD or HFD.

visceral epididymal adipose depots likewise confirmed lower macrophage infiltration into adipose isolated from HFD-fed NOX2KO mice but not from HFD-fed WT mice or mice given CD (Fig 2.4-C).

Diet-induced alterations to visceral adipocyte physiology in WT and NOX2KO mice

To determine whether the hypertrophy and inflammation noted in visceral adipose were accompanied by derangement in adipocyte physiology, markers of adipocyte function/injury in visceral adipose of WT and NOX2KO mice were examined by Western blot. Specifically, the overall expressions of PPARγ and adiponectin were evaluated, as they are both markers of mature functional adipocytes and also because loss of these factors contributes to obesity-induced metabolic decline (Aprahamian and Sam, 2011; Semple et al., 2006). Quantification and analyses of PPARγ expression revealed that HFD-fed mice had less expression of PPARγ relative to expression in CD-fed mice only in WT ($t_{(18)} = 3.72$, $p = 0.0040$) mice but not in NOX2KO mice (Fig 2.5-A). Conversely, adiponectin expression was less in HFD-fed mice in visceral fat depots collected from both WT ($t_{(18)} = 10.21$, $p < 0.0001$) and NOX2KO mice ($t_{(18)} = 5.2$, $p < 0.0001$; Fig 2.5-B), and data also showed that the effect of HFD was significantly lower in NOX2KO mice than in WT mice ($t_{(18)} = 2.79$, $p = 0.0122$; Fig 2.5-B).

Adipocyte injury was estimated by evaluating the expressions of GADD153/CHOP and GRP78, both of which are known to be increased in the context of obesity and thought to reflect endoplasmic reticulum (ER) stress in adipocytes caused by chronic and/or excessive inflammation (Oyadomari and Mori, 2004; Ozcan et al., 2004). Evaluation and statistical analysis of GADD153/CHOP blots revealed that HFD-fed mice had significantly higher GADD153 expression only in WT mice ($t_{(18)} =$

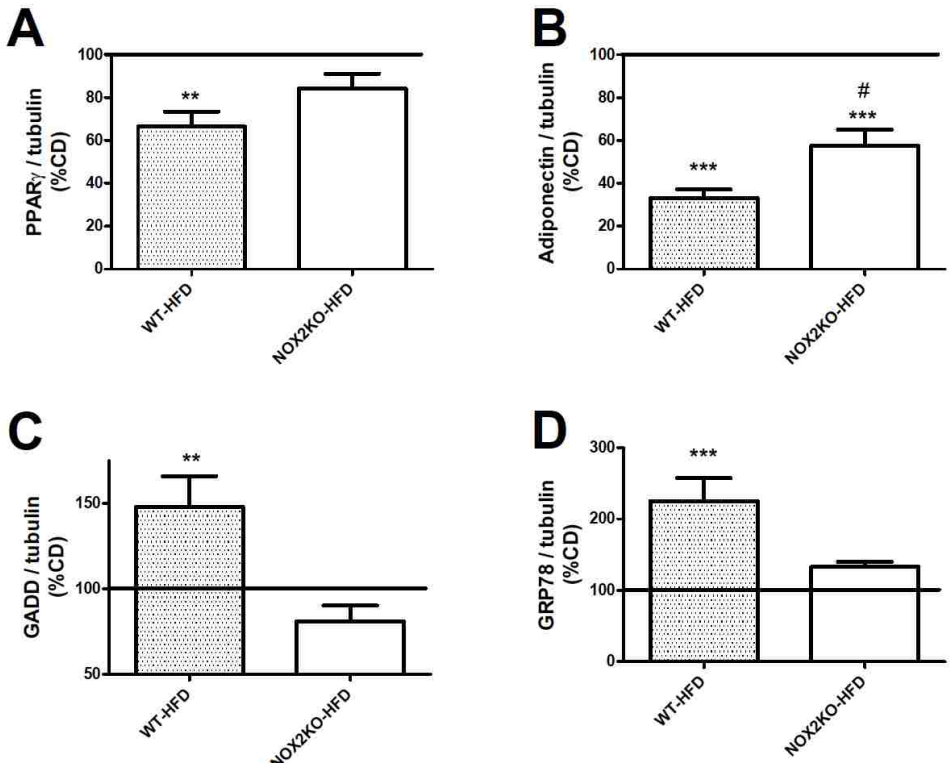


Figure 2.5: Effects of HFD on markers of adipocyte function and injury in WT and NOX2KO mice. Expression of (Figure 2.5-A) PPARγ, (Figure 2.5-B) adiponectin, (Figure 2.5-C) GADD153/CHOP, and (Figure 2.5-D) GRP78 were evaluated in tissue homogenates prepared from epididymal adipose depots. Data were collected from 10 mice in each group except for the NOX2KO/HFD group, which had 9 samples, and depict means ± SE expression in HFD mice presented as percentage values in CD mice (100% line on graph). Significant effects of diet are indicated with "*" (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$), while significant effects of genotype are indicated with "#" (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, respectively) changes in expression in HFD mice vs. CD mice; Significantly (#$p < 0.05$) higher expression in adiponectin in NOX2KO-HFD adipose vs. changes in expression in WT-HFD mice.

3.77, $p = 0.0014$) but not in NOX2KO mice (Fig 2.5-C). Likewise, expression of GRP78 was also higher in HFD-fed WT mice ($t_{(18)} = 2.5$, $p = 0.0217$; Fig 2.5-D) but not in NOX2KO mice (Fig 2.5-D).

Histological pattern of NOX2 expression in visceral adipose of WT mice

Although the above data indicate that NOX2 expression participates in HFD-induced adiposopathy, these data do not give any indication as to the cell type(s) mediating this effect. This issue is noteworthy because, while NOX2 expression has been detected in white adipose depots in both rodents (Sakurai et al., 2009) and humans (Catalan et al., 2011), NOX2 expression in differentiated 3T3-L1 adipocyte cells appears to quite low (Han et al., 2012). Indeed, cell culture data indicate that NADPH oxidase-based signaling in adipocyte cell lines may be mediated by NOX4 rather than NOX2 (Mahadev, 2004; Schroder, 2009), raising the possibility that adipose NOX2 expression may be restricted to macrophages, which are well known to be NOX2 positive (Forman and Torres, 2002). To thus better understand how NOX2 regulates adipose function, the expression of NOX2 was evaluated immunohistochemically in visceral epididymal tissue isolated from CD- and HFD-fed WT mice as described in Materials and Methods. Initial experiments verified the specificity of the selected NOX2 antibody by showing that it was unable to recognize an epitope in NOX2KO adipose tissues (Fig 2.6-A). Qualitative evaluation of NOX2 expression in the visceral adipose sections demonstrated prominent NOX2 immunoreactivity in cells with morphology typical of macrophages (Fig 2.6-B). To more accurately confirm the cell type-specific pattern of NOX2 expression, sections from CD- and HFD-fed WT mice were double-labeled for NOX2 and the macrophage markers Iba-1. Evaluation of tissue double-labeled sections showed extensive colocalization of NOX2 and Iba-1 staining (Fig 2.6-B), indicating that macrophages are the predominant cell type expressing NOX2 in the visceral adipose depot. However, an occasional and less intense pattern of

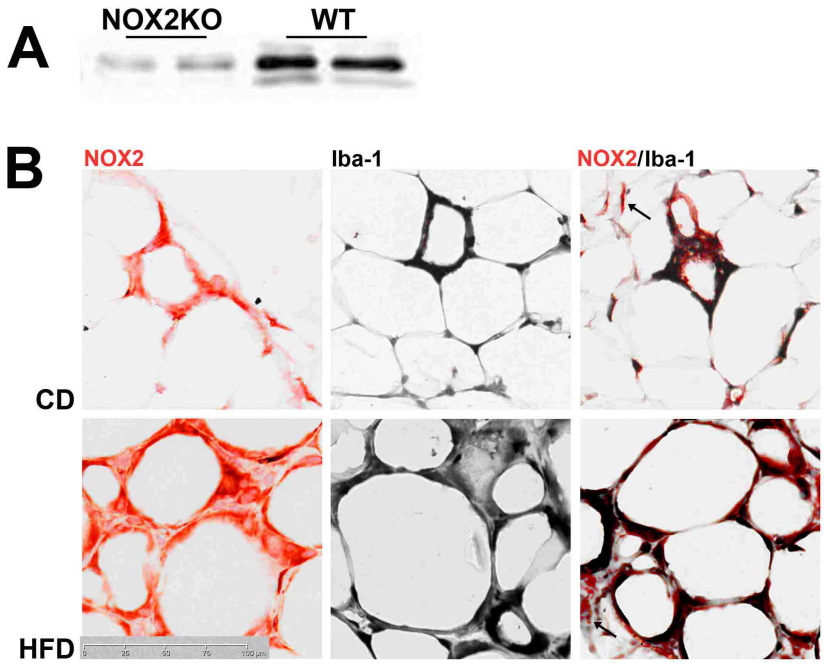


Figure 2.6: Expression of NOX2 in visceral adipose of WT mice. Figure 2.6-A: Representative images of NOX2 Western blot showing that the antibody used for immunostaining did not recognize epitopes in NOX2KO tissue. Figure 2.6-B: Representative images of NOX2 immunoreactivity (left, red), Iba-1 immunoreactivity (middle, black), and NOX2/Iba-1 double-labeled sections (right) of epididymal fat taken from WT mice given CD or HFD. Images reflect the predominant expression of NOX2 in Iba-1-positive macrophages, although NOX2-positive, Iba-1-negative cells can be observed (arrows in right) perhaps arising from preadipocytes or other stromovascular cells.

NOX2 immunoreactivity that was not associated with Iba-1-positive cells could be observed (Fig 2.6-B, arrows), perhaps arising from preadipocytes or other stromovascular cells.

Diet-induced metabolic dysfunction in WT and NOX2KO mice

To determine the extent of HFD-induced metabolic syndrome in WT and NOX2KO mice, data were collected, and for presentation purposes are thematically divided into syndromes separately describing insulin resistance and hyperlipidemia. To document insulin sensitivity and glycemic control, studies focused on regulation of

fasting glucose and glucose tolerance. At the end of the 14-wk diet regimen, mice were fasted, and blood glucose and serum insulin were measured as described in Materials and Methods. Two-way ANOVA on the effects of genotype and diet on fasting blood glucose revealed significant main effects of diet ($F_{(1,35)} = 14.44$, $p = 0.0014$) and genotype ($F_{(1,35)} = 35.67$, $p < 0.0001$), with a statistically significant interaction between diet and genotype ($F_{(1,35)} = 6.72$, $p = 0.0239$; Fig 2.7-A). Post hoc tests showed that HFD-fed mice had higher glucose levels in WT mice but not in NOX2KO mice, while planned comparisons revealed that glucose levels in HFD-fed NOX2KO mice were significantly lower than levels in HFD-fed WT mice (Fig 2.7-A). Conversely, two-way ANOVA on the effects of genotype and diet on fasting insulin revealed a significant effect of diet ($F_{(1,35)} = 14.44$, $p = 0.0014$) but no effect of genotype and no interaction (Fig 2.7-B). Glucose tolerance was measured by OGTT as described in Materials and Methods, and statistical analyses showed significant main effects of diet ($F_{(1,35)} = 42.96$, $p < 0.0001$) and genotype ($F_{(1,35)} = 5.83$, $p = 0.0401$) on glucose tolerance but no significant interaction between diet and genotype (Fig 2.7-C). Post hoc tests showed that HFD impaired glucose tolerance (i.e., increased the area under the curve) in both WT and NOX2KO mice, but planned comparisons revealed that HFD-fed NOX2KO mice had a smaller area under the curve compared with HFD-fed WT mice (Fig 2.7-C). Finally, in light of evidence of adipocyte hypertrophy and inflammation in HFD-fed WT mice, experiments were designed to reveal alterations in adipocyte glucose tolerance. Specifically, the adipose lipolytic response to glucose loading was evaluated by quantifying serum levels of NEFA at both fasting and 60 min post-glucose conditions, as described in Materials and Methods. Fasting NEFA levels (measured as meq/dl) were

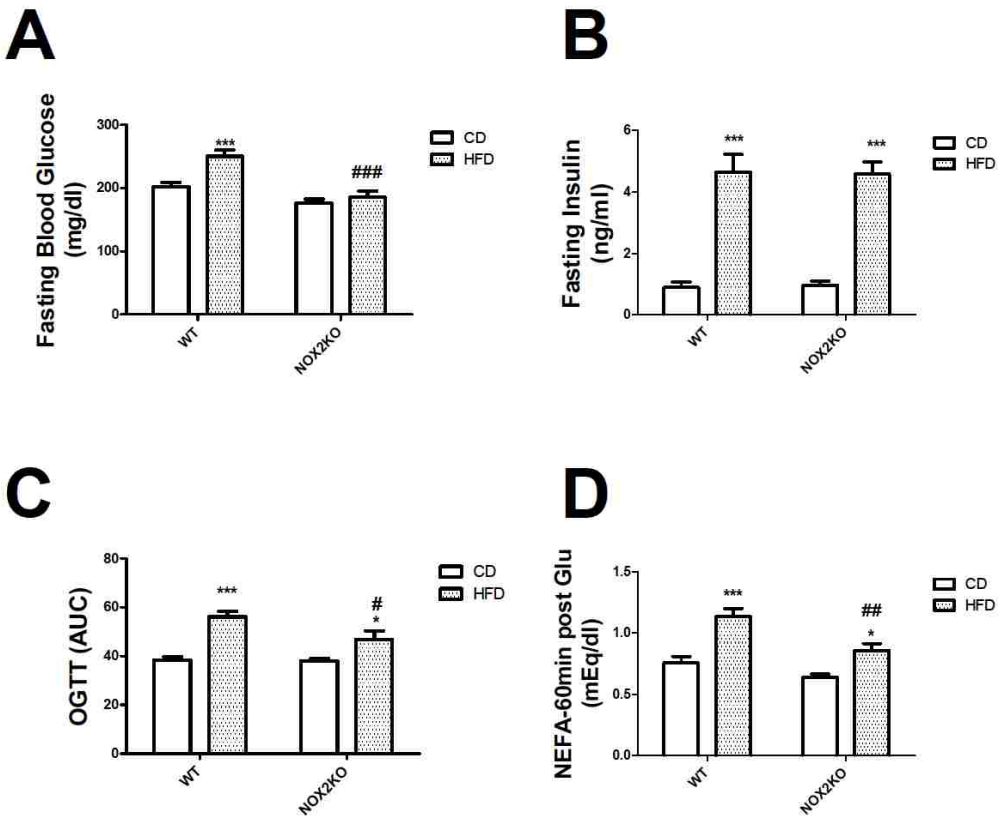


Figure 2.7: Effects of HFD on glucose regulation and tolerance in WT and NOX2KO mice. Mice were fasted for 4 h, after which an OGTT was performed to measure glucose regulation and insulin resistance. Data were collected from 10 mice in each group except for the NOX2KO/HFD group, which had 9 samples. Significant effects of diet are indicated with "*" (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$), while significant effects of genotype are indicated with "#" (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Figure 2.7-A: Fasting blood glucose levels measured in WT and NOX2KO mice after administration of a HFD or CD. Significantly (***$p < 0.001$) higher fasting blood glucose level in HFD-fed WT vs. CD-fed WT mice; Significantly (###$p < 0.001$) lower fasting glucose in HFD-fed NOX2KO vs. HFD-fed WT mice. Figure 2.7-B: Fasting insulin levels measured in WT and NOX2KO mice after administration of a HFD or CD. Significantly (***$p < 0.001$) higher fasting insulin in WT mice on HFD vs. WT mice on CD, and similarly an increase in NOX2KO mice on HFD vs. NOX2KO mice on CD. Figure 2.7-C: Oral glucose tolerance, as indicated by area under the curve (AUC). Significantly (***$p < 0.001$) larger AUC in WT mice on HFD vs. WT mice on CD; Significantly (*$p < 0.05$) larger AUC in NOX2KO mice on HFD vs. NOX2KO mice on CD. Significantly (#$p < 0.05$) smaller glucose AUC in HFD-fed NOX2KO mice vs. HFD-fed WT mice. Fig 2.7-D: NEFA levels in mice 60 min after glucose (2 mg/kg) gavage. Significantly (*$p < 0.05$ and ***$p < 0.001$, respectively) higher serum levels of NEFA in HFD mice; Significantly (##$p < 0.01$) lower levels of NEFA following glucose load in HFD-fed NOX2KO vs. HFD-fed WT mice.

not significantly affected by genotype or diet (WT-CD, 1.139 ± 0.054; WT-HFD, 1.1070 ± 0.044; NOX2KO-CD, 1.140 ± 0.027; NOX2KO-HFD, 1.060 ± 0.068). However, while glucose administration decreased NEFA in CD-fed mice, post-glucose NEFA levels in HFD-fed WT were much higher than in other groups of mice (Fig 2.7-D). Specifically, ANOVA analyses of post-glucose NEFA levels showed a significant main effect of diet ($F_{(1,33)}$ = 42.96, $p$ < 0.0001) and of genotype ($F_{(1,33)}$ = 5.83, $p$ = 0.0401) on post-glucose NEFA but no interaction (Fig 2.7-D). Post hoc tests revealed that HFD increased post-glucose levels of NEFA in both WT and NOX2KO mice, but planned comparisons showed that post-glucose NEFA were significantly lower in HFD-fed NOX2KO mice than in HFD-fed WT mice (Fig 2.7-D).

Studies next assessed a panel of bioactive serum lipids in WT and NOX2KO mice, measured under fasted conditions as described in Materials and Methods. Data showed a significant effect of diet ($F_{(1,34)}$ = 44.49, $p$ < 0.0001) on total cholesterol, but no effect of genotype, no interaction, and no significant differences between WT and NOX2KO mice (Table 2.1). Likewise, there were significant effects of diet on LDL-cholesterol ($F_{(1,34)}$ = 26.17, $p$ = 0.0013) and on HDL-cholesterol ($F_{(1,34)}$ = 30.13, $p$ = 0.0004) but no effects of genotype and no interactions. Additionally, post hoc tests showed that HFD-fed mice had more LDL- and HDL-cholesterol species in WT but not in NOX2KO mice (Table 2.1). Finally, there were no differences in levels of fasting triglycerides or NEFA in either WT or NOX2KO mice given either CD or HFD (Table 2.1).

Table 2.1. Serum lipids in WT and NOX2KO mice following CD or HFD

| | WT | | NOX2KO | |
|---|---|---|---|---|
| | **CD** | **HFD** | **CD** | **HFD** |
| Total Cholesterol (mg/dl) | 151.4 ± 8.8 | 211.5 ± 11.7*** | 159.0 ± 7.1 | 204.7 ± 11.0** |
| LDL Cholesterol(mg/dl) | 28.3 ± 1.1 | 36.5 ± 1.1* | 29.9 ± 2.1 | 35.2 ± 3.0 |
| HDL Cholesterol(mg/dl) | 80.0 ± 2.8 | 100.3 ± 4.6** | 88.1 ± 4.0 | 100.9 ± 5.0 |
| Triglycerides(mg/dl) | 29.2 ± 1.5 | 24.2 ± 3.7 | 25.2 ± 2.3 | 23.3 ± 2.7 |
| NEFA (mEq/L) | 1.13 ± 0.05 | 1.07 ± 0.04 | 1.14 ± 0.03 | 1.07 ± 0.07 |

Values are means +/- SE of data collected from 9-20 animals. Male wild-type (WT; C57BL/6) and NADPH oxidase subunit 2-deficient (NOX2KO) mice were administered control diet (CD) or high-fat diet (HFD) for 14 weeks, and then serum lipids were measured under fasted conditions as described in Materials and Methods. Data were analyzed by 2-way ANOVA followed by planned Bonferroni posttests to determine effects of HFD in NOX2KO vs. WT mice. Significant differences (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, respectively) noted in mice given HFD vs. CD mice.

Effects of HFD on brain injury in WT and NOX2KO mice

Experiments were next designed to determine the extent of brain injury caused by HFD in both strains of mice. Analyses were thematically split into evaluations of cerebrovascular integrity, synaptic density, and reactive gliosis measured in the anterior neocortex, a CNS site of increased NOX activity/expression following HFD (Bruce-Keller et al., 2010). Cerebrovascular and blood-brain barrier (BBB) integrity were evaluated by measuring the expressions of the essential tight junction proteins claudin-5, ZO-1, and occludin, as well as the matrix metalloproteinases MMP2 and MMP9, via Western blot, as described in Materials and Methods. These specific signals were chosen because lower expression of tight junction proteins is well known to accompany many neurological disorders including multiple sclerosis, stroke, Alzheimer's disease, Parkinson's disease, and epilepsy (Bednarczyk and Lukasiuk, 2011), while MMP are well known to degrade basement membranes and connective tissue of the BBB during inflammatory responses, contributing to both loss of BBB integrity and cerebral hypoperfusion (Nakaji et al., 2006). Western blot data indicated cerebrovascular injury

in WT but not NOX2KO mice following HFD (Fig 2.8). Specifically, two-tailed unpaired *t*-tests revealed significantly lower expression in HFD-fed mice of claudin-5 ($t_{(18)}$ = 2.75, $p$ = 0.0191) and occludin ($t_{(18)}$ = 6.53, $p$ < 0.0001) in WT but not NOX2KO mice (Fig 2.8-A). Likewise, MMP2 expression was higher ($t_{(18)}$ = 3.21, $p$ = 0.0048) in HFD-fed WT mice but not NOX2KO mice (Fig 2.8-A), while ZO1 and MMP9 were not affected by diet in either strain of mice (Fig 2.8-A).

Evaluations of synaptic density were based on expression of the postsynaptic marker synapse-associated protein-97 (SAP97) and total and phosphorylated forms of the presynaptic protein synapsin 1 (SYN1). Quantification of total SYN1 expression revealed no differences in expression between groups (Fig 2.8-B), but levels of phosphorylated SYN1 ($t_{(18)}$ = 2.86, $p$ = 0.0104) and SAP97 expression ($t_{(18)}$ = 2.34, $p$ = 0.0309) were significantly lower in HFD-fed WT mice but not NOX2KO mice (Fig 2.8-B).

To determine whether HFD consumption affected inflammatory gliosis in mice, the expression of astrocyte and microglial markers, as well as the proinflammatory/pro-oxidant enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2), were evaluated in cortical homogenates by use of Western blot. The intermediate filament protein glial fibrillary acidic protein (GFAP) was used to evaluate astrocyte hypertrophy (O'Callaghan and Sriram, 2005), and data showed significantly higher expression in GFAP caused by HFD in WT ($t_{(18)}$ = 7.58, $p$ < 0.0001; Fig 2.8-C) but not NOX2KO mice. Microglial reactivity was evaluated by measuring expression of Iba-1, and blots likewise revealed significantly higher Iba-1 expression following HFD in WT mice ($t_{(18)}$ = 2.62, $p$ = 0.0174; Fig 2.8-C) but not in NOX2KO mice. iNOS and COX2 were not affected by diet (Fig 2.8-C).

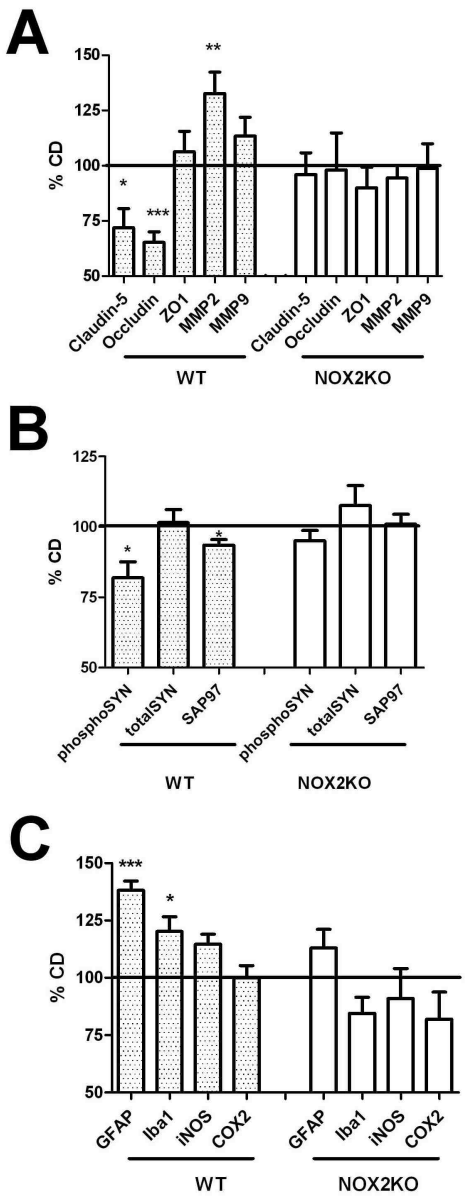


Figure 2.8: Effects of HFD on markers of brain injury in WT and NOX2KO mice. Significant effects of diet are indicated with "*" (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Figure 2.8-A: Markers of cerebrovascular integrity (expression of the tight junction proteins claudin-5, ZO-1, and occludin and matrix metalloproteinases MMP2 and MMP9). Figure 2.8-B: Synaptic density [expression of postsynaptic marker protein synapse-associated protein-97 (SAP97), presynaptic protein synapsin 1, and phosphorylated synapsin 1] were evaluated in tissue homogenates prepared from the frontal cortex. Figure 2.8-C: Reactive gliosis (expression of GFAP, Iba1, iNOS, COX2). Data were collected from 10 mice in each group except for the NOX2KO/HFD group, which had 9 samples, and depict means ± SEM expression in HFD mice presented as %values in CD mice (100% line) on graph. Significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, respectively) changes in expression in WT HFD vs. WT CD mice.

While NOX2KO mice have been used in numerous studies of brain injury, including models of aging (Park et al., 2007), stroke (Chen et al., 2011), Alzheimer's disease (Park et al., 2008), and traumatic injury (Dohi et al., 2010), systematic, side-by-side comparisons of markers of cerebrovascular integrity, synaptic density, and reactive gliosis in WT and NOX2KO mice have not been reported. This is an important issue, as not only is NADPH oxidase thought to contribute to brain injury, but data also show that NADPH oxidase participates in synaptic plasticity and memory formation (Kishida and Klann, 2007; Kishida et al., 2005; Tejada-Simon et al., 2005), raising the possibility that compensatory changes in CNS physiology caused by genetic deletion of NOX2 could confound comparisons between WT and NOX2KO animals. Thus, to determine whether NOX2 deletion results in significant alterations in the murine CNS, tissue homogenates were prepared from the anterior cerebral cortex of WT and NOX2KO mice maintained on CD and were evaluated for markers of cerebrovascular integrity, synaptic density, and reactive gliosis, as described in Materials and Methods. Data show that claudin-5 ($t_{(18)}$ = 2.42, $p$ = 0.0264) and occludin expression ($t_{(18)}$ = 4.27, $p$+ 0.0005) were significantly lower in NOX2KO mice compared with WT mice (Table 2.2). Conversely, the expressions of all other cerebrovascular, synaptic, and glial/inflammatory markers were not significantly altered in NOX2KO mice compared with WT mice (Table 2.2).

**2.4 Discussion**

Our findings strongly support a key role for NOX2 in the detrimental effects of diet-induced obesity. Specifically, we found that, while both WT and NOX2KO mice became obese following HFD administration, NOX2KO mice had attenuated adipose pathology and preserved adipose function, particularly in visceral adipose deposits.

Table 2.2. Effects of NOX2KO on baseline values for markers of brain injury

| | % WT |
|---|---|
| **Markers of Cerebrovascular Injury** | |
| Claudin-5 | 79.6 ± 5.4$^{\#}$ |
| Occludin | 71.3 ± 6.3$^{\#\#\#}$ |
| ZO-1 | 121.1 ± 12.6 |
| MMP2 | 111.7 ± 7.4 |
| MMP9 | 126.4 ± 17.8 |
| **Markers of Synaptic Injury** | |
| Phosphorylated Synapsin | 96.0 ± 4.3 |
| Total Synapsin | 109.7 ± 7.6 |
| SAP97 | 94.8 ± 3.2 |
| **Markers of Reactive Gliosis** | |
| GFAP | 116.7 ± 13.8 |
| Iba-1 | 108.7 ± 6.9 |
| iNOS | 83.1 ± 9.2 |
| COX2 | 79.1 ± 9.5 |

Data show means ± SEM expression in NOX2KO mice presented as % WT and were analyzed by 2-tailed, unpaired t-tests. Tissue homogenates were prepared from the anterior cerebral cortex of male WT (C57BL/6) and NOX2KO mice maintained on CD were evaluated for markers of cerebrovascular integrity, synaptic density, and reactive gliosis as described in Materials and Methods. MMP, matrix metalloproteinase; SAP97, synapse-associated protein-97; GFAP, glial fibrillary acidic protein; COX2, cyclooxygenase 2. Significant differences ($^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$, respectively) noted in NOX2KO mice vs. WT mice.

Additionally, glucose tolerance was normalized in NOX2KO mice compared with WT mice following HFD, and HFD-induced brain injury was prevented in NOX2KO mice. Overall, these data are consistent with previous studies demonstrating higher NADPH oxidase activity/expression in models of obesity (Bruce-Keller et al., 2010; Chinen et al., 2007; Zhang et al., 2005) and are also in agreement with the growing body of literature describing the sensitivity of the brain to obesity-induced metabolic dysfunction (Bruce-Keller et al., 2009; Middleton and Yaffe, 2009). Moreover, these data significantly enhance the findings of previous studies with the demonstration that NOX2 is a specific and powerful mediator of pathogenic effects of diet-induced obesity that extend from adipocytes to brain cells. These detrimental effects could arise from NOX2-positive

visceral adipose macrophages, whose action precipitates loss of adipocyte function and the development of ER stress within adipose tissues, triggering pathways that may ultimately result in loss of metabolic and neurological function. Collectively, our findings raise the possibility that NOX2-based therapies could be used clinically to preserve both metabolic and neurological function in the context of obesity.

These data support a key role for NOX2 in adipose responses to HFD. Visceral adipose deposits from HFD-fed NOX2KO mice were smaller, with less hypertrophy and macrophage infiltration, compared with WT mice given HFD. These data also show that NOX2 deletion attenuates the development of ER stress in adipose tissues and also preserves the expression of mature adipocyte markers, suggesting that sustained expression/activity of NOX2 leads to an overall destabilization of adipocyte physiology through ER stress. These data are in agreement with existing literature on the role of free radicals in general, and NADPH oxidase specifically, in adipocyte physiology. For example, many studies have established that HFDs increase adipose NADPH oxidase (Coate and Huggins, 2010; Furukawa et al., 2004; Matsuzawa-Nagata et al., 2008) and that NADPH oxidase regulates adipocyte chemokine expression (Furukawa et al., 2004; Han et al., 2012), which could explain our observations of less macrophage infiltration into NOX2-deficient adipose. Data also support an important role for NADPH oxidase in long-term adipocyte responses to insulin, such as proliferation and differentiation (Schroder et al., 2009); however, this paper is the first to provide evidence for a specific role for NOX2 in these pathways. The human genome contains five NOX members, NOX1 through NOX5 (Geiszt and Leto, 2004; Lambeth, 2004). And while NOX2 expression is found in white adipose deposits in both rodents (Sakurai et

al., 2009) and humans (Catalan et al., 2011), NOX2 expression in differentiated 3T3-L1 adipocytes appears to be quite low (Han et al., 2012), raising questions as to which adipose-resident cells express NOX2. Indeed, cell culture data indicate that many of the free radical-based pathways involved in adipocyte insulin sensitivity and proliferation may be mediated by NOX4 rather than NOX2 (Mahadev et al., 2004; Schroder et al., 2009). However, in contrast to the protective effects of NOX2 deletion reported here, NOX4-deficient mice have recently been shown to have enhanced susceptibility to diet-induced obesity, with accelerated insulin resistance, enhanced adipocyte hypertrophy, and increased adipose tissue hypoxia, inflammation and apoptosis (Li et al., 2012). These findings thus suggest differential roles for the NADPH oxidase subunits in adipose tissue, with NOX4 involved in maintaining physiological events such as insulin receptor signaling and adipocyte proliferation/differentiation, and NOX2 driving sustained inflammatory changes in response to stimuli like fatty acids or oxidized lipoproteins.

These data also suggest that NOX2 might play a particularly deleterious role specifically in visceral adipose. Subcutaneous and visceral adipocytes derive from different progenitor cells that exhibit a different gene expression pattern and thus may respond quite differently to the effects of diet or NADPH oxidase inhibition. Indeed, visceral fat tissue is strongly associated with insulin resistance, diabetes mellitus, dyslipidemia, hypertension, atherosclerosis, hepatic steatosis, and overall mortality, whereas subcutaneous fat seems to have intrinsic beneficial metabolic properties (Gil et al., 2011). While the reasons for this are not yet clear, several studies have documented that visceral adipose is distinguished by increased inflammation. For example, the

number of macrophages has been estimated at two- to four-fold higher in visceral compared with subcutaneous fat irrespective of adiposity levels (Harman-Boehm et al., 2007); likewise, the expression of proinflammatory cytokines is elevated in visceral compared with subcutaneous fat (Atzmon et al., 2002). This relatively enhanced level of inflammation in the visceral fat has been repeatedly and directly linked to obesity-related insulin resistance and type 2 diabetes (Hotamisligil and Erbay, 2008; Shoelson et al., 2006). For example, the cytokine TNFα has been demonstrated to mediate obesity-induced insulin resistance (Hotamisligil, 1999), while the chemokine monocyte chemotactic protein-1 (MCP-1) has also been shown to impair adipocyte insulin sensitivity (Sartipy and Loskutoff, 2003). Increased proinflammatory cytokines can induce insulin resistance by several mechanisms, including via suppression of cytokine signaling-3 (SOCS3) expression (Emanuelli et al., 2001) and/or the activation of numerous intracellular serine kinases such as c-Jun $NH_2$-terminal kinase (JNK) and inhibition of κB kinase (IKK) (Hirosumi et al., 2002). Finally, it is important to note that data from our laboratories and others' have firmly established the critical role that NADPH oxidase plays in directing macrophage inflammation, particularly the release of proinflammatory cytokines including TNFα, IL-6, and IL-1β (Clark and Valente, 2004; Robertson et al., 1990; Turchan-Cholewo et al., 2009). Thus, the data in this paper raise the possibility that inhibition of NOX2 just within visceral adipose may be sufficient to prevent the pattern of macrophage accumulation and inflammation that precipitates metabolic and neurological decline. This finding is both highly clinically significant and easily translated into new therapies.

While obesity is known to predispose individuals to a myriad of diseases (Haslam and James, 2005), the brain may be one of the more critical sites in light of the increasing costs associated with cognitive impairment and dementia. These data indicate that NOX2 may be a key aspect of diet-induced brain injury, in agreement with a large body of literature supporting a role for NADPH oxidase in neurodegenerative pathways (Block, 2008; Lambeth, 2007; Shimohama et al., 2000). For example, the same NOX2KO mice are protected from postischemic neuroinflammation and inflammatory cytokine-mediated brain damage (Chen et al., 2011) and also from microglia-mediated injury in mouse models of traumatic brain injury (Dohi et al., 2010), indicating that NOX2 mediates neurotoxic brain inflammation. In further support of this potential mechanism, data from our laboratories and others' have established the critical role that NOX2 plays in the release of proinflammatory cytokines including TNFα, IL-6, and IL-1β (Clark and Valente, 2004; Robertson et al., 1990; Turchan-Cholewo et al., 2009), all of which could underlie neurological impairment (Akiyama et al., 2000; Arvin et al., 1996; Lane et al., 1996; Parnet et al., 2002; Tyor et al., 1992). Although cytokine expression was not documented in this study, data show that HFD-induced reactive gliosis was prevented in NOX2KO mice, and previous studies from our laboratory show a tight association of reactive gliosis with both CNS cytokine release and cognitive impairment following HFD (Pistell et al., 2010). Interestingly, however, NADPH oxidase also participates in physiological events including neuronal signaling and memory formation (Kishida and Klann, 2007; Kishida et al., 2005; Tejada-Simon et al., 2005). Cognitive function was not measured in this study, since both humans and mice with similar NOX2 mutations have been shown to have some degree of cognitive dysfunction

(Kishida et al., 2006; Pao et al., 2004). In this regard, the lack of significant differences in basal expression of synaptic markers suggests that cognitive deficits associated with NOX2KO likely reflect alterations in synaptic signaling rather than decreases in synaptic number, which is in keeping with current thoughts on the role of NADPH oxidase in cognition (Ali et al., 2011; Knapp and Klann, 2002). Collectively, these data raise the possibility that neurological function would be best preserved if the proinflammatory, detrimental consequences of glial NOX activation could be inhibited while preserving physiological neuronal NOX signaling. Indeed, genetically engineered mice with LoxP sites flanking the NOX2 gene are currently under development, and these mice may significantly advance understating of the complex and nuanced role of NOX2 in the brain. It should also be pointed out that commercially available NOX2KO mice have been available as homozygous breeders for many years, raising the possibility of subtle genetic drift between commercially available NOX2KO mice and the parental C57Bl/6 strain, which could potentially contribute to reported differences between strains. Indeed, it is possible that genetic drift and/or compensatory changes in the expression of other NADPH oxidase subunits could be partially responsible for the alterations in basal claudin-5 and occludin expression noted in NOX2KO mice (see Table 2). The use of newly developed, tissue-specific NOX2 knockout mice could thus also circumvent any artifacts introduced by subtle genetic drift and/or compensatory changes in the NOX2KO and C57Bl/6 strains of mice.

One key site whereby aberrant and/or sustained NADPH oxidase activation could undermine brain function is in the cerebrovascular compartment. Indeed, data in this paper reveal that HFD-induced perturbations to cerebrovascular integrity (lower tight

junction protein/higher MMP expression) are blunted in NOX2KO mice. While direct cerebrovascular function was not measured in this study, numerous reports have confirmed that alterations in these markers faithfully reflect physiological impairments in BBB function (Liu et al., 2011; McColl et al., 2010). In addition to loss of BBB integrity, there is increasing evidence that hypoperfusion and/or loss of neurovascular coupling may be a key pathway whereby oxidative stress connects vascular-related diseases to cognitive impairment (Kim et al., 2012; Liu and Zhang, 2012). Indeed, evidence suggests that diabetes-induced cognitive decline may be induced via disruption of neurovascular coupling, with physiological impairment arising from both cerebrovascular elements and also glial cells (Mogi and Horiuchi, 2011; Serlin et al., 2011). In this light, it is important to note that NADPH oxidase is a major player in both endothelial and glial physiology. Overall, therefore, these data collectively support the hypothesis that excessive or sustained NADPH oxidase activation could disrupt brain homeostasis through any of several different cerebrovascular mechanisms, raising the possibility that targeted NOX2 inhibition could be a viable therapeutic strategy to preserve neurological function in the context of pathogenic obesity.

While NOX2KO mice were protected from many of the metabolic and neurological effects of HFD, it is clear that both strains of mice became obese in response to HFD. Thus, these data reiterate that obesity per se is not sufficient to precipitate metabolic or neurological decline. This scenario is also reflected in clinical settings, but it remains unclear why increased adiposity appears to cause disease in some people but not in others (Bays et al., 2006). Recently devised theories posit that obesity in susceptible individuals is uniquely associated with pathological dysfunction in

adipocytes (sick fat or adiposopathy) and that these abnormalities in fat function, rather than increases in fat mass, precipitate physiological decline (Appachi et al., 2011; Bays et al., 2006; Bays et al., 2008). Adiposopathy is generally defined anatomically by adipocyte hypertrophy and physiologically by impaired fatty acid regulation (elevated release, particularly under high-glucose conditions), disrupted adipokine secretion, and increased inflammation (Appachi et al., 2011; Bays, 2005; Bays, 2011; Heilbronn et al., 2004). Data in this paper suggest that NOX2 may mediate, at least in part, the pathological processes of adiposopathy. As obesity remains increasingly prevalent and seemingly resistant to clinical remediation (Padwal et al., 2004), these data suggest that new therapies to preserve health in the presence of obesity could be based on manipulation of NOX2.

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# Chapter 3. Deletion of Macrophage NOX2 Preserves Metabolic and Neurologic Health in Diet-Induced Obese Mice

## 3.1 Introduction

High fat diets and diet-induced obesity are major drivers of metabolic syndrome, which is associated with dramatically enhanced risk for many diseases including type 2 diabetes, cardiovascular disease, stroke, and cancer (reviewed in (Haslam and James, 2005)). While the pathogenesis of metabolic syndrome is not fully understood, obesity is closely associated with a pattern of chronic inflammation in adipose tissue (Shoelson et al., 2007), from which cytokines, acute-phase reactants, and other inflammatory mediators originate to elicit systemic inflammation (Berg and Scherer, 2005; Chandalia and Abate, 2007; Hotamisligil, 2006). For example, infiltration of macrophages into adipose tissue is well established following high-fat feeding (Weisberg et al., 2003; Apovian et al., 2008), and has been linked to increased inflammation as well as dyslipidemia and insulin resistance (Apovian et al., 2008). Activation of systemic inflammation is also associated with impaired brain function (Dziedzic, 2006; Trollor et al., 2012), and indeed, diet-induced obesity has also been linked to brain pathology, cognitive dysfunction, and Alzheimer's disease (reviewed in (Olufadi and Byrne, 2008)). For example, data show impaired learning, memory, and executive function in obese as compared to nonobese patients (Elias et al., 2003; Elias et al., 2005; Waldstein and Katzel, 2006), and regression studies suggest that increased body weight might decrease brain volume (Ward et al., 2005) or disrupt the balance of white to grey matter, particularly in the frontal lobe (Pannacciulli et al., 2006).

While the molecular link(s) between excess adiposity and inflammation has not yet been identified, recent reports have implicated activation of the proinflammatory

enzyme NADPH oxidase in the detrimental effects of diet-induced obesity. NADPH oxidase is a superoxide-producing complex consisting of membrane (NOX2/gp91phox and p22phox) and cytosolic (p47phox, p67phox, and p40phox) components that assemble at the plasma membrane, where NOX2 transfers electrons from NADPH to molecular oxygen, producing a burst of superoxide (Babior, 1991; DeLeo and Quinn, 1996). Studies show that NADPH oxidase activity/expression is increased in laboratory animals given high fat diet (Bruce-Keller et al., 2010; Coate and Huggins, 2010; Zhang et al., 2005; Furukawa et al., 2004; Matsuzawa-Nagata et al., 2008), and further indicate that diet-induced increases in NADPH oxidase mediate obesity-related proinflammatory cytokine and chemokine release as well as insulin resistance, hyperlipidemia, and liver steatosis (Furukawa et al., 2004; Han et al., 2012). Finally, mice deficient in NOX2 have been shown to be resistant to the detrimental effects of high fat diets, with attenuated adipose pathology and inflammation, and preserved adipose function, glucose tolerance, and cerebral homeostasis (Pepping et al., 2013).

While existing data support a role for NADPH oxidase in obesity-related inflammation, NADPH oxidase-based signaling pathways also play important roles in physiologic processes (Infanger et al., 2006; Sorce and Krause, 2009). Indeed, well-established effects of systemic NOX2 deletion on cognitive function (Infanger et al., 2006; Sorce and Krause, 2009) rule out broad-based NADPH oxidase inhibition as a viable therapeutic approach in obesity. Furthermore, recent expansion of the genome databases has identified several homologues of NOX2 (NOX1-5, DUOX1-2) that differ in their activation requirements and mediate diverse and pleiotropic actions (Lambeth, 2004; Geiszt and Leto, 2004). Cell culture and animal data indicate that adipocyte

function and insulin sensitivity requires NOX4 activity (Mahadev et al., 2004; Schröder et al., 2009; Li et al., 2012), suggesting opposing roles for NOX2 and NOX4 in obesity pathogenesis. To reveal the therapeutic potential of targeted NADPH oxidase inhibition, we generated mice with a floxed allele of the NAPDH oxidase subunit NOX2 and crossed these with mice expressing LysM-Cre to generate mice lacking NOX2 in all myeloid lineage cells, including monocytes, macrophages, and microglia. To reveal the role of macrophage-specific NADPH oxidase in the pathophysiology of obesity, a C57Bl/6 wild type (WT-FL) and macrophage-deficient NOX2 (macNOX2KO) mice were given high fat diet for 16 weeks, and subject to comprehensive metabolic, behavioral, and biochemical analyses.

**3.2 Materials and Methods**

Transgenic Animal Generation and Treatment

The Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center approved all experimental protocols, which were compliant with NIH guidelines on the use of experimental animals. Novel transgenic mice with LoxP sequences on either side of exon 5 of the NOX2 gene (*cybb*) were generated on a C57Bl/6 background using standard procedures. Mice homozygous for floxed NOX2 (WT-FL) were generated by serial crossings, and the presence of the LoxP sites was confirmed by genomic sequencing (data not shown). Female WT-FL were then crossed with male LysM-Cre transgenic mice purchased from Jackson Laboratory (Bar Harbor, ME, USA), to generate male F1 progeny lacking NOX2 specifically in myeloid lineage cells (macNOX2KO). Male macNOX2KO and WT-FL mice were then used for experimentation, and housed in standard caging with a 12:12 light:dark cycle and ad

libitum access to food and water. WT-FL and macNOX2KO mice were separated into groups given either high fat diet (HFD- 60% fat) or low fat control diet (CD- 10% fat) for 16 weeks. Both diets are open source, were purchased from Research Diets (New Brunswick, NJ; HFD: D12492; CD: D12450B), and were provided in pelleted form. Data were compiled from 2 separate experiments, each composed of both WT-FL and macNOX2KO mice given CD or HFD, with 18-20 total animals in each group.

Body weight, food intake, and body composition (measured using a Bruker minispec LF90 time domain Nuclear Magnetic Resonance (NMR) analyzer, Bruker Optics, Billerica MA) were measured 2 times a month. Fasting blood glucose was measured in tail blood using a glucometer (Ascensia Elite, Bayer, Mishawaka, IN). All mice were humanely euthanatized via isoflurane inhalation and cardiac exsanguination after a brief (6 hr) fast, and blood, brain (anterior 1/3 of cerebral cortex), visceral (epididymal), and subcutaneous (inguinal) adipose tissue depots were collected.

Clinical Chemistry

Whole blood was collected by cardiac exsanguination of terminally anesthetized mice, and was allowed to clot at 4ºC overnight and then centrifuged at 3000xg for 30 minutes. Serum was collected and either analyzed immediately or aliquoted and stored at -80ºC. Levels of total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and nonesterified fatty acids (NEFA) in sera were measured colorimetrically using commercially available kits (Wako Chemicals, Richmond, VA). Insulin levels were evaluated by ELISA in accordance with the manufacturer's assay protocol (Crystal Chem Inc., Downers Grove IL).

Histological and biochemical analyses of adipose tissue

The inguinal and epididymal adipose depots were collected for histological and biochemical analyses. For histology, the tissues were immersion-fixed in 10% neutral buffered formalin for 2-3 days, after which they were processed for paraffin embedding. Sections (5 μm) were then cut, stained with hematoxylin and eosin, and digitized. Adipocyte size was measured by an investigator blinded to the experimental grouping in 40X microscope fields by counting the total number of adipocytes within predefined area grids, and then dividing the area by the total number of adipocytes within the grids to calculate average adipocyte size. For each sample, 3 tissue sections were analyzed, with 3 fields counted in each section, for an average of 9 fields per sample. For immunohistochemical analyses of macrophage crowns in adipose tissues, tissue sections were processed using anti-Iba-1 (1:100, Wako Chemicals, Richmond, VA). Sections were incubated with biotinylated-linked secondary antibodies, and then visualized using diaminobenzidine (Vector Laboratories, Burlingame, CA). For Western blot, adipose tissue samples were homogenized in RIPA buffer (G biosciences, St. Louis, MO), and then cleared by centrifugation at 5000 x g for 10 minutes at 40C. Samples were denatured in SDS, and equivalent amounts of protein were electrophoretically separated in polyacrylamide gels and blotted onto nitrocellulose. Blots prepared from adipose tissue were processed using anti-GADD153/CHOP (1:5000, Abcam PLC), anti-GRP78 (1:500, Novus Biologicals LLC), and anti-total STAT5 (1:1000, Abcam PLC). After incubation with primary antibodies, blots were washed and exposed to horseradish peroxidase-conjugated secondary antibodies, and visualized using a chemiluminescence system (Amersham Biosciences, Pittsburgh,

PA). Blot images were scanned and densitometrically analyzed for quantification. To ensure accurate quantification across multiple blots, samples from all groups (HFD and CD in both WT-FL and macNOX2KO) were included in each individual blot. Data were calculated as a ratio of expression over tubulin expression, which was included as an internal loading control. Protein expression in HFD mice was then calculated and presented as percent expression relative to CD mice of the same genotype.

Fear conditioning memory task

Each mouse was individually evaluated for fear conditioning using an automated, video-based fear conditioning system (Med-Associates, St. Albans, VT) as described previously (Bruce-Keller et al., 2015; Pepping et al., 2014). The apparatus consists of a "startle chamber" used on days 1 and 2, which is an 8×15×15-cm acrylic and wire mesh cage located within a custom designed 90×70×70 ventilated sound-attenuating chamber, and the unique context is reinforced with an anise-based scent applied to each cage before testing. Animal movement within the apparatus results in displacement of an accelerometer (model U321AO2; PCB Piezotronics, Depew, NY, USA). Acquisition of fear conditioning on day 1 consists of 5 minutes acclimation to the startle chamber, followed by five consecutive 30 second auditory stimuli (85 db, 4 KHz) co-terminating with a mild footshock (0.5 mA × 1 sec), with 30 second recovery periods between tones. On day 2, mice return to the same chambers, but no stimuli are applied to evaluate freezing responses to context. On day 3, mice are placed in an entirely separate chamber located in a different room to remove all contextual cues, and after 5 minute habituation, a continuous tone (85 db, 4 KHz) is applied for 5 minutes. Freezing behavior is recorded as a measure of memory of the conditioned response to the tone.

Statistical analyses

All data are shown as mean ± standard error of measurement. Body weight and composition data, adipocyte size, and all metabolic data were all analyzed with 2-way analyses of variance (ANOVA), followed by planned Bonferroni post-tests to determine the effects of HFD in both WT-FL and macNOX2KO mice. Additional planned comparisons of WT-FL and macNOX2KO mice under both CD and HFD conditions were carried out to determine the effects of genotype under both diet conditions. Significant effects of diet are indicated with "*" (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$), while significant effects of genotype are indicated with "#" (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Protein expression values generated by Western blot (ratios of expression over tubulin) were normalized to percent CD for each genotype to reconcile data from multiple blots, and were analyzed by 2-tailed, unpaired t-tests to determine if statistically significant differences exist between HFD and CD groups within each genotype.

**3.3 Results**

Generation and characterization of macNOX2KO mice

To determine the therapeutic potential of cell type-specific NOX2 deletion in diet-induced obesity, newly engineered mice in which LoxP sequences were placed on either side of exon 5 of the NOX2 gene were generated (WT-FL). WT-FL mice were bred to homozygosity, and female WT-FL mice were mated with male LysM-Cre transgenic mice to generate male mice lacking NOX2 in all myeloid lineage cells (macNOX2KO). Male progeny from WT-FL crosses were used as controls, and there

were no differences in pregnancy rates, litter sizes, or sex balance of offspring between WT-FLxWT-FL and WT-FLxLysMCre breeding pairs (data not shown).

Effects of HFD on body weight and composition in WT-FL and macNOX2KO mice

4-month-old male WT-FL and macNOX2KO mice were fed either high fat diet (HFD) or low fat control diet (CD) for 16 weeks as described in Materials and Methods. During this time, body weights progressively diverged such that HFD-fed mice weighed significantly more than CD mice after 4 weeks (Fig 3.1-A). Statistically, ANOVA for genotype X diet at the end of exposure revealed a significant main effect of diet on body weight ($F_{(1,68)}$ = 104.3, $p$ < 0.0001). The effect of genotype on body weight was also significant ($F_{(1,68)}$ = 20.02, $p$ = 0. 0001), but the interaction was not. Planned comparisons of macNOX2KO and WT-FL mice revealed that while there were no significant differences between body weights in mice given CD, HFD-fed macNOX2KO mice weighed significantly less than HFD-fed WT-FL mice after 4 weeks of diet consumption (Fig 3.1-A). In addition to body weight, total body fat was measured using NMR as described in Materials and Methods. Similar to what was observed for body weight, total body fat (expressed as % total body weight) was higher in all mice over the 16-week feeding trial, but mice given HFD had significantly more fat by 4 weeks of diet consumption (Fig 3.1-B). Statistically, ANOVA to measure the effects of genotype and diet on % body fat at the end of diet exposure revealed a significant main effect of diet ($F_{(1,68)}$ = 52.34, $p$ < 0.0001), but no effect genotype and no significant interaction between diet and genotype on body fat. Planned comparisons of macNOX2KO and WT-FL mice revealed no genotype-based differences in % body fat in mice given CD, but did reveal a lower body fat percentage in macNOX2KO as compared to WT-FL mice

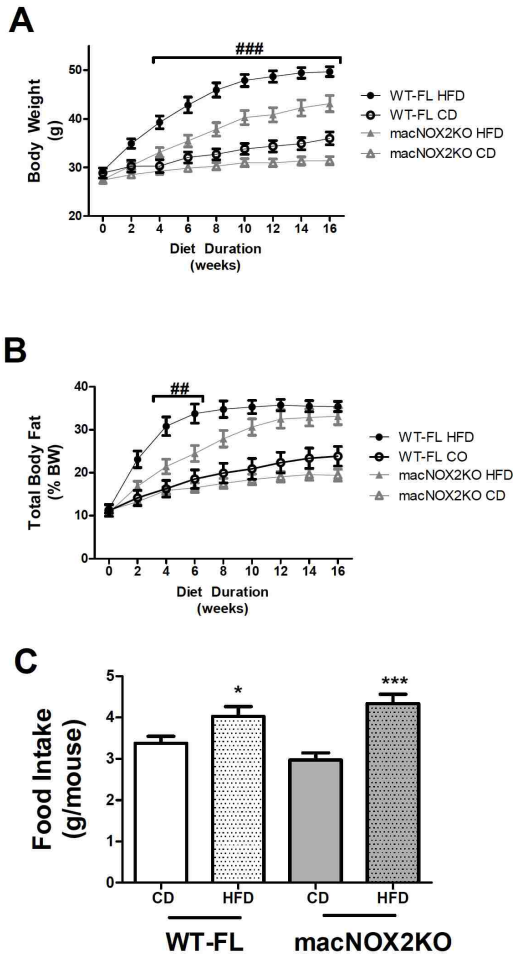


Figure 3.1: Effects of high-fat diet (HFD) on body weight and composition in wild-type mice with floxed exon 5 of NADPH oxidase subunit 2 (WT-FL) and macrophage-deficient NADPH oxidase subunit 2 knockout (macNOX2KO) mice. Four-month-old male WT-FL mice and macNOX2KO mice were placed for 16 wk on HFD or the nutritionally matched low-fat control diet (CD) with 18-20 mice in each group. Significant effects of diet are indicated with "*" (*$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001), while significant effects of genotype are indicated with "#" (#$p$ < 0.05, ##$p$ < 0.01, and ###$p$ < 0.001). Figure 3.1-A: Body weight in WT-FL and macNOX2KO mice over time following administration of CD or HFD. Significantly (###$p$ <0.001) lower body weight noted in macNOX2KO mice vs. WT-FL mice on HFD after 4 wk. Figure 3.1-B: Body fat as %total body weight in WT-FL and macNOX2KO mice over time following administration of CD or HFD. Significantly (##$p$ < 0.01) lower body fat in macNOX2KO mice vs. WT-FL mice on HFD after 4 and 6 wk. Figure 3.1-C: Average food intake per mouse for WT-FL and macNOX2KO mice following administration of CD or HFD. Significantly (*$p$ < 0.05) higher food intake for WT-FL mice on HFD vs. CD. Significantly (***$p$ < 0.001) higher food intake for macNOX2KO mice on HFD vs. CD.

4 and 6 weeks of HFD (Fig 3.1-B). Analysis of food intake during this period was also conducted, and while data show that both strains of mice ate more HFD as compared to CD, there were no differences between WT-FL and macNOX2KO mice with regards to diet (CD or HFD) ingestion (Fig 3.1-C), indicating that NOX2 is not involved in feeding behavior and that the increased body weight in WT-FL mice was not caused by increased food intake.

Diet-induced adipocyte inflammation, hypertrophy, and injury in WT-FL and macNOX2KO mice

Accumulation of macrophages in adipose tissues elicits inflammation, which leads to both local and systemic insulin resistance and metabolic impairment (Weisberg et al., 2003; Apovian et al., 2008). As our recent data suggest that NOX2-positive macrophages might mediate obesity-induced inflammatory and pathogenic changes in adipose tissue, we next investigated the impact of conditional NOX2 deletion on macrophage infiltration into visceral adipose tissue by measuring expression of Iba-1, a calcium binding protein specifically expressed in macrophages that is unregulated with activation (Hilton et al., 2008; Lee et al., 2008; Zecca et al., 2008) and can be used in immunohistological analyses in paraffin-embedded tissues (Ahmed et al., 2007; Vega-Avelaira et al., 2007). Blinded quantification of Iba-1-positive macrophages in crown-like structures (CLSs) within epididymal adipose depots was markedly increased following HFD, particularly in WT-FL mice (Fig 3.2-A). Specifically, ANOVA for the effects of genotype and diet on epididymal CLS revealed a significant main effect of diet ($F_{(1,66)}$ = 50.11, $p < 0.0001$) and of genotype ($F_{(1,66)}$ = 16.20, $p < 0.0001$), with a significant interaction ($F_{(1,66)}$ = 11.02, $p = 0.0015$). Planned comparisons revealed no differences in

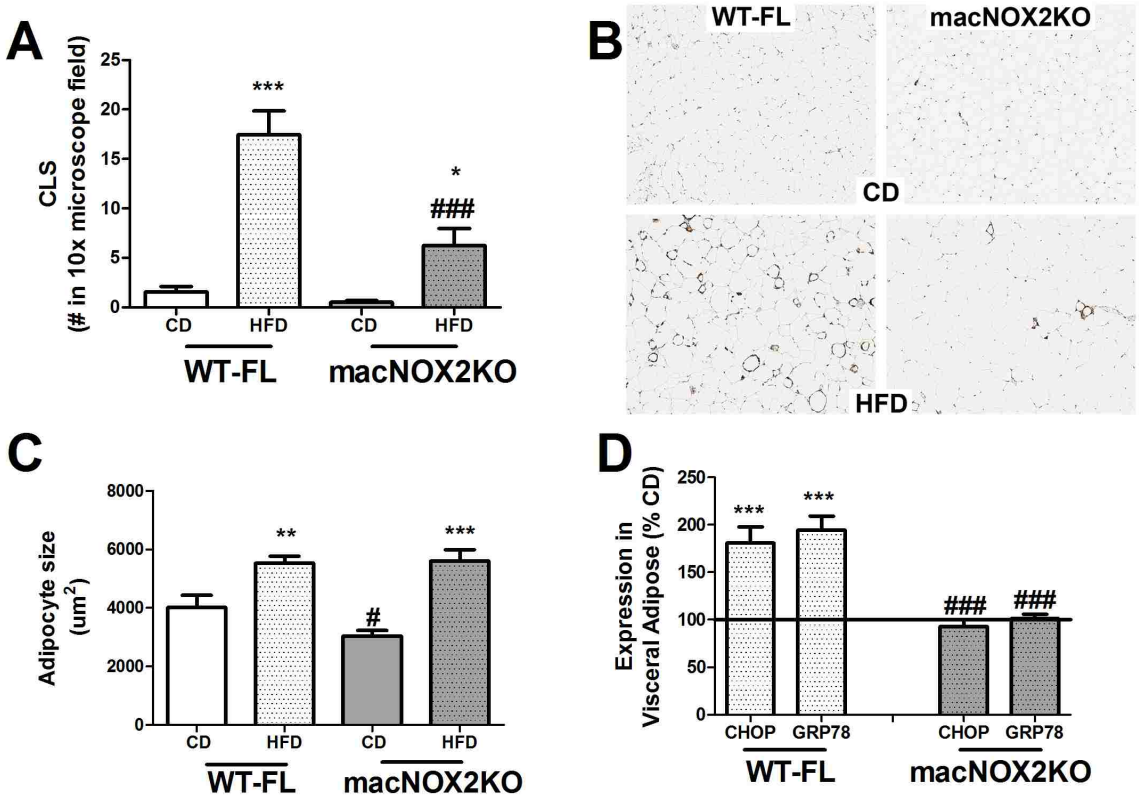


Figure 3.2: Effects of HFD on adipocyte inflammation, hypertrophy, and injury in WT-FL and macNOX2KO mice. Visceral epididymal fat pads were collected from WT-FL and macNOX2KO mice at the end of the 16-wk feeding trial and processed for analyses. Significant effects of diet are indicated with "*" (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$), while significant effects of genotype are indicated with "#" (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Figure 3.2-A: Number of Iba-1 positive macrophages in crown-like structures (CLS) in WT-FL and macNOX2KO mice after administration of CD or HFD. Significantly (***$p < 0.001$) higher number of CLS in WT-FL mice on HFD vs. CD. Significantly (*$p < 0.05$) higher number of CLS in macNOX2KO mice on HFD vs. CD. Significantly (###$p < 0.001$) lower number of CLS in macNOX2KO mice on HFD vs. WT-FL mice on HFD. Figure 3.2-B: Representative images of Iba-1 stained adipocytes used to determine number of CLS and adipocyte size. Figure 3.2-C: Size of visceral adipocytes in WT-FL mice and macNOX2KO mice after administration of CD or HFD. Significantly (**$p < 0.01$) larger adipocyte size in WT-FL mice on HFD vs. CD. Significantly (***$p < 0.001$) larger adipocyte size in macNOX2KO mice on HFD vs. CD. Significantly (#$p < 0.05$) smaller adipocyte size in macNOX2KO mice on CD vs. WT-FL mice on CD. Figure 3.2-D: Effects of HFD on markers of adipocyte injury in WT-FL and macNOX2KO mice. Significantly (***$p < 0.001$) higher levels of expression of CHOP and GRP78 in WT-FL mice on HFD vs. CD. Significantly (###$p < 0.001$) lower levels of expression of CHOP and GRP78 in macNOX2KO mice vs. WT-FL mice.

the epididymal CLS number in CD-fed macNOX2KO compared to CD-fed WT-FL mice, but did show that HFD significantly increased the numbers of CLS in both WT-FL and macNOX2KO mice (Fig 3.2-A). However, the number of CLS in HFD-fed macNOX2KO mice was significantly less that in HFD-fed WT-FL mice (Fig 3.2-A). Representative images of Iba-1 stained tissue sections reveal obviously increased Iba-1 positive CLS in visceral epididymal adipose depots isolated from HFD-fed WT-FL mice, but not from HFD-fed macNOX2KO mice or mice given CD (Fig 3.2-B).

As obesity and metabolic dysfunction are frequently associated with adipocyte enlargement or hypertrophy (Bays et al., 2008; Heilbronn et al., 2004), the size of individual adipocytes within visceral epididymal adipose depots was evaluated in tissue sections as described in Materials and Methods. Such measures revealed that HFD-fed mice had significantly larger visceral adipocytes (Fig 3.2-C). Specifically, ANOVA for the effects of genotype and diet on epididymal adipocyte size revealed a significant main effect of diet ($F_{(1,66)} = 38.61$, $p < 0.0001$), but no effect of genotype and no interaction. Planned comparisons revealed that epididymal adipocytes in CD-fed macNOX2KO mice were significantly smaller than adipocytes in CD-fed WT-FL (Fig 3.2-C). However, HFD consumption resulted in larger visceral adipocytes similarly in both WT-FL and macNOX2KO mice (Fig 3.2-C). To determine if the hypertrophy and inflammation noted in visceral adipose was accompanied by derangement in adipocyte physiology, markers of adipocyte injury in visceral adipose of WT-FL and macNOX2KO mice were examined by Western blot. Specifically, adipocyte injury was estimated by evaluating the expression GADD153/CHOP and GRP78, both of which are known to be elevated in the context of obesity and thought to reflect endoplasmic reticulum (ER) stress in

adipocytes caused by excessive inflammation (Ozcan et al., 2004; Oyadomari and Mori, 2004). Evaluation and statistical analysis of GADD153/CHOP blots revealed that HFD-fed macNOX2KO mice had significantly lower levels of GADD153 expression ($t_{(31)}$ = 4.55, $p < 0.0001$) as compared to WT-FL mice (Fig 3.2-D). Likewise, expression of GRP78 was also lower in HFD-fed macNOX2KO mice ($t_{(27)}$ = 6.70, $p < 0.0001$; Fig 3.2-D), than in WT-FL mice (Fig 3.2-D), suggesting that although adipocytes in macNOX2KO mice became hypertrophic, these cells did not appear to be subject to ER stress.

Diet-induced metabolic dysfunction in WT-FL and macNOX2KO mice

To determine the extent of HFD-induced metabolic syndrome in WT-FL and macNOX2KO mice, data were collected, and for presentation purposes are thematically divided into syndromes separately describing insulin resistance and hyperlipidemia. To document insulin sensitivity and glycemic control, studies focused on regulation of fasting glucose and glucose tolerance. At the end of the 16-week diet regimen, mice were fasted and blood glucose and serum insulin were measured as previously described. 2-way ANOVA on the effects of genotype and diet on fasting blood glucose revealed significant main effects of diet ($F_{(1,35)}$ = 14.44, $p$ = 0.0014) and genotype ($F_{(1,35)}$ = 35.67, $p < 0.0001$), with a statistically significant interaction between diet and genotype ($F_{(1,35)}$ = 6.72, $p$ = 0.0239; Fig 3.3-A). Post-hoc tests showed that HFD increased glucose levels in WT-FL mice but not in macNOX2KO mice, while planned comparisons revealed that glucose levels in HFD-fed WT-FL mice were significantly higher than levels in HFD-fed macNOX2KO mice (Fig 3.3-A). Conversely, 2-way ANOVA on the effects of genotype and diet on fasting insulin revealed a significant

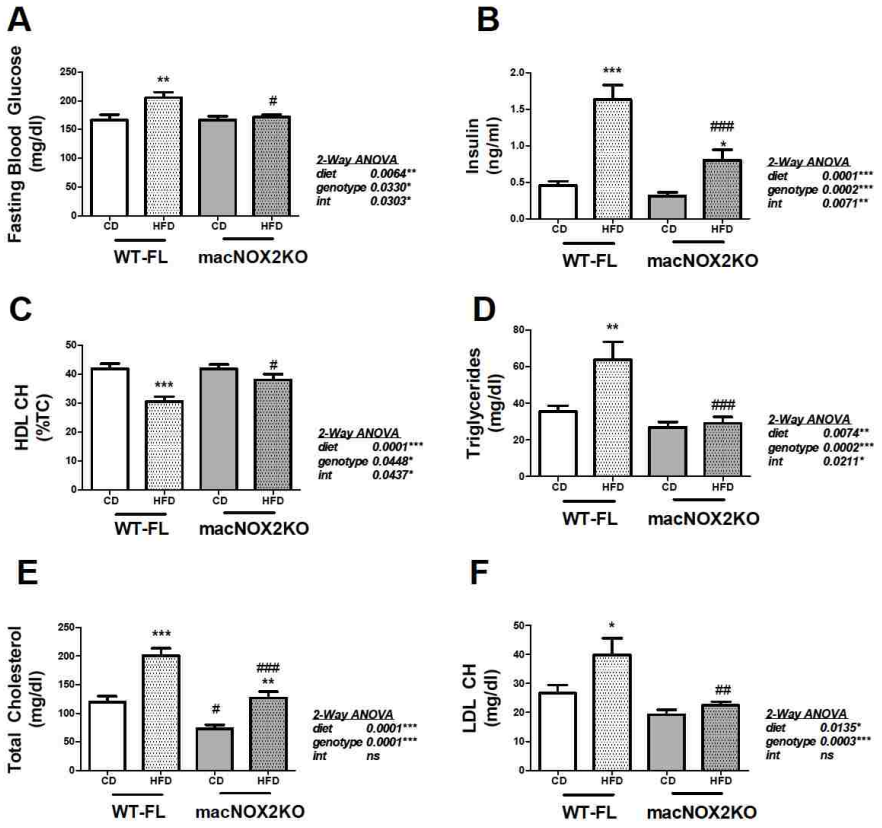


Figure 3.3: Effects of HFD on metabolic parameters in WT-FL and macNOX2KO mice. At the end of the 16-wk feeding trial mice were fasted and blood glucose, serum insulin, and serum lipids were measured. Significant effects of diet are indicated with "*" (*$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001), while significant effects of genotype are indicated with "#" (#$p$ < 0.05, ##$p$ < 0.01, and ###$p$ < 0.001). Figure 3.3-A: Fasting blood glucose levels. Significant (**$p$ < 0.01) increase in fasting blood glucose in WT-FL mice on HFD vs. CD. Significant (#$p$ < 0.05) decrease in fasting blood glucose in macNOX2KO mice vs. WT-FL mice on HFD. Figure 3.3-B: Fasting insulin levels. Significant (***$p$ < 0.001 and *$p$ < 0.05, respectively) increases in fasting insulin in WT-FL mice on HFD and macNOX2KO mice on HFD. Significant (###$p$ < 0.001) decrease in fasting insulin in macNOX2KO mice vs. WT-FL mice on HFD. Figure 3.3-C: HDL cholesterol levels. Significantly (***$p$ < 0.001) lower HDL in WT-FL mice on HFD vs. CD. Significant (#$p$ < 0.05) increase in HDL in macNOX2KO mice vs. WT-FL on HFD. Figure 3.3-D: Triglyceride levels. Significant (**$p$ < 0.01) increase in triglycerides in WT-FL on HFD vs. CD. Significantly (###$p$ < 0.001) lower triglycerides in macNOX2KO mice vs. WT-FL on HFD. Figure 3.3-E: Total cholesterol levels measured in WT-FL and macNOX2KO mice after administration of HFD or CD. Significant (***$p$ < 0.001 and **$p$ < 0.01, respectively) increases in total cholesterol in WT-FL mice on HFD and macNOX2KO on HFD. Significantly (#$p$ < 0.05) lower total cholesterol in macNOX2KO mice vs. WT-FL mice on CD. Significantly (###$p$ < 0.001) lower total cholesterol in macNOX2KO mice vs. WT-FL mice on HFD. Figure 3.3-F: LDL cholesterol levels. Significant (*$p$ < 0.05) increase in LDL in WT-FL mice on HFD. Significantly (##$p$ < 0.01) lower LDL in macNOX2KO mice vs. WT-FL mice on HFD.

effect of diet ($F_{(1,35)}$ = 14.44, $p$ = 0.0014), but no effect of genotype and no interaction (Fig 3.3-B). Studies next assessed a panel of bioactive serum lipids in WT-FL and macNOX2KO mice, measured under fasted conditions as described in Materials and Methods. Data showed a significant effect of diet ($F_{(1,34)}$ = 44.49, $p$ < 0.0001) on total cholesterol, but no effect of genotype, no interaction, and no significant differences between WT-FL and macNOX2KO mice (Fig 3.3-C). Likewise, there were significant effects of diet on LDL cholesterol ($F_{(1,34)}$ = 26.17, $p$ = 0.0013) and on HDL cholesterol ($F_{(1,34)}$ = 30.13, $p$ = 0.0004), but no effects of genotype and no interactions. Additionally, posthoc tests showed that HFD increased LDL and HDL cholesterol species in WT-FL, but not macNOX2KO mice (Fig 3.3-D & Fig 3.3-E). Finally, there were no differences in levels of fasting triglycerides or NEFA in either WT-FL or macNOX2KO mice given either CD or HFD (Fig 3.3-F).

Effects of HFD on cognitive function and brain injury in WT-FL and macNOX2KO mice

We have previously shown that HFD administration causes significant impairments in memory performance in mice (Pepping et al., 2013; Freeman et al., 2013). To determine if conditional deletion of NOX2 from macrophages can preserve cognitive function, CD- and HFD-fed WT-FL and macNOX2KO mice were evaluated using the fear conditioning assay as described in Methods. No significant differences in behavioral responses across groups were observed on day 2 (data not shown), indicating that all mice retained the basic memory of the conditioned context. However, differences in freezing behavior were observed on the third day of the fear conditioning test, when the ‘‘tone test” conducted in an entirely novel environment provides a measure of associative learning.

Specifically, freezing behavior in response to the tone was more in HFD-fed macNOX2KO mice as compared to HFD-fed WT-FL mice (Fig 3.4), suggesting improved memory of the tone cue.

### 3.4 Discussion

Data in this manuscript strongly support NOX2-based signaling in macrophages as being a key contributor to the detrimental effects of diet-induced obesity. In this study specifically, data show that, while both WT-FL and macNOX2KO mice became obese following HFD administration, macNOX2KO mice had attenuated adipose pathology and preserved adipose function. Additionally, glucose tolerance was normalized in macNOX2KO mice compared to WT-FL mice following HFD, and HFD-induced brain injury was prevented in macNOX2KO mice. Thus, the macNOX2KO mice were protected from the HFD effects. Overall, these data are consistent with previous studies demonstrating increased NADPH oxidase activity/expression in models of obesity (Bruce-Keller et al., 2010; Chinen et al., 2007; Zhang et al., 2005) and are also in agreement with the growing body of literature describing the sensitivity of the brain to obesity-induced metabolic dysfunction (reviewed in (Bruce-Keller et al., 2009; Middleton and Yaffe, 2009)). Moreover, these data significantly enhance the findings of previous studies with the demonstration that NOX2 is a specific and powerful mediator of pathogenic effects of diet-induced obesity that extend from adipocytes to brain cells. These data indicate that NOX2-signaling in visceral adipose macrophages precipitates loss of adipocyte function and the development of ER stress within adipose tissues, triggering pathways that ultimately result in loss of metabolic and neurologic function.

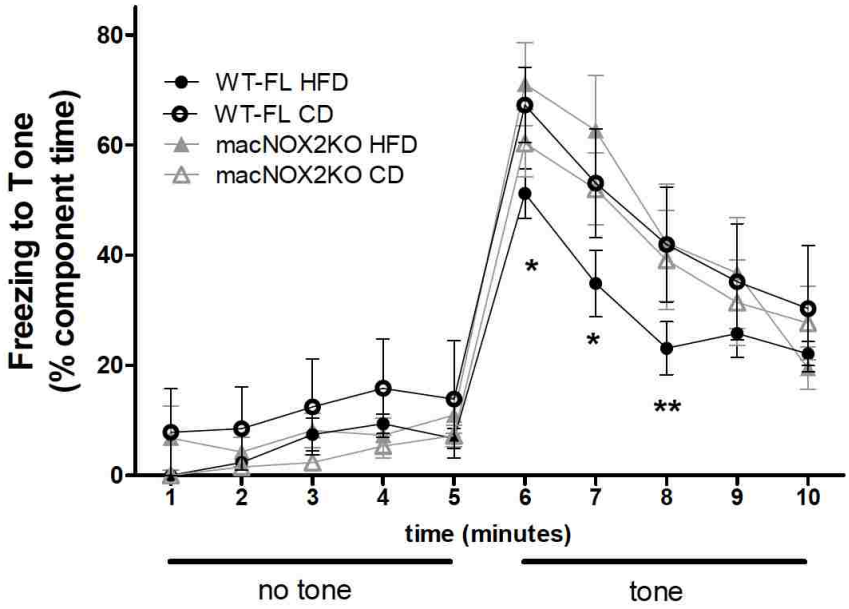


Figure 3.4: Effects of HFD on cognitive function in WT-FL mice and macNOX2KO mice. Mice were evaluated using the fear conditioning assay as described in Materials and Methods. Significantly (*$p < 0.05$, *$p < 0.05$, **$p < 0.01$) more freezing to tone at 6 min, 7 min, and 8 min in NOX2KO mice vs. WT mice on HFD.

Collectively, these data raise the possibility that macrophage-specific NOX2-based therapies could be used clinically to preserve both metabolic and neurologic function in the context of obesity.

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# Chapter 4. Conclusions

The intent of our first study was to determine if deletion of NOX2 does indeed provide protection from the deleterious effects of high-fat diet-induced adiposopathy. After establishing this to be true, we set out to develop a cre-lox knockout mouse model with deletion of NOX2 restricted to macrophages. After completing this, the intent of our second study was to determine if macrophage-specific NOX2 deletion also provides protection while also preserving the beneficial roles of NOX2, namely in cognitive functioning. Both studies that were conducted resulted in significant developments in the investigation of NOX2 as a key contributor to high-fat diet-induced adiposopathy and neurologic impairment. The first study looked at the implications of a whole-body deletion of NOX2, as well as where the majority of NOX2 was expressed. It was important for us to focus our studies on looking at NOX2 in macrophages, because prior studies have suggested that NOX2 is localized to macrophages and not adipocytes (Han et al., 2012, Mahadev et al., 2004; Schroder et al., 2009)

A limitation of the first study that may have played a minor role in affecting the results was that we were unable to house the NOX2KO mice and the WT mice in the same room under the same conditions. This was due to the fact that the NOX2KO mice have increased susceptibility to pathogens (Pollock, 1995), and based on our pilot study, it was discovered that these mice can develop sterile granulomas in response to being housed in a conventional room. Thus, we had to house the NOX2KO mice under sterile conditions.

In the first study, the results provided evidence that NOX2KO mice on a high-fat diet have less visceral adipose tissue pathology and improved glucose tolerance.

Additionally, these mice have less brain injury. Numerous studies have shown that NADPH oxidase plays a role in the pathogenesis of neurodegenerative diseases (Block, 2008; Lambeth, 2007; Shimohama et al., 2000). NOX2 does play a role in brain inflammation, but it also plays a role in physiologic cognitive and memory functioning as well (Kishida and Klann, 2007; Kishida et al., 2005; Tejada-Simon et al., 2005). In light of this, and because the whole-body NOX2KO mice are cognitively impaired (Kishida et al., 2006; Pao et al., 2004)., we were unable to assess cognitive functioning via behavioral testing, since the mice would be unable to perform the tests appropriately due to cognitive deficits. A future area of investigation would be to use cre-lox knockout mice to target certain areas and cells in the brain so as to inhibit pathological effects but preserve physiologic effects.

Lack of effective antibodies for the detection of NOX2 expression was another limitation of these studies. If antibodies could be developed that are specific for NOX2, this would greatly aid in validating NOX2 expression. Through genetic testing and PCR, we confirmed that NOX2 was deleted in the appropriate mice, but additional confirmation through the use of specific antibodies would be helpful. This would also allow for improved investigation as to where NOX2 is being expressed specifically.

Because adiposopathy and not obesity is the driving factor in development of metabolic impairments, therapies that look specifically at alleviating adiposopathy and not necessarily obesity should be investigated. The challenge of finding a treatment modality that effectively alleviates obesity (Padwal et al., 2004) suggests that focusing on protecting the adipose tissue by targeting NOX2 may be more effective.

Our data suggest that therapies could be developed to target NOX2 in order to preserve metabolic and neurologic function in the context of obesity.

Additional studies should be conducted to determine if inhibition of NOX2 has the potential to be therapeutic on a pharmacologic level. One way this could be done is through the use of a glucan-based encapsulation system to target visceral adipose macrophages. This encapsulation system utilizes shells extracted from *Saccharomyces cerevisiae* (baker's yeast) to deliver siRNA (Aouadi et al., 2009; Soto and Ostroff, 2008). Specifically, NOX2 siRNA could be loaded into these beta glucan microspheres and then given via intraperitoneal injection to wild type mice on a high fat diet. The expected result would be that NOX2 expression will be decreased in these mice. Exploring this option and others, could result in viable therapeutic options to treat adiposopathy.

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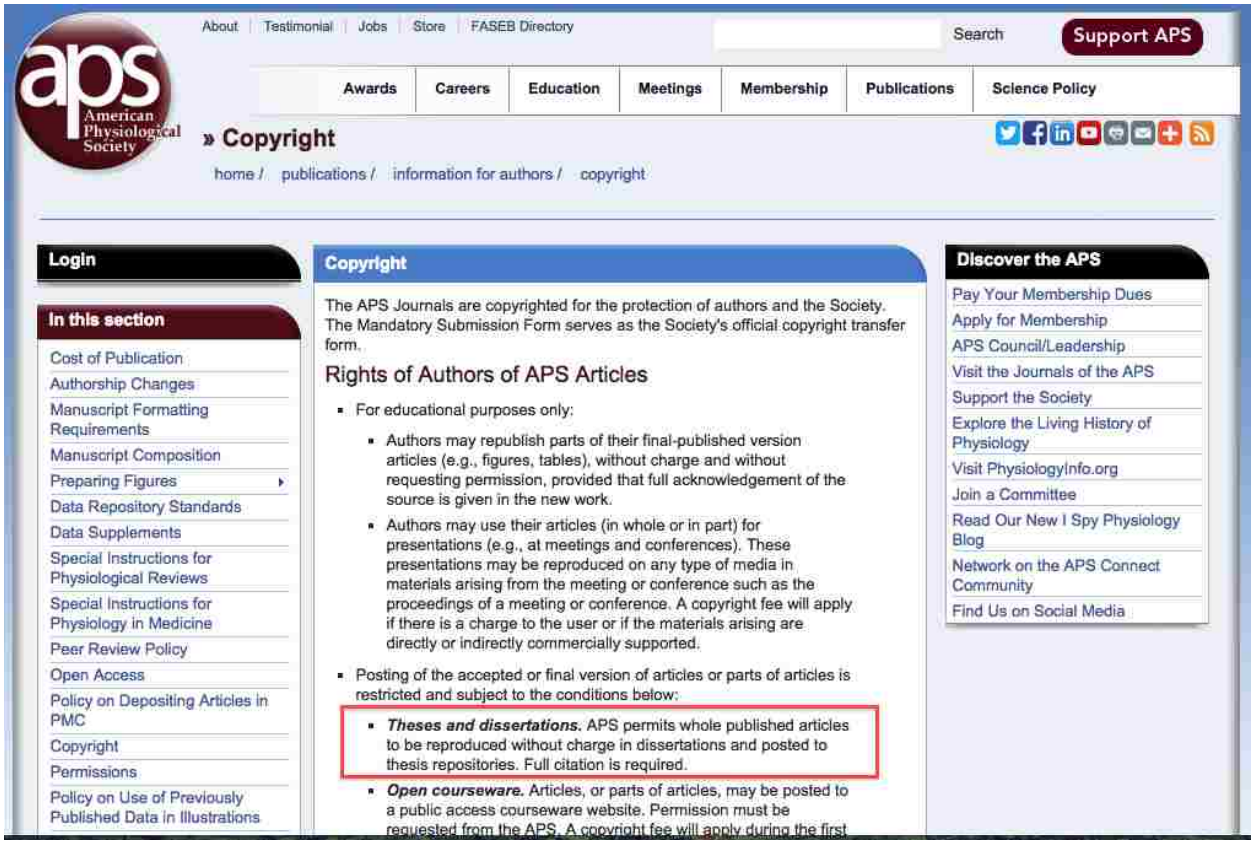

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# Vita

Jennifer Kathleen Pepping was born in Olympia Fields, Illinois. She graduated from Geneva High School in 2003. After graduation, she attended University of Illinois Urbana-Champaign where she graduated in 2007 with a Bachelor of Science in animal sciences. After undergraduate school, she continued her studies at the University of Illinois Urbana-Champaign, where she attended veterinary school at the College of Veterinary Medicine. She graduated from veterinary school in 2011. Immediately following graduation, Jennifer began a residency program in Laboratory Animal Medicine within the Division of Laboratory Animal Medicine (DLAM) at Louisiana State University School of Veterinary Medicine. During the residency, she also began her graduate studies in the Department of Pathobiological Sciences. She completed her residency program in 2014. Following the residency program, she continued to work on her graduate studies and is currently a candidate for the Master of Science degree in Veterinary Medical Sciences under the direction of Dr. Rhett W. Stout and Dr. Annadora Bruce-Keller.